Locus control regions and gene therapy

Locus control regions en gentherapie

PROEFSCHRIFT

Ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
Prof. Dr P.W.C. Akkermans M.A.
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op
Woensdag 29 september 1999 om 9.45 uur

door
Dubravka Dalibora Drabek
Geboren te Dubrovnik

Promotie commissie

Promotor	Prof. dr F. Grosveld
Overige leden:	Prof. dr B. Löwenberg
	Prof. dr E.A. Dzierzak
	Dr Ir D.N. Meijer

Omslag: Gornje Celo F. Drabek

Dit proefschrift werd bewerkt binnen de vakgroep Celbiologie en Genetica, Faculteit der Geneeskunde en Gezondheidswetenschappen, Erasmus Universiteit Rotterdam. De vakgroep maakt deel uit van het Medisch Genetisch Centrum Zuid-West Nederland.

Print: Offsetdrukkerij Ridderprint B.V., Ridderkerk

Groen en prachtig in de lente,
Rood en treurig in de herfst,
Naakt en bang in de winter,
Staat een boom in mijn tuin.
Hij wist alles.
Hij luistert wat de wind hem vertelt
En bewaart het geheim.

To my parents and
my pseudoparents (Misha and Branko)

Contents

List of abbreviations

5-FC	5-fluorocytosine
5-FU	5-fluorouracil
6-TX	6-thioxanthine
AAV	Adeno-associated viruses
ADA	adenosine deaminase
ASGPr	asialoglycoprotein receptor
BPV	bovine papilloma virus
Btk	Bruton's tyrosine kinase
CAT	chloramphenicol acetyltransferase
CEA	carcinoembryogenic antigen
CD	cytosine deaminase
CMV	cytomegalovirus
DHFR	dyhidrofolate reductase
EBV	Epstein-Barr virus
ES cells	embryonic stem cells
FISH	fluorescence in situ hybridisation
GC	glucocerebrosidase
GCV	ganciclovir
GDEPT	gene directed enzyme prodrug therapy
HAC	human artificial chromosome
HIV	human immunodeficiency virus
HPRT	hypoxanthine phosphoribosyltransferase
HS	hypersensitive site
HSC	haematopoietic stem cell
HSV	Herpes simplex virus
Ig	immunoglobulin
IL	interleukin
LCR	locus control region
LTR	long terminal repeat
MAC	mammalian artificial chromosome
MDR	multidrug resistance
MHC	major histocompatibility complex
MoMLV	Moloney murine leukaemia virus
NTR	nitroreductase
PEM	polymorphic epithelial mucin
PEV	position effect variegation
RME	receptor mediated endocytosis
SIMP	single insertion mutagenesis procedure
Sycp 1	synaptonemal complex protein 1
TK	thymidine kinase
TNF	tumour necrosis factor
UTR	untranslated region
VZV	Varicella- Zoster virus
XGPRT	xanthine-guanine phosphoribosyltransferase
Xid	X-linked immunodeficiency
XLA	X-linked agammaglobulinaemia
YAC	yeast artificial chromosome
ZP3	zona pellucida 3 gene

Chapter 1

General introduction

General introduction

Gene therapy is a procedure in which exogenous genetic material is introduced into the cells of a patient in order to correct an genetic error or to provide the cells of the patient with a new functional property. Correction can be achieved by gene targeting via homologous recombination, at present achieved with very low efficiency (reviewed in Yanez and Porter 1998), or by addition of a therapeutic gene (augmentation). For gene therapy to succeed in clinical practice, the therapeutic gene must be delivered efficiently into the appropriate cells (tissue) and, once delivered, the gene must be expressed at a therapeutical level.

In this chapter (section A) I will summarise some of the efforts currently underway for the development of practical, efficient and safe methods for gene transfer in man and the rationale underlying these approaches as well as the limitations and the problems involved (Tables 1A and 1B). I will also address the following issues: regulation of gene expression (section B), possible targets for gene therapy (section C), creation of animal models for human diseases and their relevance to somatic gene therapy (section D) and ethical and social implications of gene therapy (section E).

A. Gene delivery systems

Highly efficient gene transfer is essential for any future gene therapy protocols. It requires delivery of an intact therapeutic gene (piece of genomic DNA, cDNA, oligonucleotides or RNA) through the plasma membrane and cytoplasm into nucleus (except for RNAs), where it should be maintained as a stable plasmid or chromosomal integrant. The available techniques are based either on viral or non-viral gene delivery methods.

1. Viral gene delivery methods

Retroviruses

Retroviral vectors are widely used as gene transfer vehicles, both in research and recently in clinical gene therapy trials. They are largely based on Moloney murine leukaemia virus (MoMLV) which is an enveloped

single stranded RNA virus whose genome is composed of three regions. The long terminal repeats (LTRs) contain the viral promoter and enhancer region required for initiation and termination of transcription. The LTRs, which flank the viral genome also, contain sequences required for integration into the target genome. The second region encodes the psi sequence necessary for efficient packaging into viral particles. The third region contains the viral genes *gag, pol* and *env*, which respectively encode the viral structural proteins, the enzyme reverse transcriptase and the envelope proteins. In retroviral vectors, viral genes (*gag, pol* and *env*) are deleted and replaced by exogenous gene(s). The substitution capacity of the vector is limited to ~ 10 kb. Since they lack the genes required for the formation of a virion, retroviral vectors can not be packaged autonomously. To incorporate vector RNA into virions, "packaging cell lines" have been developed (Cone and Mulligan 1984; reviewed in Miller 1992), containing the viral genome but lacking the psi sequence required for incorporation of the viral mRNA into virions. Alternatively, all the structural viral genes are incorporated into the packaging cell line under separate promoters (Danos and Mulligan 1988). Vector DNA introduced into the packaging cell line results in a producer line. Since the vector contains the encapsidation sequence (ψ) the vector genome is preferentially packaged into retroviral vector particles. Retrovirus entry into cells is dependent on the existence of the appropriate viral receptor on the actively dividing target cells. After reverse transcription, the proviral sequences are stably integrated into the genome of a target cell. This occurs in generally random fashion but there is some preference for integration into transcriptionally active regions (Rohdewohld *et al.* 1987). This feature of retroviral integration presents a potential risk of insertional mutagenesis. Although, the risk is estimated to be low, concerns still remain. Compared to wild type retroviral titers, recombinant retroviral titers are often low (10^4-10^5 particles/ml). Furthermore recombinant retroviruses are difficult to purify without loss of infectivity, partly due to loss of the env product (reviewed in Mulligan 1993). For several reasons it is difficult to introduce retroviral vector-producing cells *in vivo* for human gene therapy. As stated already, recombinant retroviral titers are low, targeted cells may not express the membrane protein to which the retroviral envelope binds and there may be an immunological reaction to the cells by human complement. There are reports that the CD59 gene, one of the regulators of complement activation, can be used for rapid selection of transduced cells (Takizawa *et al.* 1992). Due to the transduction of the CD59 molecule, xenogeneic cells are

resistant to human complement attack (Lubin and Coyne 1991; White *et al.* 1992). To insert the CD59 gene as well as other therapeutic genes into vector-producing cells, a novel complement-resistant retroviral vector, which possesses the CD59 gene as a selection gene, was designed (Hayashi *et al.* 1998).

Recent studies have opened the possibility for gene delivery into non-dividing cells by using lentiviruses, namely pseudotyped high-titer replication defective human immunodeficiency virus type 1 (HIV-1) vectors (Reiser *et al.* 1996; Mochizuki *et al.* 1998; Uchida *et al.* 1998; Douglas *et al.* 1999). HIV based vectors are able to efficiently transduce freshly isolated, non-prestimulated $CD34^+$ cells, terminally differentiated neurons, contact-inhibited primary human skin fibroblasts and cardiac myocytes, while Moloney murine leukaemia virus-based vectors under the same conditions fail to transduce.

Adenoviral vectors

Adenoviral vectors may be particularly attractive if considering transfer into post-mitotic (non-dividing) cells. They are non-integrating vectors with a large cloning capacity (at least 8 and possibly up to 35 kb). They can be purified to high titers (10^{13} viral particles/ml). Adenoviruses used for gene therapy are deleted in the E1 and E3 regions, which code for proteins involved in the transcription of the early regions (E1, E2A, E2B, E3 and E4) and the proteins involved in "hiding" the infected cell from the immune system. However, there are well-documented cytotoxic T lymphocyte responses to the expression of virally encoded proteins after adenoviral vector administration (Kass-Eisler *et al.* 1994; Dai *et al.* 1995; Yang *et al.* 1996; Barr *et al.* 1995; Yang *et al.* 1995; DeMatteo *et al.* 1996; Reichel *et al.* 1998). Currently, this continues to be a major obstacle for adenovirus-mediated gene therapy, although completely "empty " viruses are under development. Taking the viral gene removal to extremes, helper virus dependent vectors have been produced with 25kb Cre-lox mediated deletion, containing only terminal adenovirus sequences required for replication (Schiedner *et al.* 1998; Searle and Mautner 1998).

Adeno-Associated viruses (AAV)

Considerable interest in AAV as a potential vector in human gene therapy came from the specific properties of this virus. It is a non-pathogenic human virus that integrates into the genome of the infected cell. Integration can occur in non-dividing cells (Podsakoff *et al.* 1994; Russell

et al. 1994) albeit at a lower frequency than in dividing cells, and occurs at a specific site in the human genome (the q arm of chromosome 19) (Kotin *et al.* 1990). It seems that site specific integration is related to the presence of the *rep* coding region, which is absent in vectors derived from the native form of AAV (Linden *et al.* 1996). The critical sequence for site-specific integration has been determined to reside in a 33 base pair fragment, containing two signal sequences that play essential roles in AAV DNA replication. These are a binding site for the AAV Rep protein and an appropriately positioned site that is specifically nicked by the Rep protein (Im and Muzyczka 1990). The substitution capacity of the vector is limited to ~ 5kb and production requires co-infection with a helper adeno or herpes virus (McLaughlin *et al.* 1988). Lately, there have been reports that AAV vectors can be efficiently produced without helper virus (Matsushita *et al.* 1998). The adenovirus regions that mediate AAV vector replication were identified and assembled into a helper plasmid. These included the VA, E2A and E4 regions. When this helper plasmid was cotransfected along with plasmid encoding the AAV vector, AAV vector was produced as efficiently as when using adenovirus infection as a source of help.

Other viral vectors

A number of other viral gene delivery systems are under development. Vaccinia virus is a large DNA virus that can be engineered to deliver genes into mammalian cells (Whitman *et al.* 1994). Also Herpes virus is a large DNA virus that infects and persists in cells of the nervous system. It is therefore possible that Herpes virus based vectors may provide long-term gene expression in cells of the central nervous system (Anderson *et al.* 1992). Several recent publications demonstrate that gene transfer and expression in mammalian cells can be mediated by recombinant baculoviruses (Boyce and Bucher 1996; Sandig *et al.* 1996; Barsoum *et al.* 1997; Condreay *et al.* 1999).

Replicating plasmid vectors have been derived from Bovine papilloma virus (BPV) and Epstein-Barr virus (EBV). BPV vectors are not reliably maintained as episomes (Mecsas and Sugden 1987) and all too often undergo integration, deletion, recombination and rearrangement. For this reason many researchers have turned to EBV vectors.

The Alphavirus vectors, based on Semliki Forest and Sindbis virus also have several features which make them well suited as expression vectors. Naked genomic RNA is able to start an infection when introduced into the cytoplasm of nearly all animal cell types. There is no evidence of

chromosome integration. The infecting RNA is self-replicating by its ability to code for its own replicase (Liljeström and Garoff 1991).

2. Non-viral gene delivery methods

One practical advantage of the non-viral vectors is that theoretically there is no limitation on the size of the expression cassette to be transferred. Such vectors do not require "live" packaging systems, are in principle much easier to purify and pose no risk of recombination during the production process. Unlike the vigorous host responses to some viral vectors, most non-viral vectors have a limited number of antigenic epitopes for the immune system to recognize, resulting in little or no host response to the administration of the vector. However the disadvantages of non-viral vectors are the low efficiency of gene transfer and transient expression, particularly in *vivo*.

Many physicochemical methods have been developed for the introduction of DNA into mammalian cells. These include calcium phosphate precipitation (Graham and van der Eb 1973), DEAE-dextran (Lake and Owen 1991), polybrene-DMSO (Morgan *et al.* 1980; Chaney et al. 1986), protamines (Wienhues *et al.* 1987), liposomes either alone (Fraley *et al.* 1980; Schaefer-Ridder *et al.* 1982) or in combination with reconstituted viral envelopes (Lapidot and Loyter 1990; Nussbaum *et al.* 1992), cationic liposomes (Felgner *et al.* 1987), electroporation (Chu *et al.* 1987), direct injection of DNA (Wolff *et al.* 1990), particle-bombardment technology (gene gun) (Johnston and Tang 1994; Qui *et al.* 1996) and a rapidly developing technique, which exploits the natural process of receptor mediated endocytosis (RME) and promises to combine both efficiency and specificity of cell targeting (reviewed in Guy *et al.* 1995). The aim of this approach is to combine the efficiency of viral vectors and the safety of non-viral vectors. DNA-polylysine-ligand conjugates are being investigated as such tissue-specific delivery vectors. These conjugates seek to exploit the physiological pathways by which cells internalise macromolecules such as nutrients. Ligands are recognised and bound by cell surface receptors and endocytosed via clathrin-coated pits. Most of the research on DNA delivery by this approach has been performed by targeting the liver-specific asialoglycoprotein receptor (ASGPr) (Wu and Wu 1987; Wu and Wu 1988; Wu *et al.* 1991; Wilson *et al.* 1992; Plank *et al.* 1992) and the relatively ubiquitous transferrin receptor (Zenke *et al.* 1990; Wagner *et al.* 1990; Wagner *et al.* 1992; Curiel

et al. 1991; Curiel *et al.* 1992; Cotten *et al.* 1992). DNA delivery to hepatocytes has also been achieved using insulin-polylysine conjugates (Rosenkranz *et al.* 1992) and to antigen-bearing cells using monoclonal antibodies (Trubetskoy *et al.* 1992). More complex conjugates of adenovirus-ligand-DNA combine the efficiency of targeting conferred by a specific ligand with the endosome disrupting ability of adenovirus (Curiel *et al.* 1991). The movement of the DNA-containing complex from the cytoplasm to the nucleus may be one of the most important limitations to successful gene transfer (Zabner *et al.* 1995; Wilke *et al.* 1996). The gene transfer is most effective in rapidly dividing cells where, in addition to DNA replication, the inner and outer nuclear membranes break down at mitosis. Approaches to efficient gene delivery, particularly in the absence of cell division, must therefore employ strategies that overcome the barrier of the nuclear envelope, either by incorporating elements of the cellular nuclear-cytoplasmatic trafficking machinery or by deploying components of viral nuclear entry mechanism.

Non-viral carriers are used to shuttle DNA, RNA and oligonucleotides into cells. At the moment, there is a particular interest in chimeraplasty (delivery of DNA/RNA hybrid molecules). Using this method, the site-specific correction of different genetic defects by mechanism of mismatch repair has been reported (Cole-Strauss *et al.* 1996; Yoon *et al.* 1996; Kren *et al.* 1998;).

Non-viral delivery methods in theory have no size limitation. The development of a mammalian artificial chromosome (MAC) would allow the delivery of any gene of interest with its controlling sequences and the ability to replicate at each cell division (Huxley 1994). This could be combined with stem cell targeting. The problem with MACs is not a lack of enthusiasm for the development of such vectors, but a deficiency in the understanding of the basic biology concerned with defining the three functional elements of a mammalian chromosome-telomeres, origins of replication and centromeres. The real problem lies in identifying the cis-acting chromosomal locus responsible for the segregating of chromosomes in mitosis and meiosis (the centromere). Mammalian centromeres are always associated with large amounts of species- and often chromosome-specific repetitive DNA. In humans the most abundant sequence is α satellite which can cover anywhere between 250-5000 kb of DNA. Various functional studies involving the introduction of small (less than 100 kb) regions of alphoid repeats have shown that this DNA is involved in at least some, but not all aspects of centromere function (Davies 1995). One

possible reason why this DNA does not form a genuine centromere is that a minimum threshold of alphoid repeats may be necessary to nucleate kinetochore formation. Introduction of yeast artificial chromosome (YAC) clones containing larger regions of alphoid DNA into mammalian cells would provide a useful assay system for identification of the region required. Recently, a human artificial chromosome (HAC) vector was constructed from a 1 Mb YAC. This YAC, which also included non-alpha satellite DNA, was modified to contain human telomeric DNA and a putative origin of replication from the human ß-globin locus. When transfected into human cells by lipid-mediated DNA transfection, HACs were mitotically stable in the absence of selection for at least 100 generations. Fluorescence in situ hybridization (FISH) analysis revealed that the HACs were not derived by telomeric fragmentation of the endogenous chromosomes (Henning *et al.* 1999). YAC-based approaches such as this should broaden our understanding of how to attain human centromere function. However, there will be technically demanding steps such as the intact isolation and transfection of large DNA molecules. The maximum size delivered intact *in vitro* by the use of cationic lipids is at least 600 kb, although the majority of clones contained broken DNA. Shearing and degradation of such large molecules will be an even greater problem *in vivo*.

Most of the currently used delivery methods, their advantages and disadvantages are summarised in Tables 1A and 1B.

Table 1A. Viral delivery methods: advantages and disadvantages

Delivery system	Advantages	Disadvantages
Viral vectors (in general)	- High efficiency of transduction	- Size limitation - Generally low titers - Possibility of the generation of replication-competent viruses
Retroviruses	- High efficiency, especially *ex vivo*	- Dividing cells only (exept lentivirus based) - Risk of insertional mutagenesis - Risk of oncogene activation - Fast inactivation by complement system *in vivo*
Adenoviruses	- Dividing and non-dividing cells - High titers	- Strong host immune response - Presence of neutralising antibodies
AAV	- High frequency of transduction in a variety of cells - Dividing and non-dividing cells - Non-pathogenic	- Helper virus requirement - Small insert size (4.7 kb) - Lack of stable packaging cell lines - Random integration in absence of the viral replicase (rep) function
HSV	- Stably maintained as episomes in non-dividing cells (neurones) - Packaging capacity (~35kb)	-Pathogenic?

Table 1B. Non-viral delivery methods: advantages and disadvantages

Delivery system	Advantages	Disadvantages
Non-viral methods (in general)	- No size limitation - Usually no host immune response	- Low efficiency of transfer
RME	- Potential targeting via specific receptor-ligand systems	
Direct injection of naked DNA	- Plasmid based vaccination	
Liposomes	- Easy to prepare from a pharmaceutical standpoint - Not significantly antigenic - Possibility of repeated treatment - Amenable to chemical modification aimed at improved cell specificity	-Transient gene expresssion - Low efficiency of transfer
Gene gun	- Selective bombardment	- Requires surgical intervention to reach tissues other than skin

Choice of gene-transfer strategies

Gene therapy could be applied *ex vivo* or *in vivo*. In the *ex vivo* approach, cells are removed from the body, cultured *in vitro*, genetically modified and subsequently returned to the body. Critical issues are the *in vitro* culturing of cells and their ability to be transplanted. The advantages of the *ex vivo* approach are a generally high efficiency of gene transfer and the possibility to enrich genetically modified cells if the vector has a selectable marker. Gene transfer into haematopoietic stem cells (HSC) is perhaps the best example. The pluripotent HSC, which constitutes 0.01-0.1% of human bone marrow cells, but could also be enriched from other sources such as umbilical cord and peripheral blood (Lu *et al.* 1996; Kalle

et al. 1998), is an ideal target cell because of its pluripotency and self-renewal qualities.

The *in vivo* approach involves the direct transfer of genes into cells as they reside naturally in the body. The main problems with this strategy are low efficiency of gene transfer, necessity for repeated treatment and increased potential for problems with host immune response. On the other hand, observations in the early 1990s that plasmid DNA could directly transfect animal cells *in vivo* led to exploration of its use in induction of immune responses. The effectiveness of DNA vaccines against viruses, parasites, bacteria and cancer cells has been demonstrated in numerous animal models (reviewed in Donnelly *et al.* 1997). This new approach comes as an aid for the prevention of diseases for which the conventional vaccines have failed. DNA vaccines employ genes encoding proteins of pathogens or tumours rather than using the proteins themselves, a live replicating vector, or an attenuated version of the pathogen itself. Purified plasmid DNA containing antigen coding sequences and the necessary regulatory elements to express them is introduced into the tissue by intramuscular injection or particle bombardment (Johnston and Tang 1994). Once the DNA reaches the tissue, the antigen should be expressed in sufficient quantities to induce potent and specific humoral and cellular immune responses and to confer protection against possible infection or cancer development. There is a body of evidence showing that dendritic cells are the essential antigen-presenting cell types involved in immune responses to intramuscularly administered DNA vaccines (Manickan *et al.* 1997). Protective immune responses have been reported in a number of preclinical models after challenges with relevant pathogens or tumour cells. Epitope-based plasmid DNA vaccinations were carried out against influenza virus, bovine herpes virus, human herpes simplex virus, rabies virus, hepatitis B virus, human immunodeficiency virus (HIV), Mycobacterium tuberculosis, Salmonella typhi, Leishmania major, Plasmodium yoelii, human carcinoembryogenic antigen (CEA) bearing tumours, polymorphic epithelial mucin (PEM) expressing tumour cells and against many other infectious agents, epitopes of which were coded by the plasmid DNA used for vaccinations (reviewed in Donnelly *et al.* 1997)

B. Regulating expression of introduced genes

Achieving reproducible and controlled expression of delivered genes is an important issue, if a treatment is to be used on human patients. Through the use of mouse transgenic models a very large body of information is now available concerning the mechanisms of mammalian gene regulation particularly with respect to high level tissue specific expression *in vivo*. It should be possible to use this information (promoters, enhancers and locus control regions in gene transcription) to approach the problem of optimising expression in a systematic manner.

Promoters

Promoters lie in the first 200 bp upstream of the transcriptional start site of protein-coding genes (transcribed by RNA polymerase II). They contain short sequences to which transcription factors bind with the purpose of stabilising and positioning the transcriptional apparatus (Benoist and Chambon 1981, Serfling *et al.* 1985; McKnight and Tjian 1986). The TATA box and the initiator, separated by 25-30 base pairs (Smale and Baltimore 1989) are the core promoter elements, essential for gene activation. The TATA binding protein (TBP) specifically binds the TATA element and forms the TFIID complex that contains several TBP associated factors. In this way the TATA box positions the basal transcription machinery and determines the precise site of transcription initiation. The TATA box and the initiator can drive transcription in cell free systems, upon addition of extracts containing the general components of transcription machinery, but not *in vivo* in a chromatin environment (Imbalzano 1994). Additional promoter proximal elements are needed such as CCAAT and CACC boxes that bind ubiquitous as well as specific trans-acting factors and contribute to constitutive or more tissue and stage specific gene expression. Although the promoter's role is to potentiate transcription and mediate cell-type and developmental specificity, a promoter alone is usually not sufficient to give significant levels of transcription of a gene that is integrated into chromatin.

Enhancers

Enhancers are DNA elements (between 50 bp and 1.5 kb), located upstream or downstream from the promoter sequences, which can potentiate transcription independently of their orientation. Transcriptional enhancement occurs even when the enhancer is located at varying distances

from the site of transcriptional initiation (Banerji *et al.* 1981; Moreau *et al.* 1981; Serfling *et al.* 1985; Weber and Schaffner 1985). Like promoter proximal elements, enhancers provide additional specificity to the gene that they activate by binding developmental, cell type specific factors. However, the level of expression achieved for a particular enhancer usually shows a wide variation, depending on the position of integration into the host chromatin (Wilson *et al.* 1990). This feature of enhancer function has important implications for gene therapy since it makes it very difficult to obtain reproducible expression. While an enhancer may occasionally give rise to therapeutically useful levels of expression when integrated into a favourable position, many integration events may fail to give adequate levels of expression.

Locus control regions (LCRs)

An LCR is defined as an element that gives rise to gene expression in a transgenic assay that is insensitive to the position of integration and dependent on the number of copies of the transgene. LCRs therefore seem to be able to override the suppressive position effects of surrounding closed chromatin. The human β globin LCR, which provides erythroid specific expression, was the first of these elements to be identified (Grosveld *et al.* 1987). Indications for the existence of such a region (that controls the activity of the entire β-globin gene cluster) came from the study of a human γβ-thalassaemia, namely a patient with a 100 kb deletion which eliminated the entire upstream region, but left the β-globin gene intact (Kioussis *et al.* 1983; Taramelli *et al.* 1986). A 21 kb DNA region containing DNase I hypersensitive sites (from the deleted region in patient) is capable of driving expression of the human β-globin genes in transgenic mice (Grosveld *et al.* 1987)

Subsequently, many more LCRs have been identified and described for a number of vertebrate genes expressed in a tissue specific manner. These include LCRs for the human CD2 gene (Greaves *et al.* 1989), the chicken lysozyme gene (Bonifer *et al.* 1990), the murine MHC class II Ea gene (Carson and Wiles 1993), the α/δ T-cell receptor locus (Diaz *et al.* 1994), the mouse tyrosinase gene (Montoliu *et al.* 1996), the human growth hormone gene (Jones *et al.* 1995), the human S100β gene (Friend *et al.* 1992), the human IgH Cα gene (Madisen and Groudine 1994), the human red and green visual pigment gene (Wang *et al.* 1992), the human adenosine deaminase gene (Aronow *et al.* 1992), the rat LAP gene (Talbot

et al. 1994), the human class I HLA-B7 gene (Kushida *et al.* 1997), the murine zona pellucida 3 gene (Lira *et al.* 1990) and others.

Such regions of regulatory interest have been shown to correlate with the presence of DNase I hypersensitivity sites (HS)(Tuan 1985;Grosveld 1987, Forrester 1987). These sites represent nucleosome-free regions in chromatin, which are accessible to trans-acting proteins. In the case of the β-globin LCR, they are mapped to the 200-300 bp core fragments of HS 1-4 (Talbot *et al.* 1990; Talbot and Grosveld 1991; Philipsen *et al.* 1990; Pruzina *et al.* 1991). Different HS sites might provide an LCR with distinct functions (such as enhancer activity or chromatin opening activity), but only the presence of a complete set of regulatory elements will result in a proper LCR function, as concluded from deletion studies (Bonifer *et al.* 1994; Carson and Wiles 1993; Milot *et al.* 1996; Festenstein *et al.* 1996).

The murine class II MHC Eα gene LCR

The major histocompatibility complex (MHC) cell surface glycoproteins, class I and II are members of the immunoglobulin supergene family and function as key markers in the recognition of self versus non-self by the immune system. The MHC proteins have been extensively studied in the mouse and human systems and share both structural and functional homology within classes, as well as between species. Class I MHC antigens are expressed on most somatic cells and represent the classically defined major transplantation antigens. The class II MHC antigens have two modes of expression-constitutive and inducible. MHC II genes are expressed constitutively in only a very restricted number of cell types specialised in antigen presentation, such as dendritic cells and B lymphocytes. Within these lineages, MHC II expression is also subjected to strict developmental control. For example, in the murine B-lymphocyte compartment, early pro-B cells are MHC II negative. Expression starts from the pre-B cell stage, where it is also inducible by interleukin-4 (IL-4). Mature B-cells show constitutive MHC II expression, while terminal differentiation into plasma cells is accompanied by the extinction of expression (Glimcher and Kara 1992). MHC II expression can also be induced in a large variety of other cell types, in particular by γ-interferon (IFN-γ).

The mouse class II MHC locus is a model system for studying the regulation of multigene family, during B-cell differentiation. The locus consist of six genes (Ob, Ab, Aa, Eb1, Eb2, Ea) encoding two expressed

class II MHC antigens, A (A alpha , A beta heterodimer) and E (E alpha, E beta heterodimer)(Figure 1). Using a panel of well-characterised mouse lines specific for different stages of B cell development, as well as non-B cell lines, DNase I hypersensitive sites were mapped adjacent to the mouse MHC class II Ea gene only in those cell lines which are either developmentally programmed to express class II MHC or which actually do express class II MHC at the cell surface (Carson 1991). The upstream region 5' of the Ea gene contains five defined groups of DNase I hypersensitive sites. HS1 (-50 bp from the cap site) and HS2 (-1.2 Kb) reflect regions of chromatin which are open for the binding of regulatory proteins important for the expression of class II MHC Ea. These sites are clearly present upon IFN-γ induction. They map closely to the promoter (X, Y boxes) and enhancer (W box) regions defined for Ea (Dorn *et al.* 1987; Dorn *et al.* 1988). Other groups mapped further upstream are: HS3 (-3.4 kb), HS4 (-5.4 kb) and HS5 (-8.4). There are no additional hypersensitive sites other than HS1-5 within a total of 20 kb 5' of Ea gene (Figure 1). The proposed LCR was tested in a transgenic assay, driving the expression of Ea^d gene in a mouse strain that does not express an endogenous Ea gene (Carson and Wiles 1993). Only a construct containing all 5 HS sites showed position independent, copy number-dependent expression of the Ea^d gene. The region delineated by Ea HS1 and HS2 is necessary but not sufficient as an LCR because it is subject to position-dependent effects. These results identified an immunologically important, putative LCR which can be used to target gene expression to cells of the B-lymphocyte lineage, as well as to other class II MHC expressing cells (such as dendritic cells and macrophages) (Chapter 3 and 4 of this thesis).

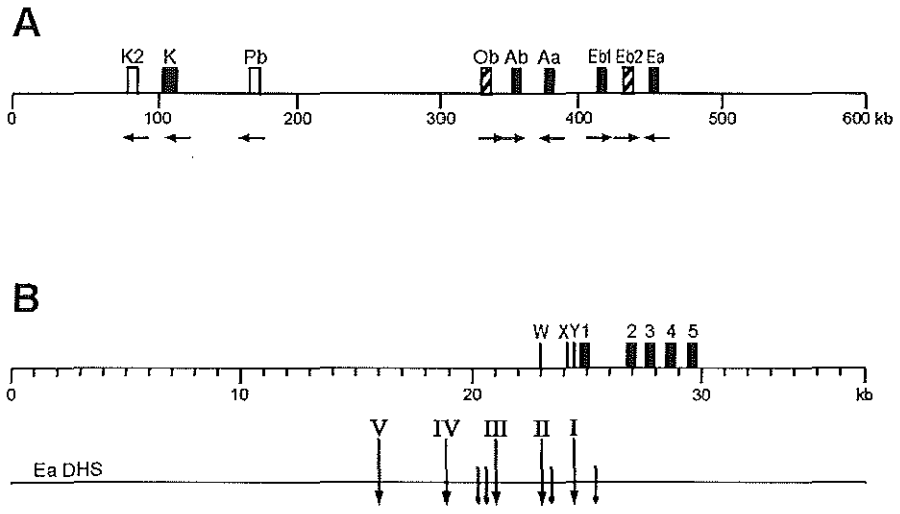

Figure 1: Mouse H-2 I Region on chromosome 17.
A.Complete H-2 I region. MHC class II region is between 300-500 kb. K is a class I gene. Arrows show direction of transcription. White boxes represent pseudogenes, shaded boxes genes without known translation product, and black boxes are expressed genes.
B. Map of the class II Ea gene locus.
Filled boxes indicate exons. The letters X, Y and W refer to control elements present in the upstream region of the Ea gene. The arrows refer to the locations of the DNase I hypersensitivity sites (DHS). The large arrows refer to the central location of HS and the smaller arrows refer to minor HS (Carson and Wiles 1993).

The human CD2 gene LCR

CD2 (LFA-2) is one of the earliest markers to appear on the surface of developing thymocytes and in man is present only on thymocytes and circulating T-cells. In mice, however, it is present on both T and B-cells (Altevogt *et al.* 1989). It is involved in adhesion to LFA-3 molecules (reviewed in Springer 1990) present on the surface of a variety of other cells as well as in the T cell activation pathway (Meuer *et al.* 1984; Shaw *et al.* 1986; Hunig *et al.* 1987). Sequences responsible for the T cell specific, position independent, copy number dependent expression of the human CD2 gene in transgenic mice were found within the 3' flanking region of

the gene (Figure 2). The LCR is located within a 5.5 kb DNA fragment which is also hypersensitive to DNase I digestion (Greaves *et al.* 1989). Within the human CD2 locus, one hypersensitive site is positioned immediately 5' of the first exon within the promoter of the gene. Two further sites are found approximately 0.5 kb and 1.0 to 1.5 kb of the polyadenylation signal of the gene. The 3' CD2 element can direct expression of heterologous genes, such as β-globin, in T cells, utilising the β-globin promoter in a copy-related and position-independent manner. In addition, the CD2 LCR is capable of exerting this effect, regardless of its orientation with respect to the promoter of the gene (Greaves *et al.* 1989). Deletion analysis of the human CD2 gene LCR, performed with the aim of establishing a minimum sequence necessary for copy-dependent transgene expression, revealed that 1.5 kb of flanking sequences immediately 3' to the polyadenylation signal of the gene are sufficient for the LCR effect (Lang *et al.* 1991). Clusters of possible binding sites for TCF1α and GATA transcription factors were found in regions where hypersensitive sites have been mapped. Recently, three clusters of sites were identified within 2 kb of the 3' flanking region (HS 1-3) (Festenstein *et al.* 1996). The upstream cluster (HS1) of strong hypersensitive sites coincides with the region that functions as a classical enhancer, whereas the weak HS2 and HS3 have no enhancer activity in transient transfection assay (Lake *et al.* 1990) but their exclusion or partial deletion from the constructs will cause a mosaic expression pattern when the transgene integrates into a heterochromatic centromeric region (Festenstein *et al.* 1996). This position effect variegation (PEV) has also been described for the human β-globin LCR (Milot *et al.* 1996).

A human CD2 minigene cassette was designed with the aim of directing expression of the gene of interest to all T cells and thymocytes of transgenic mice (Zhumabekov *et al.* 1995). This expression cassette includes 5 kb 5' and 5.5 kb 3' flanking sequences containing respectively the promoter and LCR of the human CD2 gene. The cassette also provides the transcription initiation site, the first intron of human CD2 gene and the two polyadenylation signals found in the 3' untranslated region of the human CD2 gene.

In chapter 2 of this thesis, the CD2 LCR was used to drive expression of a bacterial suicidal gene in the thymus of transgenic mice to test the killing specificity of the new NTR/CB1954 prodrug system.

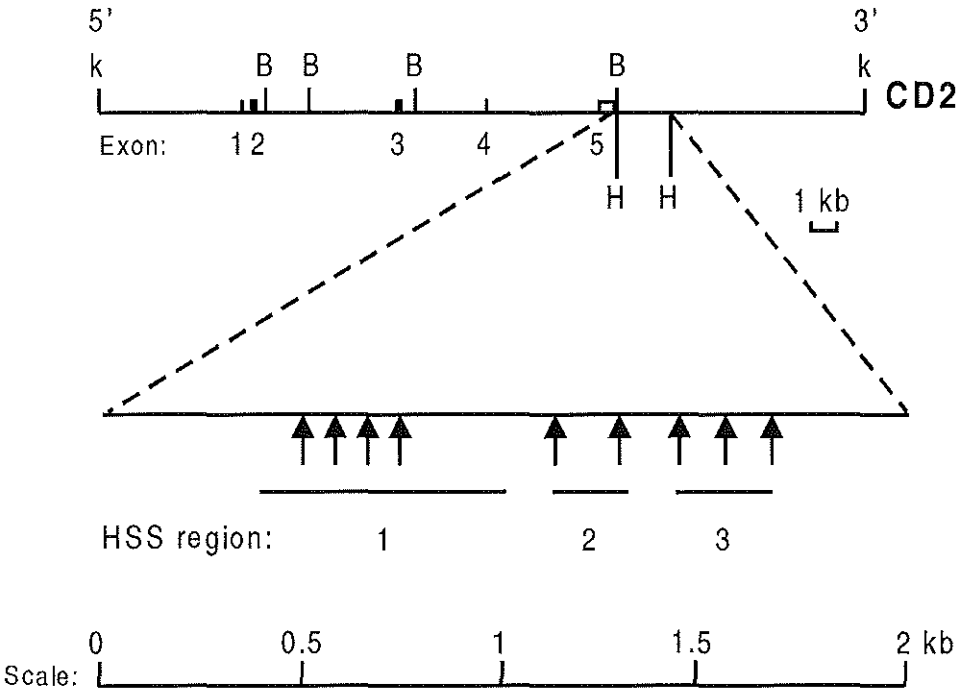

Figure 2. Human CD2 locus.
The 2-kb Hind III fragment with full LCR function is indicated. Arrows refer to DNase I hypersensitive sites arranged in three regions.

Since LCR-like elements seem to be a general phenomenon, a substantial amount of current research focuses on the isolation of the LCR elements associated with particular genes. The first step is to achieve regulated, position-insensitive expression of the gene of interest in transgenic mice using the largest piece of DNA available (usually by injection of yeast artificial chromosomes (YACs)). The second step would be to test a number of smaller constructs to locate the sequence(s) that mediate this expression. It is essential that the properties of any construct are tested in a transgenic assay, since transformed cells in culture can give

misleading results. The final aim would be to bring these sequences together in a construct that is small enough to fit into the chosen delivery vector. The control sequences for a given gene may prove to be incompatible with the particular vector. For example, efforts by several groups to incorporate LCR subfragments into β-globin retroviral vectors, have resulted in vector rearrangements, poor titers, or very low expression of the β-globin gene (Novak *et al.* 1990; Chang *et al.* 1992; Plavec *et al.* 1993; Sadelain *et al.* 1995). A similar problem was reported with a 2.1 kb CD2 LCR fragment incorporated into a retroviral vector with the human adenosine deaminase (ADA) cDNA driven by the retroviral (LTR) promoter (Kaptein *et al.* 1998). However, the idea behind the search for LCRs is that the gene will be expressed under its own control sequences. In the following chapters it will be shown that this does not have to be the case if control elements from another gene can give adequate levels of expression in the appropriate tissues. A cDNA or genomic DNA fragment can be placed under the control of a promoter and LCR elements from a heterologous gene.

C. Targets for gene therapy

1. Inherited diseases

One of the earliest disease-related genes to be cloned and characterised was the gene for human beta globin (Maniatis *et al.* 1976), an obvious target for gene transfer and gene therapy studies because of its relevance to human diseases (β-thalassaemias and sickle cell anaemia). In 1980, Cline and colleagues at UCLA reported that they could introduce the human globin gene into murine bone marrow (BM) cells by calcium phosphate transfection and, at least partially, repopulate the marrow of irradiated recipient mice with genetically modified BM cells (Cline *et al.* 1980; Marcola *et al.* 1980). The known inefficiency of the transfection procedure, absence of the regulatory sequences from the construct used, and the rarity of BM stem cells made these results rather surprising. Nevertheless, Cline and his colleagues proceeded with a, scientifically and in many other

aspects, premature gene therapy experiment in humans, which was predestined to be a failure.

Very soon it became clear that diseases of haemoglobin synthesis were in fact going to be particularly difficult to correct by gene therapy. Haemoglobin is composed of four polypeptide chains (2α and 2β encoded by genes located on two different chromosomes) that have to be present in the majority of the red blood cells in the appropriate ammounts for gene therapy to succeed.

Genetic diseases caused by single gene defects that are completely corrected by bone marrow transplantation are the leading initial candidates for bone marrow gene therapy. If transplantation with allogeneic bone marrow is able to correct all of the manifestations of a disease, then correcting the genetic defect in the patient's own bone marrow by gene transfer ought to cure the disorder as well. For gene therapy, it seemed wiser to focus initially on a genetic disease in which corrected cells might have a selective growth advantage in patient (Anderson 1984). Therefore, adenosine deaminase (ADA) deficiency as a cause of severe combined immunodeficiency (SCID) was a good candidate. ADA is a single protein chain enzyme in the purine metabolic salvage pathway that catalyses the conversion of adenosine to inosine and deoxyadenosine to deoxyinosine. Toxic metabolites that accumulate particularly affect T cells. Following extensive regulatory review of the proposed protocol to use cultured gene–modified cells to treat ADA-deficiency, the first authorised gene therapy in man began on September 14th, 1990, when a 4 year old girl with ADA deficiency began receiving transfusions of ADA gene-corrected autologous T cells. The number of clinical trials has grown at an increasing rate since then.

Ideally, preclinical studies of any potential therapy should involve both the development of suitable protocols for carrying out the key technical steps and a assessment of the therapeutic efficacy and safety in an appropriate model of the disease.

The first gene therapy models, based on genetic augmentation through grafting of *ex vivo* modified cells, have been developed for single gene disorders such as ADA deficiency (Hoogerbrugge *et al.* 1992), familial hypercholesterolaemia (Grossman *et al.* 1994), α1-antitrypsin deficiency (Ledley and Woo 1989; Rosenfeld *et al.* 1991; Kay *et al.* 1992), coagulation disorders caused by deficiencies of clotting factors IX and VIII (Axelrod *et al.* 1990, Roman *et al.* 1993 Yao *et al.* 1991), Gaucher disease (Nimgaonkar *et al.* 1994) and others.

Chapters 3 and 4 of this thesis will focus on mouse models for X-linked agammaglobulinaemia and Gaucher disease respectively and the potential use of the locus control region (LCR) of the class II MHC Eα gene in therapeutic constructs which could be used for these two disorders.

The early approaches to what might be called "classical" gene therapy were aimed at inborn enzyme defects associated with metabolic diseases. Very soon, the possibilities for gene therapy application to acquired diseases were also investigated.

2. Acquired diseases

A critical issue in considering any acquired disease as a candidate for gene therapy is the choice of the gene to be transferred. Nevertheless, there are many diseases for which gene therapy strategies are developing. These includes infectious diseases such as AIDS, cardiovascular diseases (atherosclerosis, myocardial infarct, vascular restenosis and ischaemia), neurodegenerative diseases (Alzheimer's disease, Parkinson's disease), joint disorders such as rheumatoid arthritis and various approaches to cancer therapy.

Gene therapy for HIV infection is receiving particularly intensive study. Approaches that are in development include both immunotherapy (e.g. therapeutic vaccines and adoptive transfer of $CD8^+$ T-cell clones) and direct antiviral therapy (intracellular immunisation) (reviewed in Yu et al. 1995). The later strategies include transdominant modification of HIV proteins, RNA decoys, antisense RNA, ribozymes and soluble CD4. These strategies would work by transdominant inhibition of REV or TAT proteins by "molecular traps" that bind and inactivate the REV or TAT proteins or by competitive inhibition of the binding of those proteins to their recognition elements (Sullenger *et al.* 1991; Yu *et al.* 1994; Lori *et al.* 1994; Frasier *et al.* 1998). Several of these strategies are now entering clinical trials.

Cardiovascular diseases, the prime cause of death in industrialised countries, are also target diseases for gene therapy. Strategies include: transfer of the gene encoding the low density lipoprotein (LDL) receptor to lower blood-cholesterol levels and thus decrease the likelihood of plaque formation in blood vessels, delivery of the tissue plasminogen activator to prevent blood-clot formation on cardiac stents, catheter-based delivery

systems for introducing gene sequences to blood vessel walls and others (reviewed in Dzau *et al.* 1993).

Despite many difficulties, a number of neurological disorders have emerged as promising gene therapy models (reviewed in Zlokovic *et al.* 1997). This advance has been due, in part, to the realisation that gene therapy for certain disorders may not require the use of brain cells. Instead, "surrogate cells" such as autologous fibroblasts, may be useful for delivering therapeutic gene products after genetic manipulation and transplantation to the brain (Gage *et al.* 1987). Studies using animal models for Parkinson's disease have demonstrated that intrastrial grafting of fibroblasts genetically modified to produce L-dopa may reverse the disease phenotype (Wolf *et al.* 1989). More recently, L-dopa-producing primary astrocytes were generated by retroviral transduction (Lundberg *et al.* 1996). Neurotrophic factors that improve the survival of specific neuronal types during development and after exposure to various neuronal insults have also potential for treatment of neurodegenerative diseases. In particular, brain-derived neurotrophic factor (BDNF) has been shown to exert trophic and protective effects on dopaminergic neurons (Yoshimoto *et al.* 1995).

Cancer

Nearly half of all cancers remain non-responsive to current therapeutic regimens. The progression of disease is often a result of growth of tumour cells that are resistant to any combination of surgery, radiation therapy and chemotherapy (Goldstein *et al.* 1991; Goldstein *et al.* 1992). Cytogenetic and molecular techniques have allowed the identification of genetic alterations that underline progression into malignancy. Attempts have been made to exploit this knowledge to develop new therapeutic strategies for the treatment of cancer patients. These include genetic marking, inhibition of oncogene expression, restoration of tumour suppressor genes, cancer vaccination, suppression of tumour angiogenesis and the use of suicide genes.

The identification and sequencing of oncogenes has led to the development of antisense strategies. The aim is to suppress the expression of specific oncogenes. Antisense strategies utilise short sequences of RNA, complementary to oncogene transcripts, that will bind to oncogene mRNA and thereby inhibit mRNA translation. In several in *vitro* models, this has resulted in the inhibition of proliferation and reversal of the malignant phenotype (Trojan *et al.* 1992; Zhang *et al.* 1993).

Mutation of tumour suppressor genes also contributes to the development of malignancy. Mutation of the p53 tumour suppressor gene is the most common alteration in human malignancies (Hollstein *et al.* 1991; Greenblatt *et al.* 1994). *In vitro,* the transduction of wild-type p53 has been shown to arrest the growth of the colorectal carcinoma cell line (Baker *et al.* 1990) and *in vivo* to prevent the growth of established tumours leading to their regression (Shaw *et al.* 1992). However, there are a number of problems associated with these therapies. The most obvious is that it is necessary to modify each individual malignant cell within the tumour to achieve a therapeutic effect. This is unrealistic given the low efficiency of current methods of gene delivery.

An alternative approach involves strategies designed to enhance an antitumour immune response. Although cytotoxic lymphocytes that have the capability for the recognition and killing of tumour cells can be detected in the peripheral blood of cancer patients, these cells are not sufficiently active to control the growth of the tumour cells. This state of immunoparalysis in cancer patients has been attributed to defects in the presentation of tumour antigens by the tumour cells and hence therapy is directed towards the *in vitro* introduction and expression of cytokine genes (IL2, IL4, IL7, IL12, IFN, TNF) (Hock *et al.* 1993; Belldegrun *et al.*1993; Golumbek *et al.* 1991; Gansbacher *et al.* 1990; Hock *et al.* 1991; Chong *et al.* 1998), class I HLA genes (Nabel *et al.* 1993; Nabel *et al.* 1994) or costimulatory antigens e.g. HLA-B7 (Townsend and Allison 1993; Chong *et al.* 1998) into the immune cells of the host or into the tumour cells.

In the knowledge that solid tumours cannot progress without the generation of new blood vessels, recent efforts have also been focused on understanding the biology of angiogenesis, the identification of mediators of angiogenesis and the evaluation of strategies that employ antiangiogenesis agents to inhibit the growth of tumours (Kong and Crystal 1998). An ideal antiangiogenesis strategy should be targeted only to the organs that contain tumour(s) and should not interfere with normal angiogenesis.

The remaining approaches to cancer therapy involve the insertion of genes conferring either drug resistance or drug sensitivity. Conventional chemotherapeutic regimes are often limited by systemic toxicity and myelosuppression. The production of cytotoxic-drug resistant stem cells may be a mechanism by which normal cells could be protected from treatment-related toxicity, allowing much higher doses to be used. The proposed transgenes can render stem cells resistant to some specific drugs

(e.g. methotrexate, by use of dihidrofolate reductase (DHFR) (Corey *et al.* 1990)) or make them multidrug resistant (MDR) by the expression of the MDR1 gene (Gottesman *et al.* 1994; Devereux *et al.* 1998).

Suicide gene therapy in cancer and the role of the bystander effect

Tumour cells can be modified in such a way that they become sensitive to an agent that is otherwise non-toxic. This is achieved by insertion of a gene coding for an enzyme that converts a non-toxic prodrug into a lethal compound. Administration of the prodrug results in the death of the recipient cell. This approach is known as suicide gene therapy. It is an attractive form of therapy against solid tumours, since administration of the prodrug results not only in the death of recipient cells (that express the suicide gene) but also in the death of surrounding cells. This phenomenon of the death of neighbouring untransfected cells is known as the "bystander effect". A number of theories have been proposed to explain how bystander killing occurs. Cell-cell contact appears to be required *in vitro* (Colombo *et al.* 1995; Bi *et al.* 1993). Metabolic co-operation and the release of apoptotic vesicles are both implicated in the mediation of the bystander effect. The relative contribution of these two processes is probably dependent on whether or not the cells possess gap junctions. However, there is increasing evidence to suggest that the immune system plays an important role *in vivo*. A foreign gene product can inhibit tumour formation even in the absence of selection (Tapscott *et al.* 1994). It seems that an intact immune system is required for an *in vivo* bystander effect to be seen (Ramesh *et al.* 1996). Nude mice and sub-lethally irradiated mice failed to show the regression of subcutaneous tumours when the tumours were composed of 50 % HSV-1 TK positive cells. In contrast, immunocompetent mice showed tumour rejection upon receiving the same number and proportion of HSV-1 TK positive cells. It appears that the death of positive cells following prodrug administration is associated with the generation of an immunostimulatory intratumoral cytokine cascade (TNFα, IL1, IL6) and inflammatory cell recruitment (Ramesh *et al.* 1996).

A number of prodrug or suicide genes are under investigation to confer drug sensitivity to cancer cells. Some of these are listed below (Table 2).

Table 2. Suicide Genes

Gene	Toxic metabolism	Tumourocidal	Bystander	References
HSV-TK	GCV→GCV-TP	yes	yes	21, 43, 49, 68, 79
CD	5-FC→5FU	yes	yes	108, 166
XGPRT (gpt)	6-TX→6-TX-TP	yes	?	18
VZV-TK	AraM→AraM-MP	yes	?	108
DeoD	MeP-dr→6-MeP	yes	yes	163
β-glucosidase	Amygdalin-cyanide	?	?	58
β-lactamase	Vinca-ceph. → vinca alk.	?	?	58
NTR	CB1954 → hydroxylamino forms	yes	yes	28

The most well known example of gene directed enzyme prodrug therapy (GDEPT) is the Herpes Simplex virus type 1 thymidine kinase gene (HSV1-TK)/ganciclovir (GCV) system (Culver *et al.* 1992). The viral enzyme converts GCV, which is not toxic to unmodified cells because of the low specificity of human thymidine kinase for GCV, to a number of toxic metabolites of which GCV triphosphate is thought to be the most potent. GCV triphosphate inhibits DNA polymerase; thus GCV is only toxic to cells in S-phase (Furman *et al.* 1980; Elion 1983). Retroviral vectors have been used to deliver TK to intracranial and leptomeningeal tumours in the first human GDEPT trials (reviewed in Hanania *et al.* 1995). Another system, also based on the production of a toxic nucleotide analogue, involves delivery of the cytosine deaminase gene that converts 5-fluorocytosine to the toxic 5-fluorouracil (Mullen *et al.* 1992; Huber *et al.* 1993).

Recently, a novel enzyme prodrug activating system has been described (Bridgewater *et al.* 1995), which involves the use of the *E.coli* nitroreductase (NTR) gene. CB1954, a weak monofunctional alkylating agent, is a relatively non-toxic prodrug which is activated by the

nitroreductase enzyme to form a highly toxic bifunctional alkylating agent. In contrast to the HSV1-TK/GCV suicide gene system, activated CB1954 will kill both dividing and non-dividing cells. This may be advantageous when considering the treatment of human tumours where only a relatively small proportion of cells may be actively dividing at any particular time. Chapter 2 of this thesis will focus on specific killing of cells expressing nitroreductase, both *in vitro* and *in vivo*. The expression of the bacterial enzyme was limited to specific cellular targets by placing the gene under the control of tissue-specific elements. Use of the human CD2 locus control region restricted nitroreductase expression to T cells. Upon CB1954 treatment, the targeted destruction of particular cell types allows ablation of selected tissues. Thus the CB1954 /NTR GDEPT system offers a potent means of killing targeted cell types. Given the different modes of action of CB1954/NTR and HSVTK/GCV treatments, it could be that a combination of these two approaches offers a means of obtaining additive, and potentially synergistic effects.

D. Animal models for human diseases and their relevance to somatic gene therapy

It has become obvious that *in vitro* studies, however important and useful in the beginning, have a restricted role, since diseases for which a cure is sought usually affect not only an isolated tissue or organ but very often, due to a complex cascade of events involved in pathogenesis, affect the whole organism. Many important questions can only be addressed *in vivo* and therefore animal models are needed. However, question remains whether an animal model can adequately mimic the disease in humans and provide a valid test for somatic gene therapy. Patient trials will always be the only true test of therapeutic efficacy while relevant animal models will serve to speed up safe and effective progress towards this goal.

Although naturally occurring mutations observed in several species (mice, rats, dogs, etc.) have resulted in animal models for different inherited human diseases, many more have been recently generated in laboratories by gene targeting technology (Hogan *et al.* 1994). In these cases mouse models play a particilarly important role since they are inexpensive to maintain, quick to breed and easy to handle. Comparative genetic mapping reveals a striking degree of gene and linkage group conservation between human and mouse. Provided the gene suspected as

responsible for disease has been identified and cloned, mouse models can be generated by gene targeting in embryonic stem (ES) and /or classical transgenesis. ES cells, derived from the inner cell mass of 3.5 day pre-implantation embryos, have the remarkable capacity to be propagated, manipulated and mutated in culture, while still retaining the ability to contribute to all of the somatic and, critically, the germ line tissues after re-implantation into a host blastocyst.

Most techniques that permanently introduce a DNA fragment into cells result in integration of multiple copies of the transgenes at one or a few random chromosomal locations (reviewed in Bishop 1996). Comparisons between such cell culture transfectants or transgenic mouse lines are difficult because the level of expression of the transgene depends on the number of integrated copies and the locus of integration (when appropriate LCR elements are not used). The ultimate goal for any gene therapy is integration of a single copy of a transgene, possibly at a predetermined chromosomal locus.

Site-specific recombinases and their role in creating genome alterations

Site-specific recombinases are being developed as tools for genetic engineering because of their simplicity and precise activity in a variety of organisms (reviewed in Kilby *et al.* 1993). Two well studied recombinases are Cre, from bacteriophage P1, and Flp, from *Saccharomyces cerevisiae*. Both of them have been shown to catalyse excision, integration, inversion or translocation of DNA between their distinct recognition target sites without requiring cofactors. The target sites, loxP (for Cre) and Flp recombinase target (Frt), both have a characteristic structure. They consist of a 34 bp sequence containing 13 bp inverted repeats separated by an 8 bp spacer region. Cre is optimally efficient at 37°C, while Flp has an optimum near 30°C (Buchholz *et al.* 1996) and has been generally reported to be less efficient than Cre in mammalian cells (reviewed in Rossant and McMahon 1999). A mutant, thermostable form of Flp, Flp-e, promises to overcome this problem and to enhance utility of the Flp system (Buchholz *et al.* 1998).

In the case of intramolecular recombination, DNA flanked by two directly orientated sites is efficiently excised leaving a single target site in the genome. If targets are in opposite orientation the segment is inverted. Recombinase activity also has the potential for insertion of DNA into target sites in the genome. When Cre catalyses the recombination between a

single loxP site in the genome and another site in an introduced circular plasmid, it promotes insertion of the plasmid into the chromosome, both in yeast and in cultured mouse cells (Sauer and Henderson 1990). However, this event is inefficient because the newly inserted sequence is now surrounded by target sites and subject to excision again. Recently, a novel strategy termed Recombinase-Mediated Cassette Exchange (RMCE) has been developed. The method takes advantage of loxP or Frt sites mutated in their spacer regions. Such mutated target sites can only recombine with themselves and not with the wild type sites or sites bearing different mutations (Bouhassira *et al.* 1997; Schlake and Bode 1994; Seibler and Bode 1997). Site-directed DNA integration has also been achieved when nucleotide changes were introduced into the 13 bp elements in plants (Albert *et al.* 1995) and in mouse ES cells (Araki *et al.* 1997).

To test gene therapy constructs which would be present in the host genome as a single copy, it is necessary to compare the expression level between single copy transgenic mice or single copy clones in tissue culture experiments (Ellis *et al.* 1997; Bouhassira *et al.* 1997). It has become common practice to introduce a lox P site into DNA constructs that are microinjected to obtain transgenic mice. Multiple copy transgenic lines could be reduced in their copy number by using the Cre recombinase system in a number of ways. The expression of Cre could be either transiently achieved in fertilised eggs of the multiple copy lines, or stably expressed in a different transgenic line (in a germ line cells). In the latter case, crossing of the two lines is needed for the recombination event to take place.

The transient expression of Cre in fertilised eggs was achieved by pronuclear injection of a Cre expression vector in a circular form to avoid genomic integration (Araki *et al.* 1995). The recombination of the transgene was detected by galactosidase expression after excision of a chloramphenicol acetyltransferase (CAT) gene. Recombination was completed before the morula stage. Although no mosaicism, no incomplete recombination and no integration of the Cre sequence were observed in 18 mice born with this modified transgene (Araki *et al.* 1995), we have not been able to achieve the reported 100% efficiency using the very same system. As there is still a possibility that plasmid sequences may integrate into the mouse genome and of mosaicism if the recombination event does not take place as soon as possible, we have tested the injection of Cre RNA as an alternative (Chapter 5 of this thesis).

Different transgenic lines expressing Cre have been generated with the aim of efficient *in vivo* manipulation of mouse genomic sequences at the zygote stage. Among these transgenic lines are Cre (deleter), where Cre is under the control of CMV minimal promoter (Schwenk *et al.* 1995), E II a-Cre , where Cre is under the control of adenovirus E II a promoter (Lakso *et al.* 1996), CAG-Cre, where Cre is under control of the cytomegalovirus immediate early enhancer-chicken β-actin hybrid (CAG) promoter (Sakai and Miyazaki 1997), Zp3 Cre, where Cre is under the control of murine zona pellucida 3 gene promoter and LCR (Lewandoski *et al.* 1996) and Sycp1-Cre, where Cre is driven by promoter sequences derived from gene encoding Synaptonemal Complex Protein 1 (Vidal *et al.* 1998). In the Zp3 Cre transgenic line, Cre is expressed specifically and exclusively in the growing oocytes prior the completion of the first meiotic division, following the expression pattern of the ZP3 gene (Kinloch *et al.* 1993). If a ZP3-Cre positive female is mated with the target (loxP) bearing transgenic male, in some cases sufficient Cre activity remains in mature oocytes to mediate the recombination of the paternally contributed target gene. However, in a target bearing ZP3-Cre female mouse, Cre-mediated recombination of the target gene apparently occurs in 100% of oocytes. When Cre^+/$target^+$ females are mated to wild type males, all target-bearing offspring, regardless of carrying the Zp3-Cre gene, undergo recombination as a consequence of Cre presence in growing oocytes (Lewandoski *et al.* 1996). The weak point of this method is that it needs 2 generations for the excision (recombination) to take place. The same applies to the Sycp 1-Cre transgenic line, where Cre is expressed at an early stage of male meiosis (leptotene to zygotene). Mice born after mating the double transgenic males with a wild type females showed extensive deletions of the loxP flanked sequences (Vidal *et al.* 1998). The situation with other Cre expressing lines is similarly time consuming, because the resulting F1 mice with the recombined allele of the target gene are inevitably transgenic also for the Cre gene (Schwenk *et al.* 1995; Lakso *et al.* 1996). Thus, it would be desirable to remove the Cre transgene during the subsequent crosses. The only exception is the CAG-Cre line, where Cre mRNA and /or protein are retained in mature oocytes irrespective of the transmission of the CAG-Cre transgene, resulting in the efficient Cre-mediated recombination of paternally derived target genes upon fertilisation (Sakai and Miyazaki 1997).

Interpretations of knock out phenotypes using current gene targeting technology are often limited. The presence of a selection marker may

influence the phenotype of the mutation (Fiering *et al.* 1995; Olson *et al.* 1996). The inactivation of a gene may result in intra-utero lethality, precluding analysis of the possible function(s) of the gene at later stages of development and post-natally. A conditional gene targeting method based on recombinase activity could overcome these limitations by allowing the removal of the selection cassette and the tissue specific and timed inactivation of target genes. Placing Cre under the control of either a cell-specific (Gu *et al.* 1994; Tsien *et al.* 1996; Barlow *et al.* 1997) or inducible (Kühn *et al.* 1995; Brocard *et al.* 1997) promoter can lead, through the excision of the floxed DNA segment, to spatially, temporally or spatio-temporally controlled somatic mutation. The latter system would allow generation of somatic mutation in a defined gene at a given time during the life of the animal and in a specific cell type. The most commonly used inducible system utilises the ligand-binding properties of the steroid hormone receptors. A fusion protein between the ligand binding domain of a steroid receptor and Cre recombinase is inactive except in the presence of the ligand, most likely because the ligand-binding domain is complexed with HSP90 proteins in the cytoplasm, preventing Cre from reaching its nuclear targets. The ligand binding domains used in transgenic animals have been engineered to bind synthetic ligands that do not activate the normal steroid hormone responses. Cre fusion with an oestrogen receptor mutant that binds tamoxifen (Ert) only (Feil *et al.* 1996; Brocard *et al.* 1997) and a progesterone receptor that binds RU486 only (Kellendonk *et al.* 1996) have been developed and tested in mice. Successful inducible excision with Cre-ERt fusion was reported in the epidermis cells within a few days of tamoxifen treatment of mice transgenic for both Cre-ERt and a target construct with floxed DNA (Brocard *et al.* 1997). There are other inducible systems available, such as the interferon inducible system and the tetracycline based system. The problems with these systems are often mosaic induction, background leakiness (particularly with the reverse transactivator tet system), no detectable expression of the transactivator, inefficient processing of the bacterial tetR gene in mammalian cells and others.

The Cre-loxP system can also be used to design a variety of chromosomal translocations in mouse ES cells. Recombination between lox P sites located on different chromosomes can occur, although as a rare event, since the chance of synapsis between sites is reduced as a result of their decreased proximity and because of possible physical constrains on the interaction of DNA sequences from different chromosomes as a

consequence of nuclear architecture. However, it was shown that using an appropriate orientation of loxP sites integrated into different non-homologous chromosomes, after transient expression of the recombinase, cells which have undergone a balanced chromosomal translocation event can be recovered by genetic selection. A programmed translocation between c-myc and immunoglobulin heavy chain genes on chromosome 15 and 12 was created by this method (Smith *et al.* 1995). Genetic selection was based on a reconstruction of a human minigene encoding hypoxanthine phosphoribosyltransferase (HPRT) in a HPRT deficient ES cell line. This technology should also allow the creation of mouse models for those chromosome rearrangements associated with the human cancers and developmental abnormalities.

In conclusion, the combined strategies of gene targeting and transgenesis with the use of recombinase systems will greatly extend the range of designed modifications that can be made to the mammalian genome.

Level of expression required for successful gene therapy

For future correction of genetic disease by gene therapy, it is important to have some idea about the minimum level of expression that will provide significant therapeutic benefit. The ideal outcome would be to achieve the expression levels that match those of the normal gene in question. In many diseases involving metabolic enzymes, a lower level of expression will be sufficient. For example, many individuals have been identified who have only 10-20 % of the normal levels of adenosine deaminase (ADA) enzyme and yet are immunologically normal (reviewed in Baese and Culver 1991). It should be remembered that overexpression can also present a problem. For example, higher levels (2-3 times) than normal of human β globin in transgenic mice, caused by multiple copies of the transgene, result in destruction of red blood cells and lethal anaemia (Grosveld *et al.* 1987). Obtaining an accurate assessment of the levels of expression required is likely to be a difficult exercise. Expression in some cases must be targeted to the appropriate cell type and the level has to be related accurately to the phenotype. Thus choices for regulation must be made not only at the level of transcript but also translation, cellular localisation, developmental timing, etc. The above mentioned mouse (knock out) models for human genetic diseases are likely to be of particular value in addressing these

problems through so-called "rescue" experiments. Transgenic technology can be used to reintroduce a normal gene into these animals and temporal, spatial and quantitative level of expression can then be measured. However, such quantification will need to be carried out with great care. I will now focus on two particular inherited disorders (X-linked agammaglobulinemia and Gaucher disease) and related mouse models. These examples will illustrate the complexity of the problems involved in obtaining useful animal model and suggest that the phenotypes observed (at least in these mouse models) do not necessarily provide an accurate picture of phenotypes resulting from the same mutation in humans. Thus, animal models must be carefully characterised and used with cautious consideration in assessing human gene therapy strategies.

X-linked agammaglobulinaemia (XLA) and related mouse models

X-linked agammaglobulinaemia (XLA) is an inherited X chromosome-linked humoral immunodeficiency disease (Bruton 1952) with an incidence of $5:10^6$ (reviewed in Sideras and Smith 1995). Affected males have a severe deficiency of mature B cells and circulating immunoglobulins of all isotypes and consequently suffer from recurrent infections. They are mainly pyogenic bacterial infections of the respiratory and genito-urinary tracts and skin, but also viral infections (caused by enteroviruses such as Echoviruses) and protozoal infections. Due to treatment with antibiotics and gammaglobulins patients can reach adulthood. The gene defective in XLA encodes a cytoplasmatic protein tyrosine kinase (Vetrie *et al.* 1993; Tsukada *et al.* 1993), designated Btk (for Bruton's agammaglobulinaemia tyrosine kinase). XLA patients carry mutations in the Btk gene that include deletions, insertions and point mutations, leading to amino acid substitutions or premature stop codons in virtually all of the Btk domains. There is a high degree of clinical heterogeneity among XLA patients. There are more than 200 mutations involved in individual cases, but there appears to be little (if any) correlation between the type of mutation present (genotype) and the severity of the disease (phenotype)(Vihinen *et al.* 1998). However, most of the patients show the typical severe XLA phenotype outlined above.

The mouse Btk sequence was found to be highly homologous to that of humans (99.3% conservation at the amino acid level). The coding regions

are divided into 19 exons covering approximately 43.5 kb in the mouse, and 37.5 kb in the human genome. With the exception of the exons encoding for the 5'-UTR and 3'-UTR, all the other exons are identical in length in both mouse and human genes (reviewed in Sideras and Smith 1995). Btk is expressed throughout B cell development (Figure 1), from the earliest pro-B cell stage to mature B cells, except in terminally differentiated plasma cells (de Weers *et al.* 1993; Smith *et al.* 1994). It is also expressed in myeloid and erythroid cells, but it is not required for their differentiation and maturation (reviewed in Sideras and Smith 1995).

The murine *xid* (X-linked immunodeficiency) mutation in the mouse strain CBA/N served as a model for mild X-linked B-cell intrinsic immunodeficiency for over 25 years (Amsbaugh *et al.* 1972; reviewed in Scher 1982). It involves a single amino acid substitution of a highly conserved residue (Arginine 28→Cysteine) in the pleckstrin homology (PH) domain of the Btk gene (Thomas *et al.*1993; Rawlings *et al.* 1993). In the *xid* defect, the overall number of peripheral B cells is usually 50-60% of normal, the mature B cell population in the spleen is severely reduced, $CD5^+$ B cells are not detected, levels of serum IgM and IgG_3 are low, and responses to T independent type II (TI-II) antigens are impaired (reviewed in Sideras and Smith 1995).

The milder phenotype of murine *xid* compared with human XLA cannot be explained by the nature of the mutation involved. Mutation of the same Arg 28 amino acid into histidine has been observed in patients with a classic severe XLA phenotype (de Weers *et al.* 1994). Furthermore, Btk knock out mice, generated by two different gene targeting strategies, both showed a total absence of Btk protein, but resulted only in mild *xid* phenotype (Khan *et al.* 1995; Hendriks *et al.* 1996). In conclusion, the elimination of Btk function in mice does not lead to the severe block in B cell development which is characteristic for XLA in man (Table 3). However, the Btk knock out mouse models have clearly identified the precise stage(s) in B cell development at which deficiency in Btk results in a selective disadvantage. From the analysis of *in vivo* competition between cells expressing wild type Btk and those expressing Btk^-/LacZ allele in heterozygous females, there are clear indications that development up to the large pre-B cell (fraction D) is not affected by the Btk mutation and that Btk is essential at two distinct steps, ie. at the transition from small pre B-cells to immature B cells in the bone marrow, and during the antigen-driven maturation of selected B cells in the periphery (Hendriks *et al.* 1996). Indeed, as will be described in chapter 3 of this thesis, Btk^-

phenotype can be corrected by transgenic expression of Btk (in this case human) from the pre-B cell stage onwards, which might have an important implications for the design of gene therapy strategies for XLA.

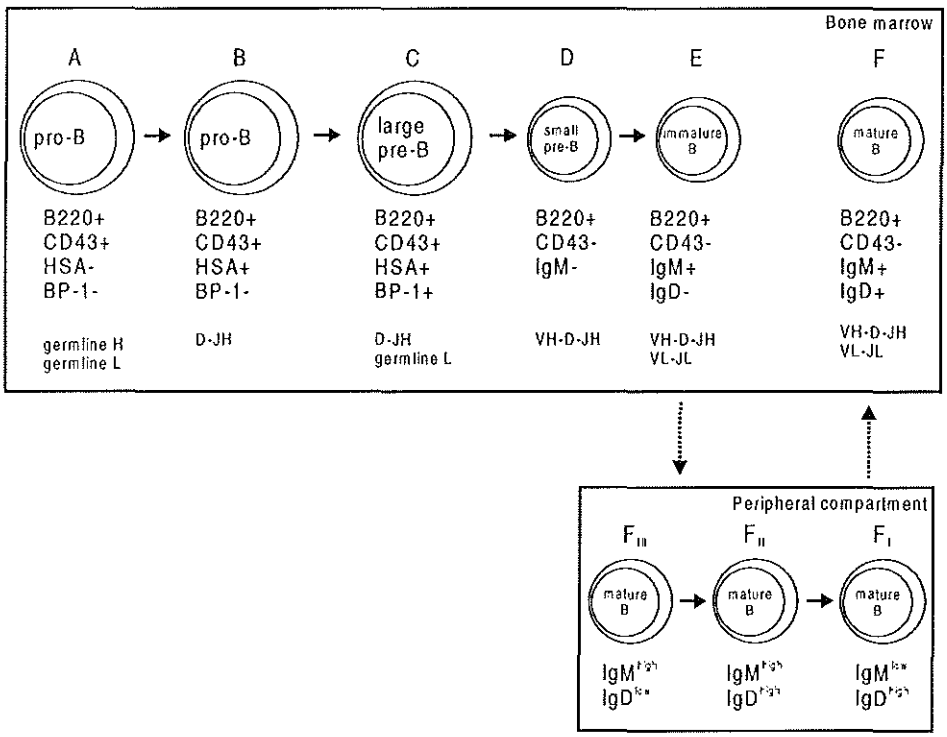

Figure 3. B cell differentiation in the mouse.
The classification of B cell lineage cells into bone marrow fractions A-F is according to the expression of cell surface molecules (Hardy *et al.* 1991) and the status of the Ig loci (Ehlich *et al.* 1993). The three mature B cell populations (FIII, FII, FI) are distinguished on the basis of their differential expression of IgM and IgD. The dotted arrows indicate that fraction F in the bone marrow reflects recirculating cells that have been selected in the periphery.
Courtesy of R. Hendriks (published in Hendriks *et al.* 1996)

Table 3. Parallels and differences between human XLA and murine *xid*

Studied features	**XLA**	*xid*
Peripheral B lymphocytes	Absent or low	Reduced
Ig response to proteins	Almost completely absent	Present
Immunoglobulin levels	All isotypes absent or low	Only IgM and IgG_3 low
Block in pre- to B transition	Almost complete	Detectable
Peripheral B lymphocyte phenotype	IgM^{high}	
Rearrangement of Ig genes	Normal	
Proportion of Ig λL chain usage	Decreased	
Ig response to T cell dependent Ag (e.g. ø174)	Present	
Stimulation with α CD40	Induces proliferation and CD23 expression	
Phenotype of heterozygous female	Normal	

References: Scher 1982; Hendriks *et al.* 1996; Nonoyama *et al.* 1998; Maas 1998.

Gaucher disease and related mouse models

Gaucher disease is the most prevalent lysosomal storage disorder in humans with an incidence of $1 : 5 \times 10^{4}$ in the general population. It results from an autosomally inherited deficiency of the enzyme β-glucocerebrosidase (GC), which is involved in sphingolipid glucocerebroside metabolism. As a consequence, the GC substrate, glucosylceramide, accumulates in cells, mainly in macrophages of the mononuclear phagocyte system. More than 50 different mutations have been found throughout the gene encoding GC, including missense, splice site, frame shift and deletion mutations (reviewed in Beutler 1993; Horowitz and Zimran 1994). Although there are cases of genotype-phenotype correlations (Zimran et al 1989; Beutler and Grabowski 1995), it is difficult to predict clinical presentation and course of disease from the genotype due to phenotypic variability that exists among patients with the same genotype and vice versa. The phenotype is heterogeneous, ranging from asymptomatic or mildly affected individuals (mainly adults), to those (mainly children) who die after a severe clinical course. The disease has been classified into three clinical types. Patients without a neurological involvement are classified as type 1, whereas patients with a neurological involvement are classified as type 2 (acute) or type 3 (subacute). Clinical manifestations involve anaemia, hepatosplenomegaly, bone lesions, seizures, trismus, strabismus, hyperreflexia, supranuclear ophtalmoplegia and others (reviewed in Frenkel 1993).

The gene for glucocerebrosidase (GC) is located on chromosome 1q21 (Ginns *et al.* 1985) and is comprised of 11 exons (Horowitz *et al.* 1989). A highly homologous pseudogene is located nearby (Choudry *et al.* 1985) and has contributed significantly to the origin of mutations in the GC gene (Tsuji *et al.* 1987). There is a high level of homology between the human and mouse GC genes. In the protein coding region of the murine cDNA, the nucleotide sequence and the corresponding deduced amino acid sequences were 82% and 86% identical to the respective human sequences (O'Neill *et al.* 1989). As attempts to mimic the disease in mice by inhibiting the enzyme were inadequate (Kanfer *et al.* 1982), and technology to generate mouse models by gene targeting became available, a mouse model was generated (Tybulewicz *et al.* 1992).

To disrupt the murine glucocerebrosidase gene, a neo cassette was inserted replacing exons 9 and 10, which encode part of the active site of the enzyme. Mice homozygous for this mutation have less than 4% of

normal glucocerebrosidase activity. They have weak irregular respiration, do not feed, and die within twenty four hours of birth. Glucocerebroside is stored in lysosomes of cells of the mononuclear phagocyte system, microglia and in brainstem and spinal cord neurones (Tybulewicz *et al.* 1992; Willemsen *et al.* 1995). Surprisingly, the cellular pathology of tissues from the Gaucher mice was very mild, and the early death could not be explained by substrate accumulation, suggesting that a toxic metabolite may be responsible for their rapid deterioration. In addition, examination of the skin showed severe disruption of the lamellar bilayer structure of the outer stratum of corneum (Holleran *et al.* 1994). The absence of glucocerebrosidase may affect functional skin integrity, since sphingolipids play an important role in skin permeability barrier homeostasis (Holleran *et al.* 1991).

Similar to the homozygous mutant mice, a group of neonates with Gaucher disease with particularly devastating clinical course was described (Sidranski *et al.* 1992). These infants present at, or shortly after birth. They have rapidly progressing fulminant disease, and many have associated ichthyotic skin and /or hydrops fetalis. In conclusion, specific clinical manifestations of the mouse, particularly its ischthyotic skin and early demise, have aided recognition of a distinct human phenotype of Gaucher 2 disease.

Neonatal death, however, obviously restricts the utility of the null mutant mouse in testing novel therapeutic strategies and studying pathogenesis causing different clinical variants of Gaucher disease. To overcome this problem, another targeted mutation (A→G in exon 9) was introduced into the GC gene by homologous recombination in embryonic stem cells, with the aim of establishing a mouse model for a mild form of Gaucher disease. Unexpectedly the mice homozygous for this mutation die early in gestation. Further careful analysis led to the discovery of the Metaxin gene. Metaxin separates GC from the thrombospondin 3 gene and is transcribed convergently. The gene targeting cassette happened to be inserted into the terminal exon of the Metaxin gene, disrupting its expression, which appears to be essential for embryonic development in mice (Bornstein *et al.* 1995). This discovery prompted a closer analysis of the sequence of the entire region surrounding the human GC gene, resulting in the identification of human metaxin and of the three additional genes contiguous to the GC locus (Winfield *et al.* 1997).

Using a single insertion mutagenesis procedure (SIMP), mice were generated either carrying the very severe Rec NciI mutation that can cause

type 2 disease or the mutation L444P, associated with type 3 disease (Liu Y *et al.* 1998). Although, there are clear differences between the two homozygous mutant mice regarding the GC enzyme activity and sites of glucosylceramide accumulation, which correlated with either Gaucher 2 or Gaucher 3 type in the human situation, both point mutation mice died within 48 hours of birth, apparently of a compromised epidermal permeability barrier caused by defective glucosylceramide metabolism in epidermis. In conclusion, not only does the contiguous gene organisation at the GC locus limit targeting strategies for the production of murine models of Gaucher disease, but the results show that the differences in skin biology between mice and humans must be taken into account in any strategy to produce a model of late onset disease. Possible approaches include preservation of epidermal GC activity by a transgene in the context of an enzyme-deficient animal or a conditional gene disruption after the formation of the epidermal permeability barrier.

In the meantime, different therapeutic constructs and delivery methods are being tested on healthy mice. Several groups have reported protein expression in macrophages differentiated from mouse hematopoietic stem cells using retroviral vectors containing human glucocerebrosidase cDNA (Weinthal *et al.* 1991; Ohashi *et al.* 1992; Correll *et al.* 1994; Krall *et al.* 1994). In chapter 4 of this thesis, a construct driving expression of the GC gene specifically in macrophages is discribed and tested in transgenic mice.

E. Ethical and social implications

There is now a general consensus that somatic cell gene therapy for the purpose of treating a serious disease is an ethical therapeutic option. However, there are related risks. The recombination between vector and host virus could produce replication competent virus leading to lethal infection, environmental spread and possible germ line integration. There is also a risk of insertional oncogenesis and concern that partial correction of an early lethal mutation could lead to years of morbidity.

A serious controversy exists as to whether the clinical use ofgermline gene therapy would be ethical. The medical concern is that genetic manipulation could produce damage in future generations. Altering the genetic information in a patient's cells may result in long- term side effects

that are unpredictable at present. Until it becomes possible to correct the defective gene itself by homologous recombination (rather than inserting a normal copy of the gene elsewhere in the genome), the danger of producing a germline mutagenic event exists. Besides the medical arguments, there are a number of philosophical, ethical and theological concerns (Anderson 1992; McGleenan 1995; Karpati and Lochmüller 1997). For instance: do infants have the right to inherit an unmanipulated genome; does the concept of informed consent have any validity for patients who do not yet exist; where do we cross the line into "playing God"? There is also concern about using gene transfer to insert genes into humans for the purposes of enhancement or "improvement" of desired characteristics. The therapeutical potential for medically related problems is obviously huge, but the potential for misuse is also great. Thus society must ensure that gene therapy research is supervised by an expert body, that it maintains ethical standards of practice and protects subjects of research from harm while simultaneously preserving their rights and liberties (Clothier Report HMSO 1992).

Aim of the thesis

Many believe the potential for human gene therapy is tremendous, eventually having applications in virtually every subspecialty of medicine (see Chapter 1). While this may be true, the reality is that at present gene therapy is in its very preliminary stages.

If we define the ideal gene therapy protocol, it would be an *in vivo* administration of a therapeutic gene via a targetable delivery system. The introduced transcriptional unit should possess all the necessary information for fully regulated expression. In the case of genetically inherited disorders, the therapeutic DNA should homologously recombine at the site of the defective gene. The cells that are corrected should include a self renewing stem cell population. In conclusion, gene therapy needs to be, as far as possible a pharmaceutical approach for it to have a wide applicability. This ideal is far from the current status of gene therapy. The available methods of delivery are generally inefficient. They lack specificity. Most of them cannot be given systemically and in most cases only *ex vivo* procedures are possible. The introduced transcriptional unit is poorly expressed. As homologous recombination is too inefficient, only gene addition is feasible. Stem cells are still difficult to target. It is obvious that improvements through further research are needed in areas of gene delivery and gene expression.

There is a substantial amount of work directed towards the development of new viral vectors other than retroviruses and non-viral delivery systems particularly receptor-mediated delivery. There is also a necessity for fundamental research into the dynamics of DNA uptake into the nucleus and its subsequent fate, in extrachromosomal maintenance of introduced DNA and in development of mammalian artificial chromosomes. There is a huge interest in methods that would improve efficiency of homologous recombination and introduce genes into specific sites in the genome.

With regard to gene expression, it is necessary to identify minimal sequences required for efficient expression of various genes. The focus of the work presented in this thesis is on the application of locus control regions (LCRs). Using different animal models we have been able to show that expression of a potential therapeutic gene can be limited to specific cellular targets by placing the gene under the control of these tissue-specific elements.

Chapter 2 describes the use of the human CD2 locus control region. The expression of the nitroreductase suicide gene is restricted to T cells of

transgenic mice. This allows selective ablation of expressing cells upon administration of the prodrug (CB1954).

In chapter 3, the class II MHC Eα gene LCR was used to target Bruton tyrosine kinase (BTK) gene expression to B cells of transgenic mice from the pre-B cell stage onwards. This resulted in correction of the BTK^- phenotype when these transgenics were crossed to null mice.

In chapter 4, the same LCR was used to drive expression of the β-glucocerebrosidase gene in transgenic mice. The possible implications for gene therapy of Gaucher disease are discussed.

Chapter 5 of this thesis describes a new method for producing single copy transgenic animals by using the Cre-recombinase system. This approach will allow testing and comparison of gene therapy constructs which would be present in the host genome as a single copy.

Taken together, the results of these experiments clearly demonstrate the importance of LCRs for tissue specific and developmentally controlled expression of potential therapeutic genes. They represent some positive achievements in our understanding of the problems involved and point out possibilities for the improvement of gene therapy strategies.

Industry will clearly play an important role in the late stage development and distribution of gene therapy products. Thus, academic medical centres should provide critical and objective leadership in the formative stages (through innovative basic research and proof of concepts in animal studies which will lead to human clinical trials), while at the same time establishing links with industry for later stages of development. Work described in this thesis is a product of such an academic link with the gene therapy company Therexsys Ltd. UK.

References

1. Albert H, Dala EC, Lee E and OW DW. Site–specific integration of DNA into wild-type and mutant lox sites placed in the plant genome. *Plant J.* 1995; **7**:649-659.
2. Altevogt P, Michaelis M and Kiewski B. Identical forms of the CD2 antigen expressed by mouse T and B lymphocytes. *Eur. J. Immunol.* 1989; **19**:1509-1512.
3. Amsbaugh DF *et al.* Genetic control of the antibody response to type III pneumococcal polysaccharides in mice. I. Evidence that an X-linked gene plays a decisive role in determining responsiveness. *J. Exp. Med.* 1972; **169**:2109-2119.
4. Anderson JK, Garber DA, Meaney CA and Breakfield XO. Gene transfer into mammalian central nervous system using herpes virus vectors: extended expression of bacterial Lac Z in neurones using the neuron specific enolase promoter. *Hum. Gene Ther.* 1992; **3**:487-499.
5. Anderson WF. Human gene therapy. *Science* 1992; **256**:808-813.
6. Anderson WF. Prospects for human gene therapy. *Science* 1984; **226**: 401-409.
7. Araki K, Araki M and Yamamura K. Targeted integration of DNA using mutant lox sites in embryonic stem cells. *Nucleic Acids Res.* 1997; **25**:868-872.
8. Araki K, Araki M, Miyazaki J and Vassalli P. Site-specific recombination of a transgene in fertilized eggs by transient expression of Cre recombinase. *Proc. Natl. Acad. Sci. USA* 1995; **92**:160-164.
9. Aronow BJ *et al.* Functional analysis of the human adenosine deaminase gene thymic regulatory region and its ability to generate position-dependent transgene expression. *Mol. Cell. Biol.* 1992; **12**:4170-4185.
10. Axelrod JH, Brinskhous KM and Verma IM. Phenotypic correction of factor IX deficiency in skin fibroblast of hemophilic dogs. *Proc. Natl. Acad. Sci. USA.* 1990; **87**:5173-5177.
11. Baker SJ, Markovich S, Fearon ER, Willson JKV and Vogelstein B. Suppression of human colorectal carcinoma cell growth by wild type p53. *Science* 1990; **249**:912-915.
12. Banerji J, Rusconi S and Schaffner W. Expression of β-globin gene is enhanced by remote SV40 DNA sequences. *Cell* 1981; **27**:299-308.
13. Barlow C *et al.* Targeted expression of Cre recombinase to adipose tissue of transgenic mice directs adipose-specific excision of loxP-flanked gene segments. *Nucleic Acid Res.* 1997; **25**:2543-2545.
14. Barr D *et al.* Strain-related variations in adenovirally mediated transgene expression from mouse hepatocytes *in vivo*; comparisons between immunocompetent and immunodeficient strains. *Gene Ther.* 1995; **2**:151-155.
15. Barsoum J, brown R McKee M and Boyece FM. Efficient transduction of mammalian cells by recombinant baculovirus having the vesicular stomatitis virus G glycoprotein. *Hum. Gene Ther.* 1997; **8**:2011-2018.
16. Belldegrun A *et al.* Human renal carcinoma line transfected with interleukin-2 and/or Interferon-α gene(s): implication for live tumour vaccines. *J. Natl. Cancer Inst.* 1993; **85**:207-216.

17. Benoist C and Shambon P. *In vivo* sequence reqirements of the SV40 early promoter region. *Nature.*1981; **290**:304-310.
18. Besnard C, Monthioux E and Jami J. Selection against expression of the Escherichia coli gene in hprt$^+$ mouse teratocarcinoma and hybrid cells. *Mol. Cell Biol.* 1987; **7**:4139-4141.
19. Beutler E and Grabowski GA. Gaucher disease. In: *The metabolic basis of inherited disease* (ed. Scriver CR, Beaudet AL, Sly WS and Valle D), 1995; pp. 2641-2670. McGraw-Hill, New York, NY.
20. Beutler E. Gaucher disease as a paradigm of current issues regarding single gene mutations of humans. *Proc. Natl. Acad. Sci .USA* 1993; **90**:5384-5390.
21. Bi WL, *et al.* In vitro evidence that metabolic co-operation is responsible for the bystander effect observed with HSV tk retroviral gene therapy. *Hum. Gene Ther.* 1993; **4**:725-731.
22. Bishop JO. Chromosomal insertion of foreign DNA. *Reprod. Nutr. Dev.* 1996; **36**:607-618.
23. Blase RM and Culver KW. Progression towards the Application of Gene Therapy. In: Clinical and Basic science Aspects of Immunohematology, ed. Nance SJ, Arlington, VA: American Association of Blood Banks, 1991; p1-11.
24. Bonifer C, Vidal M, Grosveld F and Sippel AE. Tissue specific and position independent expression of the complete gene domain for chicken lysozyme in transgenic mice. *EMBO J.* 1990; **9**:2843-2848.
25. Bonifer C, Yannoutsos N, Krüger G, Grosveld F and Sippel AE. Dissection of the Locus Control Function Located on the Chicken Lysozyme gene Domain in Transgenic Mice. *Nucleic Acids Res.* 1994; **22**:4202-4210.
26. Bornstein P *et al.* Metaxin, a gene contiguous to both thrombospondin 3 and glucocerebrosidase, is required for embryonic development in the mouse; Implications for Gaucher disease. *Proc. Natl. Acad. Sci. USA.* 1995; **92**:4547-4551.
27. Bouhassira E, Westerman K and Leboulch P. Transcriptional Behaviour of LCR Enhancer Elements Integrated at the Same Chromosomal Locus by Recombinase-mediated Cassette Exchange. *Blood* 1997; **90**:3332-3344.
28. Bridgewater JA *et al.* Expression of the Bacterial Nitroreductase Enzyme in Mammalian Cells Renders Them Selectively Sensitive to Killing by the Prodrug CB1954. *European Journal of Cancer* 1995; **31**:2362-2370.
29. Brocard J *et al.* Spatio-temporally controlled site-specific somatic mutagenesis in the mouse. *Proc. Natl. Acad. Sci.* USA 1997; **94**:14559-14563.
30. Bruton OC. Agammaglobulinaemia. *Pediatrics* 1952; **9**:722-727.
31. Buchholz F, Angrand PO and Stewart AF. Improved properties of FLP recombinase evolved by cycling mutagenesis. *Nat. Biotechnol.* 1998; **16**:657-662.
32. Buchholz F, Ringrose L, Angrand PO, Rossi F and Stewart AF. Different thermostabilities of FLP and Cre recombinases: implications for applied site-specific recombination. *Nucleic Acids Res.* 1996; **24**:4256-4262.
33. Carson S and Wiles MV. Far upstream regions of class II MHC Ea are necessary for position-independent, copy-dependent expression of Ea transgene. *Nucleic Acids Res.* 1993; **21**:2065-2072.
34. Carson S. Dnase I hypersensitive sites flank the mouse class II major histocompatibility complex during B cell development. *Nucleic Acids Res.* 1991; **19**:5007-5014.

35. Chaney WG, Howard DR, Pollard JW, Sallustio S and Stanley P. High frequency transfection of CHO cells using polybrene. *Somatic Cell Mol. Genet.* 1986; **12**:237-244.
36. Chang JC, Liu D and Kan YW. A 36-base-pair core sequence of locus control region enhances retrovirally transferred human beta-globin gene expression. *Proc. Natl. Acad. Sci. USA* 1992; **89**:3107-3110.
37. Chong H, Todryk S, Hutchinson G, Hart IR and Vile RG. Tumour cell expression of B7 costimulatory molecules and interleukin-12 or a granulocyte-macrophage colony stimulating factor induces a local antitumour immunity. *Gene Ther.* 1998; **5**:223-232.
38. Choudary PV, Ginns EI and Barranger JA. Molecular cloning and analysis of the human β-glucocerebrosidase gene. *DNA* 1985; **4**:74.
39. Chu G, Hayakawa H and Berg P. Electroporation for the efficient tranfection of mammalian cells with DNA. *Nucleic Acids Res.* 1987; **15**:1311-1326.
40. Cline MJ *et al.* Gene transfer in intact animals. *Nature* 1980; **284**: 422-425.
41. Clotier C (1992) Report of the Committee on the Ethics of Gene Therapy, HMSO.
42. Cole-Strauss A *et al.* Correction of the Mutation Responsible for Sickle Cell Anemia by an RNA-DNA Oligonucleotide. *Science* 1996; **273**:1386-1389.
43. Colombo BM, *et al.* The "bystander effect": Association of U87 cell death with ganciclovir-mediated apoptosis of nearby cells and lack of effect in athymic mice. *Hum. Gene Ther.* 1995; **6**:763-772.
44. Condreay P, Witherspoon S, Clay W and Kost TA. Transient and stable gene expression in mammalian cells transduced with a recombinant baculovirus vector. *Proc. Natl. Acad. Sci. USA* 1999; **96**:127-132.
45. Cone RD and Mulligan RC. High-efficiency gene transfer into mammalian cells: generation of helper-free recombinant retrovirus with broad mammalian host range. *Proc. Natl. Acad Sci. USA.* 1984; **81**:6349-6353.
46. Corey CA, DeSilva AD, Holland CA and Wiliams DA. Serial Transplantation of Methotrexate-Resistant Bone Marrow: Protection of Murine Recipients from Drug Toxicity by Progeny of Transduced Stem Cells. *Blood* 1990; **75**:337-343.
47. Correll PH and Karlsson S. Towards therapy of Gaucher disease by gene transfer into hematopoietic cells. *Eur. J. Haematol.* 1994; **53**:253-264
48. Cotten M *et al.* High-efficiency receptor-mediated delivery of small and large (48 kilobase) gene constructs using the endosome-disruption activity of defective or chemically inactivated adenovirus particles. *Proc. Natl. Acad. Sci. USA* 1992; **89**:6094-6098.
49. Culver KW, Ram Z, Wallbridge S, Hiroyuki I, Oldfield R and Blaese RM. *In Vivo* Gene Transfer with Retroviral Vector-Producer Cells for Treatment of Experimental Brain Tumors. *Science* 1992; **256**:1550-1552.
50. Curiel DT *et al.* High-efficiency gene transfer mediated by adenovirus coupled to DNA-polylysine complex. *Hum. Gene Ther.* 1992; **3**:147-154.
51. Curiel DT, Agarwal S, Wagner E and Cotten M. Adenovirus enhancement of transferrin-polylysine-mediated gene delivery. *Proc. Natl. Acad. Sci. USA* 199' **88**:8850-8854.
52. Danos O and Mulligan RC. Safe and efficient generation of recombinant retrovir with amphotropic and ecotropic host ranges. *Proc. Natl. Acad. Sci. USA.* **85**:6460-6464.
53. Davies N. How long should we wait for big MAC? *Gene Ther.* 1995; **2**:299-3(

54. Day Y *et al.* Cellular and humoral immune responses to adeno-viral vectors containing factor IX gene: tolerization of factor IX and vector antigens allows for long-term expression. *Proc. Natl. Acad. Sci. USA* 1995; **92**:1401-1405.
55. de Weers M *et al.* The Bruton's tyrosine kinase gene is expressed throughout B cell differentiation, from early precusor B cell stages preceding immunoglobulin gene rearrangement up to mature B cell stages. *Eur. J. Immunol.* 1993; **23**:3109-3114.
56. de Weers M, Mensink RGJ, Kraakman MEM, Schuurman RKB and Hendriks RW. Mutation analysis of Bruton's tyrosine kinase gene in X-linked agammaglobulinemia: identification of a mutation which affects the same codon as is altered in immunodeficient xid mice. *Hum. Mol. Genet.* 1994; **3**:161-166.
57. DeMatteo RP *et al.* Prolongation of adenoviral transgene expression in mouse liver by T lymphocyte subset depletion. *Gene Ther.* 1996; **3**:4-12.
58. Deodarain MP and Epenetos AA. Targeting enzymes for cancer therapy: old enzymes in new roles. *Br. J. Cancer* 1994; **5**:786-794.
59. Devereux S *et al.* Feasibility of multidrug resistance (MDR-1) gene transfer in patients undergoing high-dose therapy and peripheral blood stem cell transplantation for lymphoma. *Gene Ther.* 1998; **5**:403-408.
60. Diaz P, Cado D and Winoto A. A locus control region in the T cell receptor α/δ locus. *Immunity* 1994; **1**:207-217.
61. Donnelly JJ,Ulmer JB, Shiver JW and Liu MA. DNA vaccines. *Ann. Rev. Immunol.* 1997; **15**:617-648.
62. Dorn A *et al.* B cell control region at the 5' end of a major histocompatibility complex class II gene: sequences and factors. *Mol. Cell. Biol.* 1988; **8**:3975-3987.
63. Dorn A, Bollekens J, Staub A, Benoist C and Mathis D. A multiplicity of CCAAT box-binding proteins. *Cell* 1987; **50**:863-872.
64. Douglas J, Kelly P, Evans JT and Garcia JV. Efficient transduction of human lymphocytes and CD 34^+ cells via human immunodeficiency virus-based gene transfer vectors. *Hum. Gene Ther.* 1999; **6**:935-945.
65. Dymecki SM. Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and trensgenic mice. *Proc. Natl. Acad. Sci.* USA 1996; **93**:6191-6196.
66. Dzau V, Morishita R and Gary Gibbons. Gene therapy for cardiovascular disease. *TIBTECH* 1993; **11**:205-210.
67. Ehlich A, Schaal S, Gu H, Kitamura D, Müller W and Rayewski K. Immunoglobulin heavy and light chain genes rearrange independently at early stages of B cell development. *Cell* 1993; **72**:695-704.
68. Elion G. The biochemistry and mechanism of action of acyclovir. *J. Antimicrob. Chemoth.* 1983; **12**:9-17.
69. Ellis J *et al.* Evaluation of beta-globin gene therapy constructs in single copy transgenic mice. *Nucleic Acids Res.* 1997; **6**:2636-2643.
70. Feil R *et al.* Ligand-activated site-specific recombination in mice. *Proc. Natl. Acad. Sci.* USA 1996; **93**:10887-10890.
71. Felgner PL *et al.* Lipofection: a highly efficient, lipid-mediated DNA transfection procedure. *Proc. Natl. Acad. Sci.* USA 1987; **84**:7413-7417.
72. Festenstein R *et al.* Locus Control Region Function and Heterochromatin-Induced Position Effect Variegation. *Science* 1996; **271**:1123-1125.

73. Fiering S *et al.* Targeted deletion of 5'HS2 of the murine beta-globin LCR reveals that it is not essential for proper regulation of the beta globin locus. *Genes and Development* 1995; **9**:2203-2213.
74. Forrester WC, Takegawa S, Papayannopoulou T, Stamatoyannopoulos G and Groudine M. Evidence for a locus activation region: the formation of developmentally stable hypersensitive sites in globin-expressing hybrids. *Nucleic Acids Res.* 1987; **15**:10159-10177.
75. Fraley R, Subramani S, Berg P and Papahadjopoulos D. Introduction of liposome-incapsulated SV40 DNA into cells. *J. Biol. Chem.* 1980; **255**:10431-10435.
76. Frasier *et al.* Inhibition of TAT-mediated transactivation and HIV replication with Tat mutant and repressor domain fusion proteins. *Gene Ther.* 1998; **5**:946-954.
77. Frenkel EP. Southwestern Internal Medicine Conference: Gaucher Disease: A Heterogeneous Clinical Complex for Which Effective Enzime Replacement Has Come of Age. *Am. J. Med. Sci.* 1993; **305**:331-344.
78. Friend WC *et al.* Cell-Specific Expression of High Levels of Human S100β in Transgenic Mouse Brain is Dependent on Gene Dosage. *The Journal of Neuroscience* 1992; **12**: 4337-4346.
79. Furman PA, McGuirt PV, Keller PM, Fyfe JA and Elion GB. Inhibition by acyclovir of cell growth and DNA synthesis of cells biochemically transformed with herpes virus genetic information. *Virology* 1980; **102**:420-430.
80. Gansbacher B *et al.* Retroviral mediated γ-Interferon gene transfer into tumour cells generates potent and long lasting anti-tumour immunity. *Cancer Research* 1990; **50**:7820-7825.
81. Ginns EI *et al.* Gene mapping and leader polypeptide sequence of human glucocerebrosidase: Implications for Gaucher disease. *Proc. Natl. Acad. Sci. USA* 1985; **82**:7101-7105.
82. Glimcher LH and Kara CJ. Sequence and factors: a guide to MHC class II transcription. *Annu. Rev. Immunol.* 1992; **19**:13-49.
83. Goldstein LJ, Gottesman MM and Pastan I. Expression of the MDR1 gene in human cancers. *Cancer Treat. Res.* 1991; **57**:101-119.
84. Goldstein LJ, Pastan I and Gottesman MM. Multidrug resistance in human cancer. *Crit. Rev. Oncol. Hematol.* 1992; **12**:243-253.
85. Golumbek PT *et al.* Treatment of established renal cancer by tumour cells engineered to secrete Interleukin-4. *Science* 1991; **254**:713-716.
86. Gottesman MM, Germann UA, Aksentievich I, Sugimoto Y, Cardarelli CO and Pastan I. Gene transfer of drug resistance genes, implications for cancer therapy. *Annals NY Acad. Sci.* 1994; **716**:126-138.
87. Gracham FL and van der Eb AJ. A new technique for the assay of infectivity of human adenovirus 5. *Virology* 1973; **52**:456-467.
88. Greaves DR, Wilson FD, Lang G and Kioussis D. Human CD2 3'-Flanking Sequences Confer High-Level, T Cell-Specific, Position-Independent Gene Expression in Transgenic Mice. *Cell* 1989; **56**:979-986.
89. Greenblatt MS, Benett WP, Hollstein M and Harris CC. Mutations of p53 tumour suppressor gene: clues to cancer aetiology and molecular pathogenesis. *Cancer Res.* 1994; **54**:4855-4878.

90. Grossman M *et al.* Successful *ex vivo* gene therapy directed to liver in a patient with familiar hypercholesteolemia. *Nature Genet.* 1994; **6**:335-341.
91. Grosveld F, Blom van Assendelft G, Greaves DR and Kollias G. Position-independent, high-level expression of the human β-globin gene in transgenic mice. *Cell* 1987; **51**:975-985.
92. Gu H, Marth J, Orban PC and Rajewsky K. Deletion of polymerase β gene segment in T cells using cell type-specific gene targeting. *Science* 1994; **265**:103-106.
93. Guy J, Drabek D and Antoniou M. Delivery of DNA into Mammalian Cells by Receptor-Mediated Endocytosis and Gene Therapy. *Molecular Biotechnology* 1995; **3**:237-248.
94. Hanania E.G. *et al.* Recent Advances in the Application of Gene Therapy to Human Disease. *The American Journal of Medicine* 1995; **99**:537-552.
95. Hardy RR, Carmack CE, Shinton SA, Kemp JD and Hayakawa K. Resolution and characterization of the pro-B and pre-B cell stages in normal mouse bone marrow. *J. Exp. Med.* 1991; **173**:1213-1225.
96. Hayashi S *et al.* Establishment of complement-resistant retroviral vector by homologous restriction factor 20 gene. *Gene Ther.* 1998; **5**:282-285.
97. Hendriks RW *et al.* Inactivation of Btk by insertion of Lac Z reveals defects in B cell development only past pre-B cell stage. *EMBO* 1996; **15**:4862-4872.
98. Henning KA *et al.* Human artificial chromosomes generated by modification of a yeast artificial chromosome containing both human alpha satellite and single-copy DNA sequences. *Proc. Natl. Acad. Sci. USA* 1999; **96**:592-597.
99. Hock H, Dorsch M, Diamanstein T and Blankenstein T. Interleukin-7 induces $CD4^+$ T cell dependent tumour immunity. *J Exp. Med.* 1991; **174**:1291-1298.
100. Hock H, Dorsch M, Kunzendorf U, Qin Z, Diamantstein T and Blankenstein T. Mechanism of rejection induced by tumour cell targeted gene transfer of Interleukin-2, Interleukin-4, Interleukin-7, tumour necrosis factor or Interferon-γ. *Immunology* 1993; **90**:2774-2778.
101. Hogan B, Beddington R, Constantini F and Lacy E. Manipulation of Mouse Embryo. 1994; Cold Spring Harbor laboratory Press.
102. Holleran WM *et al.* Consequences of beta-glucocerebrosidase deficiency in epidermis *J. Clin. Invest.* 1994; **93**:1757-1764.
103. Holleran WM *et al.* Sphingolipids are required for mammalian epidermal barrier function: inhibition of sphingolipid synthesis delays barrier recovery after acute perturbation. *J. Clin. Invest.* 1991; **88**:1338-1345.
104. Hollstein M, Sidransky D, Volestein B and Harris CC. p53 mutations in human cancers. *Science* 1991; **253**:49-53.
105. Hoogerbrugge PM, Vassen JMJJ, Beusechann VW and Valerio D. Treatment of patients with SCID due to ADA deficiency by autologous transplantation of genetically-modified bone marrow cells. *Hum. Gene Ther.* 1992; **3**:553-558.
106. Horowitz M and Zimran A. Mutations causing Gaucher disease. *Human Mutation* 1994; **3**:1-11.
107. Horowitz M *et al.* The human glucocerebrosidase gene and pseudogene: Structure and evolution. *Genomics* 1989; **4**:87-96.

108. Huber BE,, Ausstin EA, Good SS, Knick VC, Tibbels T and Richards CA. *In vivo* anti tumour activity of 5-fluorocytosine on human colorectal carcinoma cells genetically modified to express cytosine deaminase. *Cancer Res.* 1993; **53**:4619-4626.
109. Huber BE, Richards CA and Krenitsky TA. Retroviral mediated gene therapy for the treatment of hepatocellular carcinoma: an innovative approach for cancer therapy. *Proc. Natl. Acad. Sci. USA* 1999; **88**: 8039-8043.
110. Hunig T, Tiefenthaler G, Meyer zum Buschefelde KH, and Meuer SC. Alternative pathway activation of T-cells by binding of CD2, to its cell-surface ligand. *Nature* 1987; **326**:298-301.
111. Huxley C. Mammalian artificial chromosomes: a new tool for gene therapy. *Gene Ther.* 1994; **1**:7-12.
112. Im DS and Muzyczka N. The AAV origin binding protein Rep 68 is an ATP-dependent site -specific endonuclease with DNA helicase activity. *Cell* 1990; **61**:447-457.
113. Imbalzano AN, Kwon H, Green MR and Kingston RE. Facilitated binding of TATA-binding protein to nucleosomal DNA. *Nature.* 1994; **370**:481-485.
114. Johnston SA and Tang DC. Gene gun transfection of animal cells and genetic immunisation. *Methods Cell Biol.* 1994; **43**: Pt A: 353-365.
115. Jones BK, Monks BR, Liebhaber SA and Cooke NE. The human Growth Hormone Gene is regulated by a Multicomponent Locus Control Region. *Molecular and Cellular Biology* 1995; **15**: 7010-7021.
116. Kalle von C, Glimm H, Schulz G, Mertelsmann R and Henschler R. New developments in hematopoietic stem cell expansion. *Current Opinion in Hematology* 1998; **5**:79-86.
117. Kanfer JN, Stephens MC, Singh H and Legler G. The Gaucher mouse. In: Gaucher disease: A century of delineation and research. Desnick RJ, Gatt S, Grabowski GA (eds.) Alan R liss Inc, New York, pp. 627-644.
118. Kaptein LCM, Breuer M, Valerio D and Beusechem VW. Expression pattern of CD2 locus control region retroviral vectors in hemopietic cells in vitro and in vivo. *Gene therapy* 1998; **5**:320-330.
119. Karpati G and Lochmüller H. The scope of gene therapy in humans: scientific, safety and ethical considerations. *Neuromuscular Disorders* 1997; **7**:273-276.
120. Kass-Eisler A *et al.* The impact of developmental stage, route of administration and the immune system on adenovirus-mediated gene transfer. *Gene Ther.* 1994; **1**:395-402.
121. Kay MA. *et al.* Expression of human α_1 antitrypsin in dogs after autologous transplantation of retroviral transduced hepatocytes. *Proc Natl. Acad. Sci. USA.* 1992; **89**: 89-93.
122. Kellendonk C *et al.* Regulation of Cre recombinase activity by synthetic steroid RU 486. *Nucleic Acids Res.* 1996; **24**: 1404-1411.
123. Khan WN *et al.* Defective B Cell Development and Function in Btk-Deficient Mice. *Immunity* 1995; **3**:283-299.
124. Kilby NJ, Snaith MR and Murray JAH. Site –specific recombinases: tools for genome engineering. *TIG* 1993; **9**:413-420.
125. Kinloch RA et al. Regulation of expression of mZP3, the sperm receptor gene, during mouse development. In: Molecular Basis of Morphogenesis. Edited by Bernfield M. New York: Wiley-Liss; 1993:19-33.

126. Kioussis D, Vanin E, deLange T, Flavell RA and Grosveld F. Beta-globin gene inactivation by DNA translocation in gamma beta-thalassaemia. *Nature* 1983; **306**:662-666.
127. Kong HL and Crystal RG. Gene strategies for Tumour Angiogenesis. *Journal of the National Cancer Institute* 1998; **90**:273-286.
128. Kotin RM *et al.* Site-specific integration by adeno-associated virus. *Proc. Natl. Acad. Sci. USA* 1990; **87**: 2211-2215.
129. Krall WJ, Challita PM, Perlmutter LS, Skelton DC and Kohn DB. Cells expressing human glucocerebrosidase from a retroviral vector repopulate macrophages and central nervous system microglia after murine bone marrow transplantation. *Blood* 1994; **83**:2737-2748.
130. Kren BT, Bandyopadhyay P and Steer C. *In vivo* site –directed mutagenesis of the factor IX gene by chimeric RNA/DNA oligonucleotides. *Nature Med.* 1998; **4**:285-290.
131. Kushida MM *et al.* A 150-Base Pair 5' Region of the MHC Class I HLA-B7 Gene Is Sufficient to Direct Tissue-Specific Expression and Locus Control Region Activity. *The Journal of Immunology* 1997; **159**:4913-4929.
132. Lake R, Wotton D and Owen M. A 3' transcriptional enhancer regulates tissue-specific expression of the human CD2 gene. *EMBO J.* 1990; **9**:3129-3136.
133. Lake RA and Owen MJ. Transfection of the chloramphenicol-acetyltransferase gene into eukariotic cells using diethil-aminooethyl (DEAE)-dextran, in *Gene Transfer and Transfection Protocols* 1991; (Murray EJ ed.), Humana, Clifton, NJ, pp.23-33.
134. Lakso M *et al.* Efficient *in vivo* manipulation of mouse genomic sequences at the zygote stage. *Proc. Natl. Acad. Sci. USA* 1996; **93**:5860-5865.
135. Lang G, Mamalaki C, Greenberg D, Yannoutsos N and Kioussis D. Deletion analysis of the human CD2 gene locus control region in transgenic mice. *Nucleic Acids Res.* 1991; **19**:5851-5856.
136. Lapidot M and Loyter A. Fusion-mediated microinjection of liposome-enclosed DNA into cultured cells with the aid of influenza virus glycoproteins. *Exp. Cell. Res.* 1990; **189**:241-246.
137. Ledley FD, and Woo SL. Molecular basis of alpha1-antitripsin deficiency and its potential therapy by gene transfer. *J. Inher. Metab. Dis.* 1989; supl.**9**:85-91.
138. Lewandoski M, Montzka K and Martin GR. Zp3-Cre, a transgenic mouse line for the activation or inactivation of loxP-flanked target genes specifically in the female germ line. *Current Biology* 1996; 7:148-151.
139. Liljeström P and Garoff H. A new generation of animal cell expression vectors based on Semliki Forest virus replicon. *Bio Technology* 1991; **9**:1356-1361.
140. Linden RM, Ward P, Giraud C, Winocour E and Berns K. Site-specific integration by adeno-associated virus. *Proc. Natl. Acad. Sci. USA.* 1996; **93**:11288-11294.
141. Lira SA, Kinloch RA Mortillo S and Wassarman P. An upstream region of the mouse ZP3 gene directs expression of firefly luciferase specifically to growing oocytes in transgenic mice. *Proc. Natl. Acad. Sci. USA* 1990; **87**:7215-7219.
142. Liu Y *et al.* Mice with type 2 and 3 Gaucher disease point mutations generated by a single insertion mutagenesis procedure (SIMP). *Proc. Natl. Acad. Sci. USA* 1998; **95**:2503-2508.

143.Lori F *et al.* Rapid protection against HIV Type 1 replication mediated by high efficiency non-retroviral delivery of genes interfering with HIV-1 TAT and GAG. *Gene Ther.* 1994; 1:27-31.
144.Lu L, Shen RN and Broxmeyer HE. Stem cell from bone marrow, umbilical cord blood and peripheral blood for clinical application: current status and future application. *Critical Reviews in Oncology/Hematology* 1996; **22**:61-78.
145.Lubin DM and Coyne KE. Phospholipid-anchored and transmembrane versions of either decay-accelerating factor or membrane cofactor protein show equal efficiency in protection from complement-mediated cell damage. *J. Exp. Med.* 1991; **174**:35-44.
146.Lundberg C, Horellou P, Mallet J, Bjorklund A: Generation of DOPA-producing astrocytes by retroviral transduction of the human tyrosine hydroxilase gene: In vivo characterisation and in vivo effects in the rat Parkinson model. *Exp. Neurol.* 1996; **139**:39-53.
147.Maas A. Studies on function of Bruton's tyrosine kinase in B cell development. PhD thesis Erasmus University Rotterdam, 1998.
148.Madisen L and Groudine M. Identification of a locus control region in the immunoglobulin heavy-chain locus that deregulates c-myc expression in plasmocytoma and Burkitt's lymphoma cells. *Genes and Development* 1994; **8**:2212-2226.
149.Maniatis T, Jim GK, Efstradiadis A and Kafatos F. Amplification and characterisation of beta-globin gene synthesized in vitro. *Cell* 1976; **8**:169-182.
150.Manickan E, Kanangat S, Rouse RJ, Yu Z and Rouse BT. Enhancement of immune response to naked DNA vaccine by immunization with transfecteted dendritic cells. *J. Leukoc. Biol.* 1997; **62**:125-132.
151.Matsushita T *et al.* Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Ther.* 1998; **5**:938-945.
152.McGleenan T. human gene therapy and slippery slope arguments. *Journal of Medical Ethics* 1995; **21**:350-355.
153.McKnight S and Tjian R. Transcriptional selectivity of viral genes in mammalian cells. *Cell.* 1986; **46**:795-805.
154.McLaughlin SK, Collis P, Hermonat PL and Muzyczka N. Adeno-Associated Virus General Transduction Vectors: Analysis of Proviral Structures. *Journal of Virology* 1988; **62**: 1963-1973.
155.Mecsas J and Sugden B. Replication of plasmids derived from bovine papilloma virus type 1 and Epstein -Barr virus in cell culture. *Annu. Rev. Cell Biol.* 1987; **3**:87-108.
156.Mercola KE, Stang HD, Browne J, Salser W and Cline MJ. Insertion of a new gene of viral origin into bone marrow cells of mice. *Science* 1980; **208:**1033-1035.
157.Meuer SC *et al.* An alternative pathway of T-cell activation: a functional role for the 50 kd sheep erythrocyte receptor protein. *Cell* 1984; **36**:897-906.
158.Miller AD. Retroviral vectors. in: *Current Topics in Microbiology and Immunology*, edited by Muzyczka N, Spring-Verlag; Berlin-Heidelberg 1992; **158**:1-119.
159.Milligan RC. The Basic Science of Gene Therapy. *Science.* 1993; **260**:926-932.
160.Milot E. *et al.* Heterochromatin Effects on the Frequency and Duration of LCR-Mediated Gene Transcription. *Cell* 1996; **87**:105-114.
161.Mochizuki H, Schwartz JP, TanakaK, Brady RO and Reiser J. High-titer human immunodeficiency virus type 1-based vector systems for gene delivery into nondividing cells. *J. Virol.* 1998; **11**:8873-8883.

162.Montoliu L, Umland T and Schütz G. A locus control region at -12 kb of the tyrosinase gene. *EMBO J.* 1996; **15**:6026-6034.
163.Moolten FL. Drug sensitivity ("suicide") genes for selective cancer chemotherapy. *Cancer Gene Ther.* 1994; **4**:279-287.
164.Moreau P *et al.* The SV40 72 base pair repeat has a striking effect on gene expression both in SV 40 and other chimeric recombinants. *Nuclei Acids Res.* 1981; **9**:6047-6068.
165.Morgan TL, Maher VM and McCormick JJ. Optimal parameters for the polybrene-induced DNA transfection of diploid human fibroblasts. *In vitro Cell. Dev. Biol.* 1980; **22**:317-319.
166.Mroz PJ and Moolten FL. Retrovirally transduced Escerichia coli gpt genes combine selectivity with chemosensitivity capable of mediating tumor eradication. *Hum. Gene Ther.* 1993; **4**:589-595.
167.Mullen CA, Kilstrup M and Blaese M. Transfer of the bacterial gene for cytosine deaminase to mammalian cells confers lethal sensitivity to 5-fluorocytosine: a negative selection system. *Proc. Natl. Acad. Sci. USA* 1992; **89**:33-37.
168.Nabel GJ *et al.* Direct gene transfer with DNA-liposome complexes in melanoma: Expression, biologic activity, and lack of toxicity in humans. *Proc. Natl. Acad. Sci. USA* 1993; **90**:11307-11311.
169.Nabel GJ. Immunotherapy for cancer by direct gene transfer into tumors. *Hum. Gene Ther.* 1994; **5**:57-77.
170.Nimgaonkar M, *et al.* Transduction of mobilized peripheral blood $CD34^+$ cells with the glucocerebrosidase cDNA. *Gene Ther.* 1994; **1**:201-207.
171.Nonoyama S *et al.* Functional Analysis of Peripheral Blood B Cells in Patients with X-Linked Agammaglobulinemia. *J. Immunol.* 1998; **161**:3925-3929.
172.Novak U, Harris EA, Forrester W, Groudine M and Gelinas R. High-level beta-globin expression after retroviral transfer of locus activation region-containing human beta-globin gene derivatives into murine erythroleukemia cells. *Proc. Natl. Acad. Sci. USA* 1990; **87**:3386-3390.
173.Nussbaum O, Rott R and Loyter A. Fusion of influenza virus particles with liposomes: requirement for cholesterol and virus receptors to allow fusion with and lysis of natural but not of negatively charged liposomes. *J. Gen. Virol.* 1992; **73**:2831-2837.
174.O'Neill R, Tokoro T, Kozak CA and Brady RO. Comparison of the chromosomal localization of murine and human glucocerebrosidase genes and of the deduced amino acid sequences. *Proc. Natl. Acad. Sci. USA* 1989; **86**:5049-5053.
175.Ohashi T *et al.* Efficient transfer and sustained high expression of the human glucocerebrosidase gene in mice and their functional macrophages following transplantation of bone marrow transduced by retroviral vector. *Proc. Natl. Acad. Sci. USA* 1992; **89**:11332-11335.
176.Olson EN, Arnold HH, Rigby PWJ and Wold BJ. Know your neighbours; three phenotypes in null mutants of the myogenic bHLH gene MRF4. *Cell* 1996; **85**:1-4.
177.Philipsen S, Talbot D, Fraser P and Grosveld F. The β-globin dominant control region: hypersensitive site 2. *EMBO J.* 1990; **9**:2159-2167.
178.Plank C, Zatloukal K, Cotten M, Mechtler K and Wagner E. Gene transfer into hepatocytes using asialoglycoprotein receptor mediated endocytosis of DNA complexed with an artificial tetra-antennary galactose ligand. *Bioconjugate Chem.* 1992; **3**:533-539.

179.Plavec I, Papayannopolou T, Maury C and Meyer F. A human beta-globin gene fused to the human beta-globin locus control region is expressed at high levels in erythroid cells of mice engrafted with retrovirus-transduced hematopoietic stem cells. *Blood* 1993; **81**:1384-1392.

180.Podsakoff G, Wong KKJ and Chatterjee S. Efficient gene transfer into nondividing cells by adeno-associated virus-based vectors. *Journal of Virology* 1994; **68**:5656-5666.

181.Pruzina S, Hanscombe O, Whyatt D, Grosveld F and Philipsen S. Hypersensitive site 4 of the human β globin locus control region. *Nucleic Acids Res.* 1991; **19**:1413-1419.

182.Qui P, Ziegelhoffer P and Yang NS. Gene gun delivery of mRNA *in situ* results in efficient transgene expression and genetic immunisation. *Gene Ther.* 1996; **3**; 262-268.

183.Ramesh R, Marrogy AJ, Munshi A, Abboud CN and Freeman SM. *In vivo* analysis of the bystander effect: a cytokine cascade. *Exp. Haematol.* 1996; **24**:829-838.

184.Rawlings DJ *et al.* Mutations of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. *Science* 1993; **261**:358-361.

185.Reichel MB *et al.* Immune responses limit adenovirally mediated gene expression in the adult mouse eye. *Gene Ther.* 1998; **5**:1038-1046.

186.Reiser J *et al.* Transduction of nondividing cells using pseudotyped replication defective high-titer HIV type 1 particles. *Proc. Natl. Acad. Sci. USA* 1996; **93**:15266-15271.

187.Rohdewohld H, Weihner H, Reik W, Jaenisch R and Breindl M. Retrovirus-Integration and Chromatin Structure: Moloney Murine Leukemia Proviral Integration sites Map near Dnase I-Hypersensitive Sites. *Journal of Virology* 1987; **61**:336-343.

188.Roman M *et al.* Circulating human or canine factor IX from retrovirally transduced primary myoblast and established myoblast cell lines grafted into murine skeletal muscle. *Somat. Cell Molec. Genet.* 1993; **18**:247-258.

189.Rosenfeld MA. *et al.* Adenovirus-mediated transfer of recombinant α1-antitrypsin gene to the lung epithelium in vivo. *Science* 1991; **252**:431-434.

190.Rosenkranz AA *et al.* Receptor-mediated endocytosis and nuclear transport of a transfecting DNA construct. *Exp. Cell. Res*, 1992; **199**:323-329.

191.Rossant J and McMahon A. "Cre"-ating mouse mutants-a meeting review on conditional mouse genetics. *Genes and Development* 1999; **13**:142-145.

192.Russel DW, Miller AD and Alexander IE. Adeno-associated virus vectors preferentially transduce cells in S phase. *Proc. Natl. Acad. Sci. USA* 1994; **91**:8915-8919.

193.Sadelain M, Jason Wang CH, Antoniou M, Grosveld F and Mulligan R. Generation of a high-titer retroviral vector capable of expressing high levels of the human β-globin gene. *Proc. Natl. Acad. Sci. USA* 1995; **92**:6728-6732.

194.Sakai K and Miyazaki J. A Transgenic Mouse Line That Retains Cre Recombinase Activity in Mouse Oocytes Irrespective of the cre Transgenic Transmission. *Biochemical and Biophysical Research Communications* 1997; **237**:318-324.

195.Sandig V *et al.* Gene transfer into hepatocytes and human liver tissue by baculovirus vectors. *Hum. Gene Ther.* 1996; **7**:1937-1945.

196.Sauer B and Henderson N. Targeted Insertion of Exogenous DNA into the Eukaryotic Genome by the Cre Recombinase. *The New Biologist* 1990; **2**:441-449.

197.Schaefer-Ridder M, Wang Y and Hofschneider PH. Liposomes as gene carriers: efficient transformation of mouse L-cells by thymidine kinase gene. *Science* 1982; **215**:166-168.
198.Scher I. The CBA/N mouse strain; an experimental model illustrating the influence of the X chromosome on immunity. *Adv. Immunol.* 1982; **33**:1-71.
199.Schiedner G *et al.* Genomic DNA transfer with a high-capacity adenovirus vector results in improved in vivo gene expression and decreased toxicity. *Nat.Gen.* 1998; **18**: 180-183.
200.Schlake T and Bode J. Use of Mutated FLP Recognition Target (FRT) Sites for the Exchange of Expression Cassettes at Defined Chromosomal Loci. *Biochemistry* 1994; **33**:12746-12751.
201.Schwenk F, Baron U and Rajewsky K. A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. *Nucleic Acids Res.* 1995; **23**:5080-5081.
202.Searle PF and Mautner V. Adenoviral vectors: not to be sneezed at. *Gene Ther.* 1998; **5**:725-727.
203.Seibler J and Bode J. Double-Reciprocal Crossover Mediated by FLP-Recombinase: A Concept and an Assay. *Biochemistry* 1997; **36**:1740-1747.
204.Serfling E, Lubbe A, Dorsch-Hasler K and Schaffner W. Metal-dependent SV40 viruses containing inducible enhancers from the upstream region of metallothionein genes. *EMBO J.* 1985; **4**:3851-3859.
205.Shaw P *et al.* Induction of apoptosis by wild type p53 in a human colon derived carcinoma cell line. *Proc. Natl. Acad. Sci. USA* 1992; **89**:4495-4499.
206.Shaw S *et al.* Two antigen-independent adhesion pathways used by human cytotoxic T-cell clones. *Nature* 1986; **323**:262-264.
207.Sideras P and Smith CI. Molecular and cellular Aspects of X-linked Agammaglobulinaemia. *Advances in Immunology* 1995; **59**:135-223.
208.Sideras P *et al.* Genomic organisation of mouse and human Bruton's agammaglobulinemia tyrosine kinase (Btk) loci. *J. Immunol.* 1994; **153**:5607-5617.
209.Sidransky E, Sheerer D and Ginns EI. Gaucher Disease in the neonate: A Distinct Gaucher Phenotype Is Analogous to a Mouse Model Created by Targeted Disruption of the Glucocerebrosidase Gene. *Paediatric Research* 1992; **32**:494-498.
210.Smale ST and Baltimore D. The "initiator" as a transcription control element. *Cell.* 1989; **57**:103-113.
211.Smith AJH *et al.* A site -directed chromosomal translocation induced in embryonic stem cells by Cre-loxP recombination. *Nature Genetics* 1995; **9**:376-385.
212.Smith CIE *et al.* Expression of Bruton's tyrosine kinase gene, BTK, is selectively down-regulated in T lymphocytes and plasma cells. *J. Immunol.* 1994; **152**:557-565.
213.Springer TA. Adhesion receptors of the immune system. *Nature* 1990; **346**:425-434.
214.Sullenger PA, Gallardo HF, Ungers GE and Gilboa E. Analysis of trans-acting response decoy RNA-mediated inhibition of human immunodeficiency virus type 1 transactivation. *J. Virol.* 1991; **65**:6811-6816.
215.Takizawa H *et al.* Species specific restriction of complement by HRF20 (CD59) generated by cDNA transfection. *Eur. J. Immunology* 1992; **22**:1943-1946.
216.Talbot D and Grosveld F. The 5' HS2 of the β-globin locus control region enhances transcription through the interaction of a multimeric complex binding at two functionally distinct NF-E2 binding sites. *EMBO J.* 1991; **10**:1391-1398.

217. Talbot D, Descombes P and Schibler U. The 5' flanking region of the LAP gene can direct high level, position independent, copy number dependent expression in multiple tissues in transgenic mice. *Genes and Development* 1994; **6**:1857-1864.
218. Talbot D, Philipsen S, Fraser P and Grosveld F. Detailed analysis of the site 3 region of the human β-globin dominant control region. *EMBO J.* 1990; **9**:2169-2178.
219. Tapscott AM, Miller AD, Olson JM, Berger MS, Groudine M and Spence AM. Gene therapy of rat 9L gliosarcoma tumours by transduction with selectable genes does not require drug selection. *Proc. Natl. Acad. Sci. USA* 1994; **91**:8185-8189.
220. Taramelli R *et al.* Gamma delta beta-thalassaemias 1 and 2 are the result of 100 kbp deletion in the human beta-globin cluster. *Nucleic Acids Res.* 1986; **14**:7017-7029.
221. Thomas J *et al.* A missense mutation in the X-linked agammaglobulinemia gene colocalizes with the mouse X-linked immunodeficiency gene. *Science* 1993; **261**:355-358.
222. Townsend SE and Allison JP. Tumour rejection after direct costimulation of $CD8^+$ cells by B7 transfected melanoma cells. *Science* 1993; **259**:368-370.
223. Trojan J *et al.* Loss of tumorigenicity of rat glioblastoma directed by episome based antisense cDNA transcription of insulin like growth factor. *Proc. Natl. Acad. Sci. USA* 1992; **89**:4874-4878.
224. Trubetskoy VS, Torchilin VP, Kennel SJ and Huang L. Use of N-terminal modified Poly (L-lysine)-antibody conjugate as a carrier for targeted gene delivery in mouse lung endothelial cells. *Bioconjugate Chem.* 1992; 3:323-327.
225. Tsien JZ *et al.* Subregion-and cell type-restricted gene knockout in mouse brain. *Cell* 1996; **87**:1317-1326.
226. Tsuji S *et al.* A mutation in the human glucocerebrosidase gene in neuronopathic Gaucher's disease. *N. Engl. J. Med.* 1987; **316**:570-575.
227. Tsukada S *et al.* Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. *Cell* 1993; **72**:279-290.
228. Tuan D, SolomonW, Li Q and London IM. The "beta-like-globin" gene domain in human erythroid cells. *Proc. Natl.Acad. Sci. USA.* 1985; **82**:6384-6388.
229. Tybulewicz VLJ *et al.* Animal model of Gaucher's disease from targeted disruption of the mouse glucocerebrosidase gene. *Nature* 1992; **357**:407-410.
230. Uchida N *et al.* HIV, but not murine leukemia virus, vectors mediate high efficiency gene transfer into freshly isolated G0/G1 human hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA* 1998; **95**:11939-11944.
231. Vetrie D. *et al.* The gene involved in X-linked agammaglobulinemia is a member of the *src* family of protein tyrosine kinases. *Nature* 1993; **361**:226-233.
232. Vidal F, Sage J, Cuzin F and Rassoulzadegan M. Cre expression in primary spermatocytes: a tool for genetic engineering of the germ line. *Mol. Reprod. Dev.* 1998; **5**:274-280.
233. Vihinen M *et al.* Btkbase, mutation database for X-linked agammaglobulinemia (XLA). *Nucleic Acids Res.* 1998; **26**:244-250.
234. Wagner E, Plank C, Zatloukal K, Cotten M and Birnstiel ML. Influenza virus hcamagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: towards a synthetic virus-like gene transfer vehicle. *Proc. Natl. Acad. Sci. USA* 1992; **89**: 7934-7938.

235. Wagner E, Zenke M, Cotten M, Beug H and Birnstiel ML. Transferrin-polycation conjugates as carriers for DNA uptake into cells. *Proc. Natl. Acad. Sci. USA* 1990; **87**:3410-3414.
236. Wang Y *et al.* A locus Control Region Adjacent to the Human Red and Green Visual Pigment Genes. *Neuron* 1992; **9**:429-440.
237. Weber F and Schaffner W. Simian virus 40 enhancer increases RNA polymerase density within the linked gene. *Nature* 1985; **315**:75-77.
238. Weinthal J. *et al.* Expression of human glucocerebrosidase following retroviral vector-mediated transduction of murine hematopoietic stem cells. *Bone Marrow Transplant* 1991; **8**:403-412.
239. White DJG *et al.* Expression of human decay accelerating factor or membrane cofactor protein genes on mouse cells inhibits lysis by human complement. *Transplan. Proc.* 1992; **24**:474-476.
240. Whitman ED, Tsung K, Paxson J and Norton JA. In vitro and in vivo kinetics of recombinant vaccinia virus cancer gene therapy. *Surgery* 1994; **116**:183-188.
241. Wienhues U, Hosokawa K, Hoveler A, Siegmann B and Doerfler W. A novel method for transfection and expression of reconstituted DNA-protein complexes in eukaryotic cells. *DNA* 1987; **6**:81-89.
242. Wilke M, Fortunati E, van den Broek M, Hoogeveen AT and Scholte BJ. Efficacy of a peptide-based gene delivery system depends on mitotic activity. *Gene Ther.* 1996; **3**:1133-1142.
243. Willemsen *et al.* A biochemical and ultrastructural evaluation of the Gaucher phenotype developing from targeted disruption of the murine glucocerebrosidase gene. *Molec. and Chem. Neurophat.* 1995; **24**:179-192
244. Wilson C, Bellen HJ and Gehring WJ. Position effects on eucaryotic gene expression. *Annu. Rev. Cell. Biol.* 1990; **6**:679-714.
245. Wilson JM, Grossman M, Cabrera JA, Wu CH and Wu GY. A novel mechanism for achieving transgene persistence in vivo after somatic gene transfer into hepatocytes. *J. Biol. Chem.* 1992; **267**:11483-11489.
246. Winfield SL, Tayebi N, Martin BM, Ginns EI and Sidransky E. Identification of Three Additional Genes Contiguous to the Glucocerebrosidase Locus on Chromosome 1q21: Implications for Gaucher Disease. *Genome Research* 1997; **7**:1020-1026.
247. Wolf JA *et al.* Grafting fibroblast geneticaly modified to produce L-dopa in trat model of Parkinson's disease. *Proc. Natl. Acad. Sci. USA*.1989; **86**:9011-9014.
248. Wolff JA *et al.* Direct gene transfer into mouse muscle in vivo. *Science* 1990; **247**:1465-1468.
249. Wu GY and Wu CH. Receptor-mediated delivery and expression in vivo. *J. Biol. Chem.* 1988; **263**: 14621-14624.
250. Wu GY and Wu CH. Receptor-mediated in vitro gene transformation by soluble DNA carrier system. *J. Biol. Chem.* 1987; **262**:4429-4432.
251. Wu GY *et al.* Receptor-mediated gene delivery in vivo. *J. Biol. Chem.* 1991; **266**: 14338-14343.
252. Yanez RJ and Porter. Therapeutic gene targeting. *Gene therapy* 1998; **5**: 149-159.
253. Yang Y, Haecker SE, Su Q and Wilson JM. Immunology of gene therapy with adenoviral vectors in mouse skeletal muscle. *Hum. Mol. Genet.* 1996; **5**:1713-1726.

254. Yang Y, Xiang Z, Ertl HCJ and Wilson JM. Upregulation of class I MHC antigens by interferon-γ is necessary for the T cell-mediated elimination of recombinant adenovirus infected hepatocytes *in vivo*. *Proc. Natl. Acad. Sci. USA* 1995; **92**:7257-7261.
255. Yao SN *et al.* Expression of human factor IX in rat capillary endothelial cells: Toward somatic gene therapy for haemophilia B. *Proc. Natl. Acad. Sci. USA* 1991; **88**:8101-8105.
256. Yoon K, Cole-Srauss A and Kmiech E. Targeted gene correction of episomal DNA in mammalian cells mediated by chimeric RNA-DNA oligonucleotide. *Proc. Natl. Acad. Sci. USA*.1996; **93**:2071-2076.
257. Yoshimoto Y *et al.* Astrocytes retrovirally transduced with BDNF elicit behavioral improvement in rat model of Parkinson's disease. *Brain Res.* 1995; **691**:25-36.
258. Yu M, Poeschla E and Wong-Staal F. Progress towards gene therapy for HIV infection. *Gene Ther.* 1994; **1**:13-26.
259. Zabner J, Fasbender AJ, Moninger T, Poellinger KA and Welsh MJ. Cellular and molecular barriers to gene transfer by cationic lipid. *J. Biol. Chem.* 1995; **270**:18997-19007.
260. Zenke M *et al.* Receptor-mediated endocytosis of transferrin-polycation conjugates: an efficient way to introduce DNA into hematopoietic cells. *Proc. Natl. Acad. Sci. USA* 1990; **87**:3655-3659.
261. Zhang Y, Mukhopadhyay T, Donehower LA, Georges RN and Roth JA. Retroviral vector mediated transduction of K-ras antisense RNA into human lung cancer cells inhibits expression of the malignant phenotype. *Hum. Gene Ther.* 1993; **4**:451-460.
262. Zhumabekov T, Corbella P, Tolaini M and Kioussis D. Improved version of a human CD2 minigene based vector for T cell-specific expression in transgenic mice. *Journal of Immunological methods* 1995; **185**:133-140.
263. Zimran AJ *et al.* Prediction of severity of Gaucher's disease by identification of mutations at DNA level. *Lancet* 1989; **2**:349-352.
264. Zlokovic BV and Apuzzo MLJ. Cellular and Nolecular Neurosurgery: Pathways from Concept to Reality-Part I: Target Disorders and Concept Approaches to gene therapy of the Central Nervous System. *Neurosurgery* 1997; **40**:789-803.

Chapter 2

The expression of bacterial nitroreductase in transgenic mice results in specific cell killing by the prodrug CB1954

D Drabek, J Guy, R Craig and F Grosveld

Gene Therapy 4: 93-100, 1997

The expression of bacterial nitroreductase in transgenic mice results in specific cell killing by the prodrug CB1954

D Drabek[1], J Guy[1], R Craig[2] and F Grosveld[1]
[1]*Department of Cell Biology and Genetics, Institute of Cell Biology, Rotterdam, The Netherlands; and* [2]*Therexsys, The Science Park, University of Keele, UK*

The enzyme nitroreductase, isolated from Escherichia coli B, converts CB1954 ((5-aziridin-1-yl)-2,4-dinitro-benzamide) into a cytotoxic DNA interstrand cross-linking agent. The E. coli B gene (nfnB, NTR) encoding nitroreductase (NTR) was cloned into eukaryotic expression vectors. When driven by a CMV promoter, 5–10% of the stably transfected mouse fibroblasts expressed the NTR enzyme. These cells were killed at a concentration of 20 μM CB1954 in comparison to nonexpressing cells which were killed at a much higher concentration (500 μM). We subsequently generated transgenic mice to test the prodrug system in vivo. Nitroreductase was expressed specifically in T cells driven by the control elements of the human CD2 locus. Upon CB1954 treatment, transgenic mice show extensive cell depletion in thymus and spleen (14–16% of normal cell numbers), whereas all other tissues are unaffected by prodrug administration. These results raise the possibility of using the NTR gene in anticancer therapy.

Keywords: *CB1954; locus control region; nitroreductase; transgene*

Introduction

Prodrugs are substances which are not toxic, but can be activated to produce cytotoxic derivatives. For the purposes of anticancer therapy, this activation should be specifically associated with tumour cells. The monofunctional alkylating agent CB1954 (5-aziridin-1-yl)-2,4 dinitrobenzamide) was synthesised in the late 1960s[1] and was found to be highly potent and selective against the Walker 256 rat tumour line.[2] DNA cross-link formation in affected cells was a result of the bioactivation of the drug by the enzyme DT diaphorase (NAD(P)H dehydrogenase (quinone)) in the Walker cells which reduces the 4-nitro group of CB1954. The product of this reaction is a difunctional alkylating agent, 5-aziridin-1-yl-4-hydroxylamino-2-nitrobenzamide. This molecule can not cross-link DNA itself, but is further activated by a nonenzymatic reaction with thioesters (such as coenzyme A).[3] However, except for a few rat hepatoma and hepatocyte cell lines, other rat cells are not known to be sensitive to CB1954, in agreement with their low levels of DT diaphorase, and human cells, even those expressing significant levels of the human DT diaphorase, are not sensitive due to differences in the kinetics of CB1954 reduction by these two forms of the enzyme.[4]

Apart from Walker cells, sensitivity to CB1954 has also been reported in bacteria.[5] The toxicity and mutagenicity of CB1954 was greatly reduced in a nitroreductase-deficient strain of *E. coli*,[6] suggesting that CB1954 can also be activated by nitroreduction in *E. coli* in an analagous manner to its bioactivation in Walker cells. A nitroreductase enzyme (NTR) has been isolated from *E. coli* B.[7] The enzyme is an FMN-containing flavoprotein with molecular mass of 24 kDa and requires either NADH or NADPH as a cofactor. Indeed, it was found that *E. coli* nitroreductase is capable of reducing CB1954, that it is a more active enzyme (k_{cat} = 360/min for CB1954 *versus* k_{cat} = 4/min for Walker cell DT diaphorase) and also has a lower K_m for NADH (6 *versus* 75 μM).[8] Thus *E. coli* nitroreductase is potentially a suitable candidate for cancer chemotherapy programmes such as antibody-directed enzyme prodrug therapy (ADEPT), where a tumour-selective monoclonal antibody is conjugated to an enzyme that is capable of bioactivating a prodrug.[9] Isolation of the gene encoding nitroreductase, *nfnB* (NTR)[10] also raises the possibility of gene-directed enzyme prodrug therapy (GDEPT), where a gene is introduced into cells, and if expressed can activate a prodrug. Expression can be directed to the tissue(s) of interest by using an appropriate locus control region (LCR), such as described for the human β-globin gene locus,[11] human CD2,[12] chicken lysozyme,[13] human S100β[14] and mouse MHC class II.[15]

Here, we describe the expression of NTR in eukaryotic cells. Initially, the enzyme was expressed in a mouse fibroblast cell line. Expressing cells could be killed by the application of the prodrug to the culture. To show that the prodrug system works *in vivo* and exclusively on targeted tissues, we generated transgenic mice expressing nitroreductase in T cells but not in other tissues. Upon prodrug treatment there is a dramatic depletion of cells in the thymus and spleen of transgenic animals, whereas nonlymphoid tissues are normal and comparable with untreated transgenic or wild-type mice.

Correspondence: F Grosveld, Department of Cell Biology and Genetics, Institute of Cell Biology, PO Box 1738, 3000DR Rotterdam, The Netherlands
D Drabek and J Guy contributed equally to this work
Received 6 September 1996; accepted 18 October 1996

Results

Transfection of mouse L cells with pCMV/NTR results in expression of nitroreductase in fewer than 10% of cells in culture

The nitroreductase gene (*nfnB*) was isolated from *E. coli* B as a PCR fragment of approximately 750 bp by N Minton (PHLS, Porton Down, UK). After improving the ribosome binding site and removing an upstream ATG we cloned this fragment into mammalian expression cassettes (see Materials and methods).

Initial tests were done on stably transfected mouse L cells with the construct pCMV/NTR (Figure 1a). After selection for 7 days at 800 μg/ml G418, 10 independent clones were picked and kept further at 400 μg/ml G418.

The clones were analysed for expression at the RNA level. An S1 nuclease protection assay using a 3′ end probe showed a protected fragment of the expected size (213 bp) in all clones (see Figure 2a). Knowing that there is a stable message, we performed indirect immunofluorescence using a rabbit polyclonal antinitroreductase antibody. By counting NTR positive cells in randomly chosen fields, it was estimated that none of the clones expressed nitroreductase in more than 10% of cells. Most of the clones showed fewer than 5% of cells expressing at one time. This phenomenon is often observed when genes are integrated in the DNA of cultured cells and has been discussed but not completely explained. It may be that in the absence of a sufficiently strong enhancer, heterochromatin can spread from flanking areas into the integrated construct leading to extensive silencing of the gene.[16]

CB1954 causes gross changes in morphology and eventual cell death in cells expressing nitroreductase

The fact that only a small percentage of cells were expressing NTR made quantitative analysis of the effect of CB1954 impossible, so we used a qualitative approach. pCMV/NTR transfected L cells were exposed to the prodrug for various times, and prodrug concentrations between 10 μM and 500 μM. Untransfected cells were not affected by CB1954 below a concentration of 500 μM, whereas cells expressing nitroreductase started to show signs of toxicity at 10 μM and were all affected at 20 μM. Effects of the prodrug on cells expressing nitroreductase

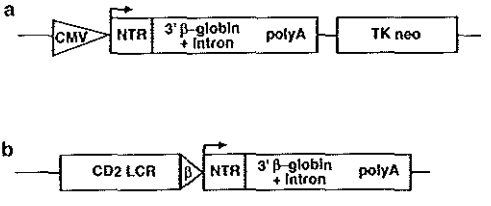

Figure 1 Nitroreductase expression constructs. (a) pCMV/NTR. The human cytomegalovirus (CMV) immediate-early gene promoter/enhancer was used to drive expression of nitroreductase. To enhance the stability of the mRNA, the 0.75-kb NTR gene was linked to a 2.8-kb fragment from the 3′ end of the human β globin gene, providing the final intron, 3′UTR and polyadenylation signals. (b) pCD2/NTR. To drive expression of nitroreductase specifically in T cells, the NTR gene was coupled to a 2.1-kb HindIII fragment containing the human CD2 locus control region and a β globin promoter fragment of approximately 500 bp.

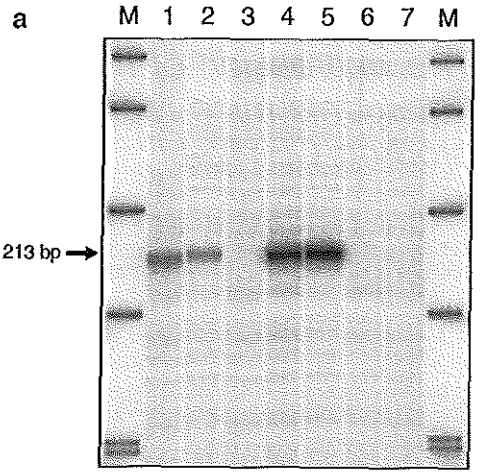

Figure 2 Expression of NTR in murine cells. (a) S1 nuclease protection analysis of NTR mRNA in transfected L cells and transgenic mouse thymocytes. The human 3′β globin EcoRI–PstI probe gives a protected fragment of 213 bp after digestion of hybridised RNA with S1 nuclease, due to the presence of 3′ β globin sequences in the constructs. For each sample 10 μg of total cellular RNA was hybridised to approximately 15 ng of ^{32}P-labelled probe. Lanes 1 and 2 pCMV/NTR transfected L cells, clones 9 and 10; lane 3, untransfected L cells; lanes 4 and 5, thymocytes from CD2/NTR 14 transgenic mice; lanes 6 and 7, thymocytes from non-transgenic mice; M, marker pUC19 cut with MspI and end-labelled with ^{32}P.

are gross changes in morphology, including 10- to 20-fold enlargement of the cells, enlargement of nuclei, multiple nuclei and ultimately nuclear disintegration (Figure 3). After 7 days of exposure to the prodrug, these cells disappear from the culture. All cells expressing nitroreductase were affected in this way, whereas nonexpressing cells in the same culture appeared to be normal.

The effect of the prodrug on the cells was irreversible after 2 days of treatment, since returning these cells to normal culture conditions did not lead to their recovery.

CD2/NTR transgenic mice express nitroreductase specifically in T cells and thymocytes

Five founder mice carrying the CD2/NTR construct (Figure 1b) were obtained, and lines of each were established. Different tissues were tested for expression of the transgene by S1 nuclease protection analysis (data not shown). The transgene expression, when detected (two out of five lines) was confined to the expected cell types: T cells and thymocytes (see Figure 2a).

Further experiments were done on line 14 which has three copies of the transgene and the highest level of nitroreductase enzyme as determined by Western blotting (data not shown). FACS analysis on single cell suspensions from thymus showed that all CD2-positive cells express NTR, but that there are two populations of cells: high and low expressors (Figure 2b). The level of NTR expression correlates with that of CD2. In the spleen, NTR is expressed only in T cells. Although CD2 positive in the mouse, B cells do not express the transgene due to the use of the human CD2 LCR. Transgene expression was detected only in B220$^-$, IgM$^-$, CD2$^+$ and CD3$^+$ cells.

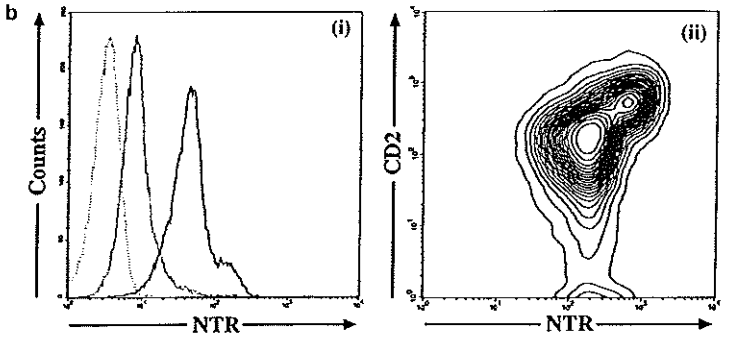

Figure 2 (b) FACS analysis of NTR expression in transgenic mouse thymus. (i) Single-staining with anti-nitroreductase polyclonal antibody on thymocytes from: CD2/NTR transgenic mouse, secondary antibody only (dotted line); non-transgenic mouse, primary and secondary antibodies (dashed line); CD2/NTR transgenic mouse, primary and secondary antibodies (solid line). (ii) Double staining of CD2/NTR transgenic mouse thymocytes with antinitroductase (FITC) and anti-CD2 (phycoerythrin, PE) antibodies.

CD2/NTR transgenic mice show depletion of T cells upon CB1954 treatment

In a pilot experiment, nontransgenic mice (FVB) were injected i.p. with 10, 20 and 50 mg/kg/day CB1954 (dissolved in 20% DMSO in PBS) for 5 consecutive days. These doses were chosen after considering the toxicology and pharmacokinetic data determined for BALB/c and C3H/He mice.[17-19] All animals receiving the highest dose were very sick with multiple organs affected. In particular the intestine (containing NTR-positive bacteria) was severely affected. The lowest dose had absolutely no effect, whereas the middle dose caused a slight reduction in weight compared to nontreated animals but no other visible problems. Hence we chose this dose of 20 mg/kg/day to test the transgenic animals.

Three-week-old transgenic and wild-type mice were injected i.p. with CB1954, solvent only (10% DMSO in PBS), or not injected at all. Two nontreated groups consisted of seven mice each, two solvent-treated groups consisted of nine mice each and two CB1954-treated groups consisted of 13 mice each. Animals received 20 mg/kg of CB1954 per day for 5 consecutive days. Three hours after the last injection mice were killed, different organs macroscopically examined and red and white blood cell counts determined.

Visually, there was an obvious difference in the size of two organs: thymus and spleen. Thymuses and spleens from CB1954-treated transgenics were much smaller than in all other controls. Single cell suspensions were made from these two organs and the total number of cells counted. CB1954-treated transgenic thymuses and spleens had 14–16% of the cell numbers present in controls (Figure 4a).

An assay for apoptosis was performed on single cell suspensions from the thymus using the *In Situ* Cell Death Detection kit, Fluorescein (Boehringer Mannheim, Mannheim, Germany). A significantly higher percentage of apoptotic cells was seen in CB1954-treated transgenic cells as compared to control groups: 15% *versus* 0.5–4% (Figure 4b). FACS analysis on thymocytes showed that there is no change in the normal ratio between CD4CD8 double positive, CD4CD8 double negative and single CD4 and CD8 positive cells in treated transgenic mice (data not shown). Surprisingly, in the spleen the B to T cell ratio is not significantly changed, implying that B cells are also killed.

The red blood cell count was normal and comparable to controls. The white blood cell count was slightly decreased, as expected.

In another experiment, animals were killed 5 days after the last prodrug or solvent treatment instead of 3 h later. It was seen that thymuses and spleens returned to their normal size during this prodrug-free period (data not shown). We therefore conclude that the progenitor cells which do not express CD2 (or the transgene) have not been affected and are able to repopulate the spleen and thymus.

Discussion

CB1954 is converted by nitroreduction from a monofunctional to a difunctional agent. Induced DNA interstrand cross-links are formed with very high frequency, and can contribute up to 70% of total lesions.[20] Interstrand or intrastrand cross-link formations are generally accepted as the most toxic lesions, inhibiting DNA function and eventually resulting in cell death.

Our results showed that eukaryotic cells are able to express the *E. coli* nitroreductase gene and judging by the end result (cell death), are capable of reducing the substrate (CB1954), inducing DNA interstrand crosslink formation leading to cell death. Changes in morphology of the cells expressing the enzyme upon CB1954 exposure can be explained by a block in DNA synthesis coinciding with a cessation of cell division, although RNA and protein synthesis continue, as evidenced by a continued increase in cell mass. A similar phenomenon was observed in *E. coli* B_{s-1}.[5]

Our data show that some L cells expressing nitroreductase were able to survive exposure to 10 μM but not 20 μM CB1954, suggesting that at a certain concentration they were no longer capable of repairing the DNA adducts formed as a result of the bioactivation of the prodrug. Cells failed to replicate their DNA and proceed through successive cycles of cell division.

It has been shown that CB1954 forms DNA interstrand

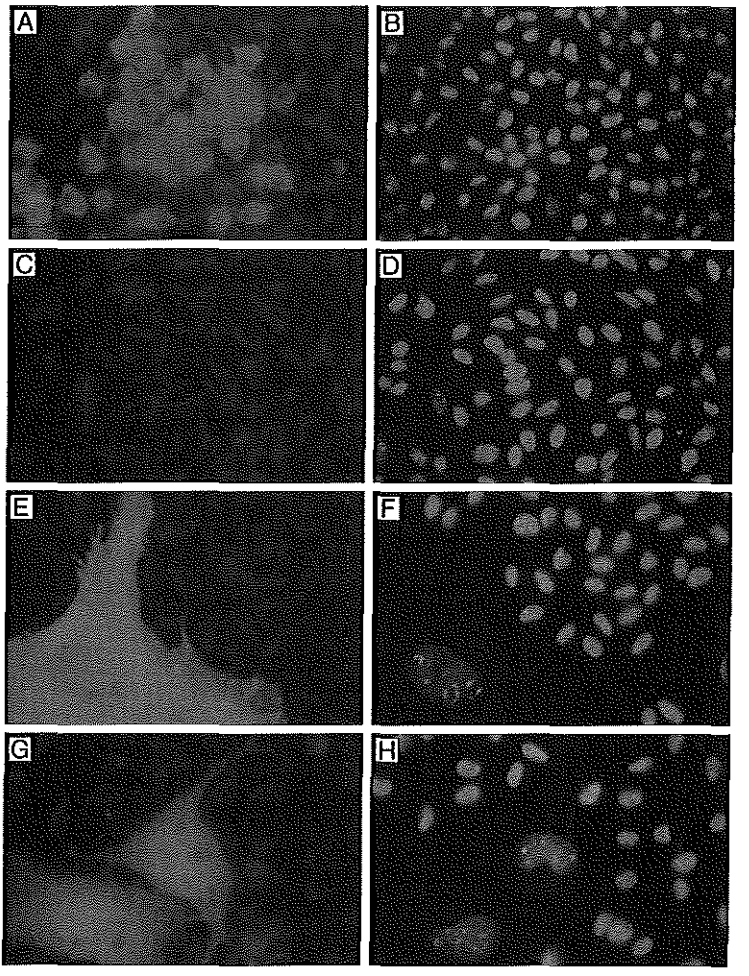

Figure 3 Treatment of NTR transfected mouse L cells with CB1954. L cells stably transfected with pCMV/NTR and untransfected L cells were treated with CB1954 for various periods of time and at various concentrations. After 5 days, cells were removed from the cultures and analysed for expression of NTR. Cells were stained, mounted and photographed using a ×63 objective lens. (a, c, e, g) Indirect immunofluorescence using a rabbit polyclonal antibody against nitroreductase, with FITC-conjugated goat-anti rabbit Ig as secondary antibody. (b, d, f, h) Nuclear staining of the same fields of cells with 4'-6-diamidino-2-phenylindole (DAPI). (a, b) L cells transfected with pCMV/NTR and grown in the absence of CB1954. (c, d) Untransfected L cells grown in the presence of 50 μM CB1954 for 5 days. (e, f) pCMV/NTR transfected L cells grown in presence of 50 μM CB1954 for 5 days. (g, h) pCMV/NTR transfected L cells grown in the presence of 50 μM CB1954 for 2 days and then in prodrug-free medium for a further 3 days.

cross-links in Walker cells but not in the insensitive Chinese hamster V79 cells. Co-culturing V79 cells with Walker cells in the presence of CB1954 results in sensitisation of the V79 cells towards CB1954.[21] The toxic metabolite was shown to be soluble and diffusible by its ability to be cytotoxic, and to cross-link DNA in V79 cells. We were expecting to see a similar effect in cell culture experiments. The effect of the prodrug on NTR nonexpressing cells due to the presence of NTR expressing cells in the very same culture was not seen. This could be explained by the very small percentage of cells expressing the enzyme (5–10%), in agreement with the results of Bridgewater *et al*[22] who found that 30–50% of NTR positive cells are required for 90% overall cell killing, using transduced NIH 3T3 cells. The relatively short half-life of the active metabolite, a lack of intimate cellular contact between expressing and nonexpressing cells or L cells being intrinsically more resistant to CB1954 than V79 cells are other possibilities.

In order to assess the proposed therapy system *in vivo*

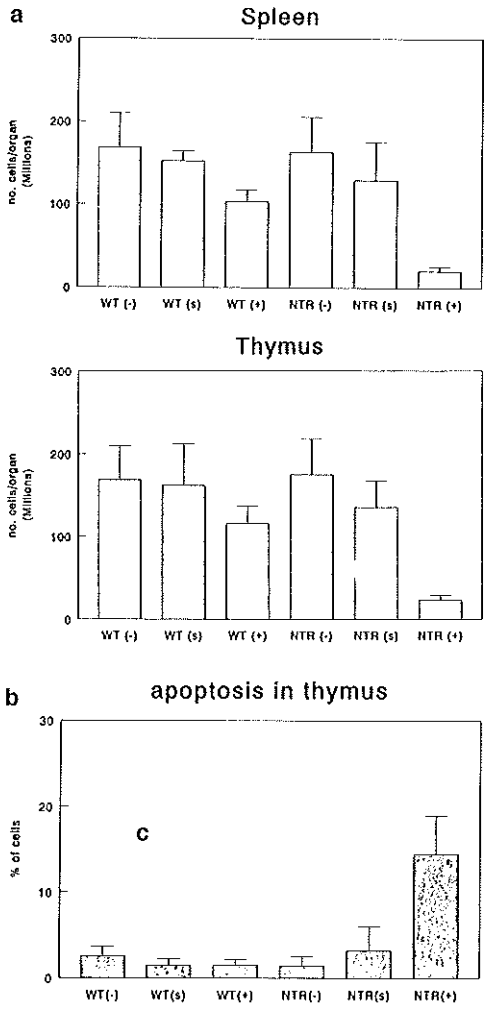

Figure 4 Reduction in cell numbers and an increase in apoptosis is seen after treatment of CD2/NTR transgenic mice with CB1954. (a) Cell numbers. Thymuses and spleens from transgenic and nontransgenic mice were made into single-cell suspensions and counted on a Coulter cell counter. (b) Apoptosis. Cells from the thymuses of treated and untreated mice were assayed for apoptosis using the In Situ Cell Death Detection Kit (Boehringer) which labels breaks in DNA, such as occur during apoptosis, with FITC. The percentage of positively labelled cells was determined using Cell Quest version 2.0 (Graphpad Software, San Diego, CA, USA). NTR, CD2/NTR transgenic mice; WT, nontransgenic mice; (–) no treatment; (s) treatment with solvent only (10% DMSO in PBS); (+) treatment with CB1954 at 20 mg/kg/day. The confidence limits were determined using a two-tailed Student t test, with $P = 0.05$.

an animal model was tested. The aim was to direct the expression of NTR to a specific tissue and to achieve killing of the chosen target. We directed NTR expression to T cells by the use of human CD2 LCR elements, previously shown to be sufficient for T cell-specific, copy number-dependent, integration site-independent expression in transgenic mice.[23] The transgenic mice which were generated express the nitroreductase in T cells, as predicted. However, it was not expressed in all of the transgenic lines, which may indicate that the 2.1 kb 3′ LCR fragment of the CD2 gene is not sufficient for integration-independent expression of the transgene. Recently, an improved version of a human CD2 mini-gene-based vector has been made for T cell-specific expression in transgenic mice which in addition to the 3′ LCR includes 5′ sequences and the first intron of the CD2 gene.[24] CD2 is one of the earliest markers to appear on the surface of developing thymocytes and in man it is present only on thymocytes and circulating T cells. In the mouse, however, it is present on both T and B cells. As we have used human CD2 locus control elements, expression of the transgene is detected only in T cells.

Toxicity studies on CB1954 were carried out to establish acceptable doses for single and multiple administration in BALB/c and C3H/He mouse strains.[18] Our pilot experiments indicated that the FVB mouse strain used in this study is somewhere between BALB/c and C3H/He in its sensitivity towards CB1954. We used two-thirds of the LD_{10} for a multiple-dose regimen which was established in BALB/c mice (31 mg/kg/day). Weight loss due to enteritis would be the first sign of toxicity, as previously described in rats[2] and in mice.[18] More than half of our experimental animals started to lose weight on day 4 of the experiment, and when killed on day 5 these showed a loss of 5 to 15%, suggesting that the threshold of toxicity was reached. The toxic effect is seen in the spleen of wild-type mice treated with the prodrug where, although the appearance is normal, there is a slight reduction in cell numbers when compared with untreated mice. Since the prodrug was administered intraperitoneally, the spleen was accessible almost immediately after the injection and will have experienced a much higher local concentration of prodrug than the thymus. Nevertheless, our results clearly demonstrate selective killing in lymphoid organs of CD2/NTR transgenic mice. As mentioned before, in fibroblast transfection studies it has been shown that 30–50% expressing cells are enough to kill 90% of all cells.[22] In the spleen, T cells contribute about 40% of the total number of cells, and we speculate that the reduction in B cell numbers is due to a similar (bystander) effect *in vivo*.

It was previously shown that several different chemotherapeutic agents can trigger the apoptotic process.[25] Apoptosis, by morphological criteria, involves chromatin condensation and margination, cell shrinkage, membrane blebbing and formation of nuclear fragments or apoptotic bodies. To see whether CB1954 induces apoptosis in the thymus of transgenic CD2/NTR mice, a TUNEL assay was performed. The results show that only the activated prodrug induces apoptosis in thymocytes.

By using transgenic mice we have shown that only cells expressing the NTR enzyme are able to convert the nontoxic prodrug CB1954 to a cytotoxic drug, resulting in highly specific killing of T cells and thymocytes, while other tissues are unaffected. The apparent killing of NTR

negative B cells in the spleen may result from the high proportion of expressing cells and the proximity of expressing and nonexpressing cells in this organ. The specific killing by nitroreductase/CB1954 is in contrast with cytotoxic agents used in chemotherapy, which in addition to killing tumour cells also affect normal tissues. Nitroreductase is therefore an excellent candidate for gene therapy against tumours. GDEPT has a number of advantages over ADEPT (where the enzyme itself is delivered to cells). The enzyme requires a cofactor (NADH or NADPH) which is present only inside the cell, being rapidly oxidixed and degraded by serum enzymes.[26] Therefore the enzyme needs to be present inside the cell before it can activate the prodrug. Also, specificity can be introduced both by targeted delivery of the gene to the required tissue, and by using tissue-specific elements (LCRs) to drive expression of the enzyme only in the target cells.

Materials and methods

Plasmids (constructs)
The plasmid pJG-1 was constructed by ligation of a 6.1 kb *NotI–ScaI* fragment from PEV-3 (Clare Gooding, Biotechnology Dept, Zeneca, Macclesfield, UK) and a 1.4 kb *NotI–ScaI* fragment from pRc/CMV (Invitrogen BV, Leek, The Netherlands). The resulting plasmid carries a CMV promoter upstream of a multiple cloning site, an intron with RNA splicing sites and a polyadenylation signal from the human β globin gene, β lactamase, and the neomycin resistance gene driven by a HSV-TK promoter. The plasmid pJG-1/NTRΔ*NotI* was constructed by inserting a 0.8 kb *HindIII* fragment carrying the nitroreductase gene from pPM 26 (kindly provided by N Minton) into the *HindIII* site of pJG-1, followed by deletion of a 140 bp *NotI* polylinker fragment between the NTR gene and the splice/polyA site. The final pCMV-NTR construct (Figure 1a) used in tissue culture experiments was created by the removal of a *HindIII–EcoRV* fragment from pJG/NTRΔ*NotI* and replacement by an oligo (aagcttgcggccgccgccagccatggatatc), thereby (1) removing an extra upstream ATG; (2) inserting a consensus ribosome binding site; and (3) introducing a new *NotI* site. A 0.8-kb *NotI* fragment from pCMV/NTR containing the nitroreductase gene was subcloned into the *NotI* site of PEV-3 and a 7.3 kb *ClaI–ScaI* fragment from the resulting plasmid was ligated to a 3.4-kb *ClaI–ScaI* fragment from GSE 1502b (a 2.1-kb *HindIII* fragment of the human CD2 LCR in pBluescript KS$^+$) giving the final pCD2/NTR construct (Figure 1b) used to generate the transgenic mice.

Cell culture, transfection
Mouse L cells were seeded in 10 cm dishes at 1.5×10^6 cells per dish in DMEM/10% FCS. The next day, 3 ml of OPTIMEM-1 (GibcoBRL Life Technologies, Paisley, UK), containing 50 μg of the plasmid pCMV/NTR linearised with *ScaI* was mixed with 3 ml of OPTIMEM-1 containing 100 μl of Lipofectin (GibcoBRL) and added to the cells. Five hours later, 3 ml of DMEM/20% FCS was added to each dish. The next day, the medium was removed and replaced with DMEM/10% FCS containing G418 at a concentration of 800 μg/ml. After 7 days of selection several independent clones were picked and analysed for expression at the RNA and protein level. For further growth the G418 concentration was redued to 400 μg/ml.

CB1954 assay
Cells were seeded at 1×10^5 cells per dish in 10 cm dishes containing sterile glass coverslips, in triplicate. CB1954 was dissolved in DMSO (100 mM stock) and added to the cells at a final concentration of 0, 10, 20, 50, 100, 250 and 500 μM in DMEM medium. Cells were observed for a period of 7 days. As a control untransfected L cells were used under the same conditions. After 2 days a medium change was performed and half the cultures received the original concentration of the prodrug and the other half were grown in prodrug-free medium. Coverslips with cells attached were removed from the culture at day 5 and immunocytochemistry was performed.

Immunofluorescence
The rabbit polyclonal anti-nitroreductase antibody was kindly provided by R Melton and N Michael, Porton Down, UK. Goat anti-rabbit Ig-FITC was purchased from Sigma (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands).

In situ staining: Cells were fixed in 3.7% paraformaldehyde (PFA) in CSK buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES pH 6.0, 3 mM $MgCl_2$, 1 mM EGTA) for 10 min, washed twice and permeabilised for 15 min in 0.5% Triton X-100 in CSK buffer. After two washes in PBS/TWEEN 0.02%, cells were incubated with the anti-nitroreductase antibody (1:200) for 30 min, washed in PBS/TWEEN 0.02% again and incubated with the goat anti-rabbit Ig-FITC antibody (1:100) for 30 min. The cells were washed, the coverslips mounted in DAPI/DABCO/glycerol and analysed under the fluorescence microscope (Leitz).

FACS protocol: A single cell suspension was made in ice cold phosphate-buffered saline pH 7.4 (PBS). Cells were washed in PBS/1%BSA and aliquoted at 10^6 cells per well in a 96-well plate. Cells were first stained with a PE-conjugated anti-CD2 monoclonal antibody (PharMingen, San Diego CA, USA) diluted 1:60 in 50 μl for 10 min at room temperature (RT). They were then washed and fixed in 100 μl 2% PFA for 30 min at RT. Cells were washed in PBS/1%BSA and permeabilised in 0.1% Triton X-100/0.1% sodium citrate for 2 min on ice. After a PBS/1%BSA wash, the cells were incubated with the anti-NTR antibody at 1:100 dilution in PBS/1%BSA for 15 min at RT (10^6 cells per 50 μl in 96-well plate). Cells were washed again and incubated with the goat anti-rabbit Ig-FITC antibody at 1:100 dilution for 15 min at RT (10^6 cells per 50 μl). Cells were washed in PBS/1%BSA, resuspended in 200 μl of PBS/1%BSA/0.02% NaAzide and analysed on a FACS analyser (Becton Dickinson, Sunnyvale, CA, USA). For detection of cell surface markers the following monoclonal antibodies were used on unfixed cells: anti CD2-PE (PharMingen), anti-CD3-FITC (PharMingen), anti-CD4-PE (Becton Dickinson, anti-CD8-biotin,[27] anti-B220-cychrome (PharMingen), anti-IgM-biotin (PharMingen). Cells were incubated for 30 min on ice, washed and incubated where appropriate for 30 min with secondary antibodies (tricolor or PE conjugated streptavidin; Caltag Laboratories, Burlingame, CA, USA).

RNA extraction and S1 protection analysis

Total cellular RNA was extracted as described.[28] Specific hybrid message was detected by S1 nuclease protection analysis using a ^{32}P end-labelled DNA probe (700 bp *EcoRI–PstI* fragment from the human β globin gene) protecting 213 nucleotides. Each hybridisation consisted of 10 μg total RNA and 10–20 ng of probe in a reaction volume of 20 μl. After denaturation at 90°C for 5 min, hybridisation was performed at 53°C for at least 16 h. Subsequent digestion with 100 U S1 nuclease (Boehringer Mannheim) was for 2 h at 25°C in a final volume of 270 μl.

Generation of CD2/NTR transgenic mice

A 6-kb *XbaI* fragment was purified from plasmid sequences by gel electrophoresis, prepared for injection as previously described[29] and injected into fertilised oocytes (FVB × FVB) at a concentration of 4 ng/μl. Injected eggs were transferred into pseudopregnant mice and transgenic offspring identified by Southern blot analysis of tail DNA.[30]

In vivo CB1954 protocol

Three-week-old mice (heterozygous for the transgene, and nontransgenic FVB) were injected i.p. with CB1954 for 5 consecutive days at 20 mg/kg/day. CB1954 was dissolved in 10% DMSO in PBS. Control animals from both groups were injected with solvent only, or not injected at all. Mice were killed 3–4 h after the last injection. Single cell suspensions were made from thymuses and spleens in PBS as described.[31] The total number of cells per organ was determined using a Coulter cell counter.

Detection of apoptotic cells

$1–2 \times 10^6$ cells were washed twice in PBS/1% BSA in a V-bottomed 96-well plate. Cells were fixed in 2% paraformaldehyde in PBS, permeabilised with 0.1% Triton X-100/0.1% sodium citrate and labelled by a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) reaction using the *In Situ* Cell Death Detection Kit, Fluorescein (Boehringer Mannheim). Apoptotic cells were detected by flow cytometry (FACS analyser, Becton Dickinson).

Acknowledgements

We thank R Hendriks for help with the FACS analysis, N Minton, N Michael and R Melton for providing the NTR PCR clone and antiserum, Caroline Springer and the Cancer Research Campaign, UK, for provision of CB1954 and L Braam for care of experimental animals. D.D. and J.G. are supported by Therexsys, UK.

References

1 Khan A, Ross WCJ. Tumour growth inhibitory nitrophenylaziridines and related compounds: structure-activity relationships. *Chem Biol Interact* 1969; 1: 27–47.

2 Cobb LM. Toxicity of the selective antitumour agent 5-aziridino-2,4 dinitrobenzamide in rat. *Toxicol Appl Pharamcol* 1970; 17: 231–238.

3 Knox RJ, Friedlos F, Marchbank T, Roberts JJ. Bioactivation of CB1954: reaction of the active 4-hydroxylamino derivative with thioesters to form the ultimate DNA–DNA interstrand crosslinking species. *Biochem Pharmacol* 1991; 42: 1691–1697.

4 Boland MP, Knox RJ, Roberts JJ. The differences in kinetics of rat and human DT diaphorase result in a differential sensitivity of derived cell lines to CB1954 (5-(aziridin-1yl)-2,4-dinitrobenzamide). *Biochem Pharmacol* 1991; 41: 867–875.

5 Venitt S. The differential response of resistant and sensitive strains of *Escherichia coli* to the cytotoxic effects of 5-aziridino-2,4-dinitrobenzamide (CB 1954). *Chem Biol Interac* 1971; 3: 177–191.

6 Venitt S, Crofton-Sleigh C. The toxicity and mutagenicity of the anti-tumour drug 5-aziridino-2,4-dinitrobenzamide (CB1954) is greatly reduced in a nitroreductase deficient strain of *E. coli*. *Mutagenesis* 1987; 2: 371–381.

7 Anlezark GM *et al*. The bioactivation of 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954)-I. *Biochem Pharmacol* 1992; 44: 2289–2295.

8 Knox RJ *et al*. The bioactivation of 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954)-II. *Biochem Pharmacol* 1992; 44: 2297–2301.

9 Bagshawe KD. Antibody-directed enzyme prodrug therapy (ADEPT). *Biochem Soc Transac* 1990; 18: 750–751.

10 Michael NP, Brehm JK, Anlezark GM, Minton NP. Physical characterisation of the *Escherichia coli* B gene encoding nitroreductase and its overexpression in *Escherichia coli* K12. *FEMS Microbiol Lett* 1994; 124: 195–202.

11 Grosveld F, Blom van Assendelft G, Greaves DR, Kollias G. Position-independent, high-level expression of the human β-globin gene in transgenic mice. *Cell* 1987; 51: 975–985.

12 Greaves DR, Wilson FD, Lang G, Kioussis D. Human CD2 3'-flanking sequences confer high-level, T cell-specific, position-independent gene expression in transgenic mice. *Cell* 1989; 56: 979–986.

13 Bonifer C, Vidal M, Grosveld F, Sippel AE. Tissue specific and position independent expression of the complete gene domain for chicken lysozyme in transgenic mice. *EMBO J* 1990; 9: 2843–2848.

14 Friend WC *et al*. Cell-specific expression of high levels of human S100β in transgenic mouse brain is dependent on gene dosage. *J Neurosci* 1992; 12: 4337–4346.

15 Carson S, Wiles MV. Far upstream regions of class II MHC Eα are necessary for position-independent, copy-dependent expression of Eα transgene. *Nucleic Acids Res* 1993; 21: 2065–2072.

16 Walters MC *et al*. Transcriptional enhancers act in *cis* to suppress position-effect variegation. *Genes Dev* 1996; 10: 185–195.

17 Workman P *et al*. CB1954 revisited. I: disposition kinetics and metabolism. *Cancer Chemother Pharmacol* 1986; 16: 1–8.

18 Workman P *et al*. CB1954 revisited. II: toxicity and antitumour activity. *Cancer Chemother Pharmacol* 1986; 16: 9–14.

19 Sheard CE, Double JA, Berenbaum MC. The sensitivity to chemotherapeutic agents of a rat tumour grown in immunosuppressed mice. *Br J Cancer* 1971; 25: 838–844.

20 Friedlos F, Quinn J, Knox RJ, Roberts JJ. The properties of total adducts and interstrand crosslinks in the DNA of cells treated with CB 1954. *Biochem Pharmacol* 1992; 43: 1249–1254.

21 Knox RJ, Friedlos F, Jarman M, Roberts JJ. A new cytotoxic, DNA interstrand crosslinking agent, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, is formed from 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) by a nitroreductase enzyme in Walker carcinoma cells. *Biochem Pharmacol* 1988; 37: 4661–4669.

22 Bridgewater JA *et al*. Expression of the bacterial nitroreductase enzyme in mammalian cells renders them selectively sensitive to killing by the prodrug CB1954. *Eur J Cancer* 1995; 31: 2362–2370.

23 Lang G *et al*. Deletion analysis of the human CD2 gene locus control region in transgenic mice. *Nucleic Acids Res* 1991; 19: 5851–5856.

24 Zhumabekov T, Corbella P, Tolaini M, Kioussis D. Improved version of a human CD2 minigene based vector for T cell-specific expression in transgenic mice. *J Immunol Meth* 1995; 185: 133–140.

25 Hickman JA. Apoptosis induced by anticancer drugs. *Cancer Metas Rev* 1992; 11: 121–139.

26 Knox RJ, Friedlos F, Boland MP. The bioactivation of CB1954

and its use as a prodrug in antibody-directed enzyme prodrug therapy (ADEPT). *Cancer Metas Rev* 1993; **12**: 195–212.

27 De Bruijn MFTR *et al.* Distinct mouse bone marrow macrophage precursors identified by differential expression of ER-MP12 and ER-MP20 antigens. *Eur J Immunol* 1994; **24**: 2279–2284.

28 Antoniou M. Induction of erythroid-specific expression in murine erythroleukemia (MEL) cell lines. In: Murray EJ (ed). *Methods in Molecular Biology: Gene Transfer and Expression Protocols*. The Humana Press: Clifton, NJ, 1991, pp 421–434.

29 Ellis J *et al.* A dominant chromatin-opening activity in 5′ hypersensitive site 3 of the human β-globin locus control region. *EMBO J* 1996; **15**: 562–568.

30 Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 1975; **98**: 503–517.

31 Slieker WAT, de Rijk-de Bruijn MFTR, Leenen PJM, Ewijk W. ER-MP12 antigen, a new surface marker on mouse bone marrow cells with thymus-repopulating ability. I. Intrathymic repopulating ability of ER-MP12-positive bone marrow cells. *Int Immunol* 1993; **5**: 1093–1098.

Chapter 3

Correction of the X-linked immunodeficiency phenotype by transgenic expression of human Bruton tyrosine kinase under the control of the class II major histocompatibility complex Ea locus control region

Dubravka Drabek, Selina Raguz, Ton de Wit, Gemma Dingjan, Huub Savelkoul, Frank Grosveld and Rudolf Hendriks

Proc. Natl. Acad. Sci. USA 95:610-615, 1997

Correction of the X-linked immunodeficiency phenotype by transgenic expression of human Bruton tyrosine kinase under the control of the class II major histocompatibility complex Ea locus control region

(B cell development/X-linked agammaglobulinemia)

DUBRAVKA DRABEK*, SELINA RAGUZ†, TON P. M. DE WIT*, GEMMA M. DINGJAN*, HUUB F. J. SAVELKOUL‡, FRANK GROSVELD*, AND RUDOLF W. HENDRIKS*§¶

Departments of *Cell Biology and Genetics and ‡Immunology, Faculty of Medicine, Dr Molewaterplein 50, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands; †Department of Experimental Pathology, University Medical and Dental School, Guy's Hospital, London SE1 9RT, United Kingdom; and §Department of Immunohaematology, University Hospital Leiden, Rijnsburgerweg 10, Leiden, The Netherlands

Communicated by C. Milstein, Medical Research Council, Cambridge, United Kingdom, November 11, 1996 (received for review July 30, 1996)

ABSTRACT **Bruton tyrosine kinase (Btk) is essential for the development of pre-B cells to mature B cell stages. *Btk*-deficient mice manifest an X-linked immunodeficiency (*xid*) defect characterized by a reduction of peripheral IgM^{low} IgD^{high} B cells, a lack of peritoneal $CD5^+$ B cells, low serum levels of IgM and IgG3, and impaired responses to T cell independent type II (TI-II) antigens. We have generated transgenic mice in which expression of the human *Btk* gene is driven by the murine class II major histocompatibility complex Ea gene locus control region, which provides gene expression from the pre-B cell stage onwards. When these transgenic mice were mated onto a Btk^- background, correction of the *xid* B cell defects was observed: B cells differentiated to mature $IgM^{low}IgD^{high}$ stages, peritoneal $CD5^+$ B cells were present, and serum Ig levels and *in vivo* responses to TI-II antigens were in the normal ranges. A comparable rescue by transgenic *Btk* expression was also observed in heterozygous $Btk^{+/-}$ female mice in those B-lineage cells that were *Btk*-deficient as a result of X chromosome inactivation. These findings indicate that the Btk^- phenotype in the mouse can be corrected by expression of human *Btk* from the pre-B cell stage onwards.**

Bruton tyrosine kinase (Btk) is a cytoplasmic protein tyrosine kinase that is essential for B cell development. The *Btk* gene encodes a 659-amino acid protein that contains a single catalytic domain, the *src* homology domains SH2 and SH3, and a unique N-terminal region with a pleckstrin homology domain and proline-rich sequences (1, 2). Btk belongs to the Tec subfamily of tyrosine kinases, together with the homologous *Tec*, *Itk*, and *Bmx* genes (3).

Mutations in the *Btk* gene (4) lead to X-linked agammaglobulinemia (XLA) in humans, which is characterized by an almost complete failure of pre-B cells to differentiate into mature B cells (5–8). In the mouse, Btk defects result in a mild B cell disorder, X-linked immunodeficiency (*xid*), both in CBA/N mice carrying an Arg-28 pleckstrin homology domain mutation, and in mice with targeted disruptions of *Btk* in their germ line (9–13). These mice have ≈50% fewer peripheral B cells than normal, with a specific deficiency of mature surface $IgM^{low}IgD^{high}$ cells (14, 15). In the peritoneum $CD5^+$ B cells are absent. Serum levels of IgM and IgG3 are low; responses to T cell independent type II (TI-II) antigens are severely impaired. *xid* B cells show an aberrant response to B cell receptor cross-linking, interleukin 5 (IL-5), IL-10, CD38, or CD40 (5–8). It has been shown that Btk activity and tyrosine phosphorylation increase upon stimulation of the B cell receptor, and the receptors for IL-5 and IL-6 (16–20).

The defect in XLA and *xid* is intrinsic to the B cell, as heterozygous females manifest a unilateral X chromosome inactivation in the mature B cell populations (13, 21–23), due to a selective disadvantage of cells that have the defective *Btk* gene on the active X chromosome. Btk is expressed throughout B cell differentiation, except in plasma cells (8, 13, 24). Although Btk is already present in pro-B cells, the first selective disadvantage of Btk-deficient cells only becomes apparent at the transition from small pre-B cells to immature B cells in the bone marrow (13). Furthermore, Btk is critical during the antigen-driven maturation of selected B cells in the periphery (9, 12, 13).

We generated transgenic mice in which *Btk* expression is driven by the class II major histocompatibility complex (MHC) Ea gene locus control region (LCR) (25), and mated them onto a Btk^- background. We show that the Btk^- phenotype can be corrected by transgenic expression of human Btk from the pre-B cell stage onwards.

MATERIALS AND METHODS

Generation of Transgenic Mice. Using a human *Btk* (h*Btk*) cDNA clone ph*Btk*2.55, which was isolated from a pro-B cell cDNA library (24), the 5′ end of the cDNA up to the *Eco*RI site at cDNA position 1453 (1) was subcloned by *Eco*RI digestion and religation (ph*Btk*1.45). A 670-bp *Eco*RI-blunted *Hin*dIII fragment (positions 1453–2123) from ph*Btk*2.55 was cloned into *Eco*RI and *Eco*RV digested ph*Btk*1.45. From the resulting plasmid a 2.1-kb blunted *Not*I–*Sal*I fragment, containing the h*Btk* cDNA with 16 bp of 3′ untranslated region, was ligated to a 6-kb blunted *Aat*II–*Sal*I fragment from pEV3 (Clare Gooding, ICI Pharmaceuticals), containing 2.8 kb of 3′ hβ-globin sequences. Subsequently, a *Pvu*I linker was introduced at a unique *Mlu*I site in the polylinker just 3′ of

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

0027-8424/97/94610-6$2.00/0
PNAS is available online at http://www.pnas.org.

Abbreviations: Btk, Bruton tyrosine kinase; h, human; KLH, keyhole limpet hemocyanin; LCR, locus control region; MHC, major histocompatibility complex; MLN, mesenteric lymph node; TD, T cell dependent; TI-II, T cell independent type II; TNP, trinitrophenol; XLA, X-linked agammaglobulinemia; xid, X-linked immunodeficiency; HPRT, hypoxanthine phosphoribosyltransferase; PE, phycoerythrin; DNP, 2,4-dinitrophenol.

¶To whom reprint requests should be addressed at: Department of Cell Biology and Genetics, Faculty of Medicine, Dr Molewaterplein 50, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

hβ-globin. Using a unique *Pvu*I site at position +33 in h*Btk*, a 4.9-kb *Pvu*I fragment containing h*Btk*–hβ-globin was cloned into a unique *Pvu*I site at position +14 in the MHC class II Eα gene on cosmid 32.1 (25). This cosmid (BALB/c H-2^d haplotype) contained 23 kb of 5′ Eα sequence, with the 5 DNase I hypersensitive sites of the Eα LCR. A 29-kb *Mlu*I fragment (see Fig. 1*A*) was injected into pronuclei of FVB × FVB fertilized oocytes at a concentration of 2 ng/μl.

Protein Analyses and Reverse Transcription–PCR (RT-PCR). For RT-PCR analysis, RNA was isolated from sorted cell populations using the Ultraspec RNA isolation system (Biotecx Laboratories, Houston) and cDNA was synthesized using reverse transcriptase (Super RT; HT Biotechnology, Cambridge, U.K.) and an oligo(dT) primer. PCRs were performed in 50 μl PCR buffer (Life Technologies, Paisley, U.K.) with 1.5 mM $MgCl_2$, 100 ng of each primer, and 5 μCi (1 Ci = 37 GBq) of [α-^{32}P]dATP. Amplification was for 27 cycles with denaturation at 94°C for 30 s, annealing at 55°C (*Btk* transgene) or 60°C (hypoxanthine phosphoribosyltransferase; HPRT) for 30 s, and extension at 72°C for 45 s. PCR products were visualized by electrophoresis through an 6% polyacrylamide/0.5× TBE gel and autoradiography. Quantifications were performed using IMAGEQUANT (Molecular Dynamics). Primers specific for the h*Btk*-transgene were in *Btk* exon 19 (5′-ATGGA TGAAG AATCC TGAGC-3′) and β-globin exon 3 (5′-TGGAC AGCAA GAAAG CGAG-3′). HPRT-specific primers were 5′-CACAG GACTA GAACA CCTGC-3′ and 5′-GCTGG TGAAA AGGAC CTCT-3′.

Western blotting analysis was performed as described (13).

Flow Cytometric Analyses and Cell Sorting. Preparation of single-cell suspensions, loading cells with fluorescein di-β-D-galactopyranoside, three-color flow cytometric analysis, and mAbs have been described (13). For analysis of Ki-67 expression, cells were lysed in lysis buffer (Becton Dickinson) after staining with surface markers. For cell-sorting experiments, 10^8 bone marrow cells were incubated with phycoerythrin (PE)-conjugated anti-CD43, fluorescein isothiocyanate-labeled anti-B220, biotin-conjugated IgM (PharMingen) and streptavidin-TriColor (Caltag, South San Francisco, CA) as a second step. Cell sorts were performed on a FACS Vantage cell sorter (Becton Dickinson): the purity of the obtained cell populations was ≈95%, as determined using CELLQUEST (Becton Dickinson) software.

Immunizations, Ig ELISA, and *in Vitro* Stimulations. To measure TI-II responses, 4- to 6-month-old mice were injected intraperitoneally with 50 μg 2,4-dinitrophenol (DNP)-Ficoll in PBS and trinitrophenol (TNP)-specific IgG3 was analyzed at day 7 by ELISA. To measure T cell-dependent (TD) responses, mice were immunized with 100 μg TNP keyhole limpet hemocyanin (TNP-KLH) precipitated with alum (26), and TNP-specific IgM was analyzed at day 7. A booster dose of 100 μg TNP-KLH was given at day 14, and TNP-specific IgG1 and IgG2a was analyzed at day 21. In the TNP-specific sandwich ELISA assays, plates were coated with isotype-specific antibodies and serum dilutions were incubated for 3 h. Subsequent steps were peroxidase labeled TNP-KLH and azino-bis-ethylbenz-thiazoline-sulfonic acid. Serum Ig subclasses were determined by isotype-specific ELISA (13, 26).

For *in vitro* stimulations, total spleen cell populations were cultured with 2 μg/ml goat-anti-mouse IgM (Southern Biotechnology Associates) or bacterial lipopolysaccharide (5 μg/ml) for 3 days, and analyzed for expression of Ki-67 by flow cytometry.

RESULTS

MHCII–h*Btk* Transgenic Mice. A transgenic construct (Fig. 1*A*) was generated in which the h*Btk* gene is put under the control of the murine class II MHC Eα gene LCR—i.e., a 23-kb genomic DNA fragment that contained the promoter proximal and B cell control regions (27), as well as more upstream DNase I hypersensitive sites required for position-independent, copy number-dependent expression (25). The h*Btk* 3′ untranslated region was replaced by a 3′ hβ-globin genomic fragment. The transgenic construct was injected into fertilized oocytes and six founder mice were obtained, as identified by genomic Southern blotting analyses using probes specific for 3′

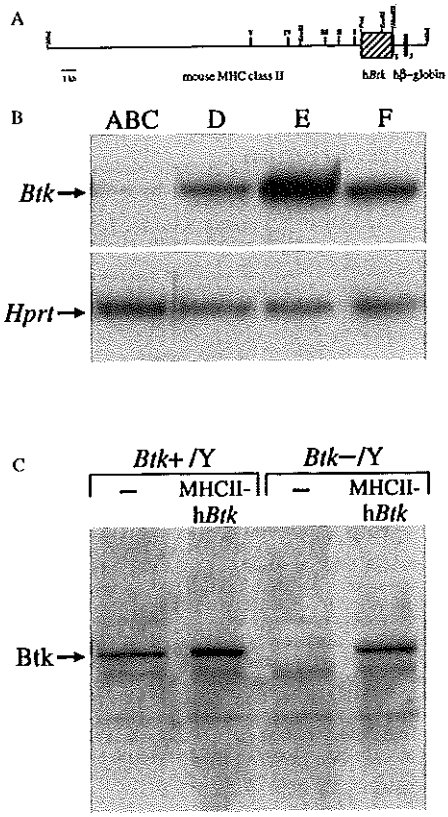

FIG. 1. Structure and expression of the MHCII–h*Btk* transgene. (*A*) Map of the transgene construct, showing the locations of five DNase I hypersensitive sites present in the 23-kb *Mlu*I–*Pvu*I mouse MHC class II upstream Eα gene fragment, the 2.1-kb *Pvu*I–*Hin*dIII h*Btk* cDNA fragment (hatched box), as well as a 2.8-kb hβ-globin fragment containing part of exon 2, exon 3, and 3′ untranslated region. (*B*) RT-PCR analyses of MHCII–h*Btk* transgene expression during B cell development. cDNA samples were prepared from sorted bone marrow populations of MHCII–h*Btk* transgenic *Btk*$^-$/Y mice and yields were normalized using ≈250 bp HPRT RT-PCR products. The MHCII–h*Btk* transgene primers generated single ≈230-bp bands, with the following densities (relative to fraction E): lane ABC, B220$^+$CD43$^-$IgM$^-$ pro-B cells (28): 1.8%; lane D, B220$^-$CD43$^-$IgM$^-$ pre-B cells: 25%; lane E, B220$^+$IgM$^+$ immature B cells: 100%; and lane F, B220^{hi}IgM$^-$ mature B cells: 35%. Data are shown as a representative of three mice examined. (*C*) Western blot analysis of Btk expression in total cell lysates from spleen of 3-month-old male wild-type mice (*Btk*$^+$/Y) or Btk-deficient mice (*Btk*$^-$/Y), either nontransgenic or MHCII–h*Btk* transgenic. The position of the 77-kDa Btk band is indicated.

hβ-globin and for h*Btk*. Five of these showed germ-line transmission of the transgene and in two lines expression of the transgene was observed by S1 analyses. No differences were found between these two lines in any of the performed analyses. The offspring did not exhibit developmental defects or any increased susceptibility to infectious diseases or malignancies, for over 1 year of age.

Consistent with the expression of MHC class II molecules in B lymphocytes and thymic epithelium (27), the highest expression levels of the transgene construct were found in spleen and thymus by S1 protection assays (data not shown). The transgene was also found to be expressed at a low level in brain, liver, and kidney. To confirm that the MHCII–h*Btk* transgene is transcribed in the B cell lineage only from the pre-B cell stage onwards, RT-PCR experiments were performed on sorted bone marrow cell populations. Transgene transcripts were readily detected in pre-B cells, immature and mature B cells (Fig. 1*B*). In contrast, expression in the sorted pro-B cells [$B220^+/IgM^-/CD43^+$ cells, fraction ABC (28)] was very low, with densities of the amplified signals that were <2% of those in fraction E [immature $B220^+IgM^+$ B cells (28)]. These faint *Btk* signals may well originate from contaminating cells that were present (up to 5%) in the pro-B cell preparations.

Male mice with one allele of the MHCII–h*Btk* transgene were mated to female $Btk^{-/-}$ mice—i.e., mice in which on one allele the *Btk* gene is inactivated through a targeted in-frame insertion of a *lacZ* reporter in exon 8 (13). In the offspring the four possible genotypes among males, wild-type (Btk^+/Y) and knock-out (Btk^-/Y), either nontransgenic or carrying the MHCII–h*Btk* transgene, were found in the expected ratio of 1:1:1:1. Protein expression of the MHCII–h*Btk* transgene construct was analyzed by Western blot experiments on spleen total cell lysates (Fig. 1*C*). Using a polyclonal rabbit antiserum specific for Btk amino acids 69–88 which are 100% conserved between human and mouse, a 77-kDa protein was detected in the spleen of normal male mice (Btk^+/Y), but not in the mice with the targeted disruption (Btk^-/Y). In the Btk^-/Y MHCII–h*Btk* transgenic mice, Btk protein expression in the spleen was restored, apparently to levels similar to those found in normal mice. When sorted bone marrow cell suspensions were analyzed in these mice, Btk protein was not detected in pro-B cells, but was found to be expressed from the pre-B cell stage (fraction D) onwards (data not shown).

Transgenic MHCII–h*Btk* Expression Corrects B Cell Numbers and Surface Ig Profiles in Btk^- Mice. The four groups of male littermates described above were investigated to assess lymphocyte surface phenotypes and numbers in spleen, mesenteric lymph node (MLN), bone marrow, thymus, and peritoneal cavity by flow cytometry (Table 1 and Fig. 2). No significant differences were found in the T cell compartment nor in the myeloid lineage (Table 1 and data not shown).

In the B cell lineage no significant differences were detected between normal and MHCII–h*Btk* transgenic mice. As previously described (13) the Btk^-/Y mice had fewer $B220^+$ B cells and, more particularly, a decrease in mature IgM^{low}/IgD^{high} B cells in spleen, MLN, and bone marrow. In the peritoneum, the numbers of conventional B cells were reduced (≈40-50% of normal) and $CD5^+$ B cells were virtually absent. However, correction of this B cell deficiency was observed in the Btk^-/Y mice that expressed the MHCII–h*Btk* transgene. In the spleen and MLN the B cell numbers reached close to normal values and mature IgM^{low}/IgD^{high} B cells were present in normal numbers in the spleen, MLN, and bone marrow (Table 1 and Fig. 2). In the peritoneum the numbers of $CD5^+$ and conventional B cells were in the normal ranges. The percentages of the pro-B, pre-B, and immature B cell subsets in the bone marrow were not significantly different between the four groups (data not shown).

Correction of Btk-Deficient Cells in $Btk^{+/-}$ Heterozygous Females. As the expression of the disrupted *Btk* allele could be identified by *lacZ* activity, in $Btk^{-/-}$ females the competition between B cells that express wild-type *Btk* and those expressing Btk^-/*lacZ*—due to the phenomenon of X chromosome inactivation—was assessed. Cell samples from spleen and MLN were evaluated for *lacZ* activity using fluorescein di-β-D-galactopyranoside as a substrate in conjunction with surface expression of IgM and IgD by flow cytometry.

In the absence of any selective disadvantage, *lacZ*-expressing cells from $Btk^{+/-}$ females would be expected to represent 50% of any B cell developmental population. However, because of the proliferative disabilities of Btk-deficient cells, the fraction of *lacZ*-expressing cells in the heterozygous females decreased during B cell maturation in the periphery from ≈35% in $IgM^{high}IgD^{low}$ (fraction III) to 10% in IgM^{high} IgD^{high} (fraction II) and to almost undetectable levels in $IgM^{low}IgD^{high}$ (fraction I) B cell stages (ref. 13 and Fig. 3*A*). Also in $Btk^{+/-}$ females that express the MHCII–h*Btk* transgene, a selective disadvantage of *lacZ*-expressing cells was observed, although the development of these cells did not appear to be compromised to such a large extent as in nontransgenic $Btk^{+/-}$ females. Significant fractions (≈10%) of

Table 1. Flow cytometric analysis of the B lymphocyte compartment in MHCII–h*Btk* transgenic mice

Tissue	Cell population	Btk^-/Y	Btk^+/Y MHCII-h*Btk*	Btk^-/Y	Btk^-/Y MHCII–h*Btk*
Spleen	Nucleated cells, $\times 10^6$	142 ± 45	138 ± 43	96 ± 30	113 ± 34
	$B220^-$ cells, %	59 ± 3	54 ± 4	45 ± 6*	52 ± 9‡
	$IgM^{low}IgD^{high}$ cells, %	30 ± 4	26 ± 4	9 ± 5*	20 ± 7†
	$CD4^+$ cells, %	18 ± 2	21 ± 3	22 ± 4	23 ± 3
	$CD8^-$ cells, %	9 ± 2	8 ± 1	11 ± 2	11 ± 2
MLN	$B220^-$ cells, %	38 ± 10	33 ± 6	20 ± 5*	31 ± 14†
	$IgM^{low}IgD^{high}$ cells, %	31 ± 8	22 ± 7	6 ± 1*	20 ± 10†
	$CD4^+$ T cells, %	36 ± 6	40 ± 9	48 ± 7	40 ± 8
	$CD8^+$ T cells, %	19 ± 4	20 ± 6	28 ± 4	22 ± 3
Peritoneum	$CD5^-$ B cells, %	4 ± 2	6 ± 4	0.3 ± 0.3*	4 ± 2†
	$CD5^-$ B cells, %	13 ± 4	11 ± 5	5 ± 3*	14 ± 5†
	$CD5^+$ T cells, %	5 ± 3	4 ± 2	7 ± 3	7 ± 4
Bone Marrow	$B220^+$ cells, %	24 ± 1	22 ± 1	19 ± 1	22 ± 1
	$IgM^{low}IgD^{high}$ cells, %	2.5 ± 0.6	3.8 ± 1.2	0.7 ± 0.2*	3.0 ± 1.4†

Mice were 4–6 months old. Numbers of mice analyzed are 10–14 (spleen), 3–6 (MLN), 7–10 (peritoneum), and 4 (bone marrow) for all groups.
*$P < 0.05$, compared with Btk^-/Y mice, in the Mann–Whitney U test.
†Not significantly different, compared with Btk^-/Y mice.

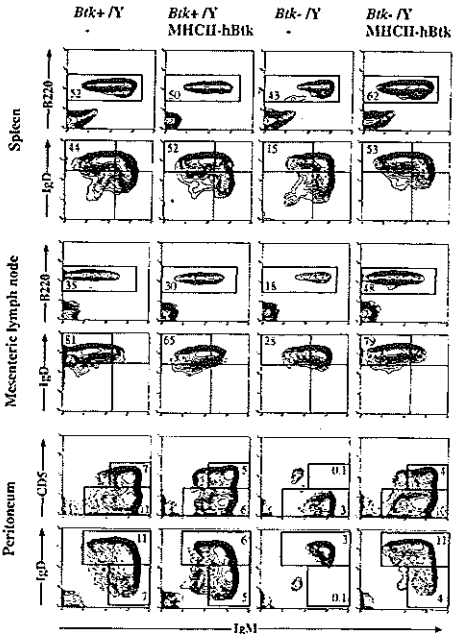

FIG. 2. The effect of the MHCII–h*Btk* transgene on peripheral B lymphocytes. Three-color flow cytometric analysis of spleen, MLN, and peritoneum from 4-month-old normal and transgenic *Btk*$^{-}$/Y males, as well as nontransgenic and transgenic *Btk*$^{-}$/Y males. Spleen and MLN cell suspensions were stained with biotinylated anti-IgM and streptavidin-TriColor, anti-IgD-PE, and anti-B220-fluorescein isothiocyanate. (*Top*) Percentages of cells displayed that are B220^{+}/IgM^{-} are indicated. (*Bottom*) Percentages of gated B220^{+} cells displayed that are IgM^{low}IgD^{high} (fraction I; refs. 14 and 15) are indicated. Peritoneal cells were stained with anti-B220, anti-IgM, and either anti-CD5-PE or anti-IgD-PE; B220^{+} cells are displayed. The percentages of total cells (including peritoneal macrophages) that are CD5^{+} (and IgD^{low}) B cells or conventional (CD5^{-} and IgD^{high}) B cells are given. Data are shown as 5% probability contour plots representative of the mice examined; dead cells were gated out, based on forward and side scatter characteristics.

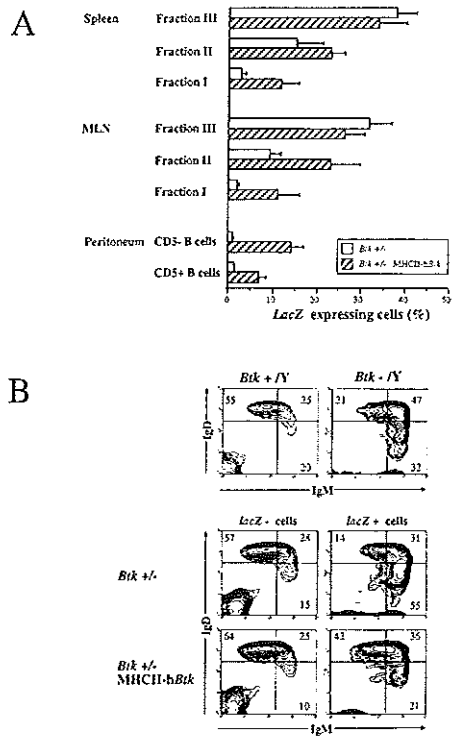

FIG. 3. The effect of the MHCII–h*Btk* transgene in *Btk*$^{+/-}$ female mice. Cell suspensions were stained for *lacZ* activity, anti-IgM (biotinylated)/streptavidin-TriColor and anti-IgD-PE (spleen and MLN) or anti-CD5-PE (peritoneum). (*A*) The percentages of *lacZ*-expressing cells as mean values and standard deviations in the three peripheral B cell compartments (fraction III, IgM^{high}IgD^{low}; fraction II, IgM^{high}IgD^{high}; and fraction I, IgM^{low}IgD^{high}; refs. 14 and 15). Values are corrected for individual variations in X chromosome inactivation ratios, using *lacZ* expression values from peritoneal macrophages (13). Data for spleen and MLN are from 6-week-old mice, and for peritoneum they are from 4-month-old mice. (*B*) Surface expression of IgM and IgD in gated *lacZ*$^{-}$ and *lacZ*$^{-}$ spleen cells from nontransgenic and MHCII–h*Btk* transgenic *Btk*$^{+/-}$ female mice. (*Upper*) For comparison the profiles in (*lacZ*$^{-}$) *Btk*$^{-}$ males and in (*lacZ*$^{+}$) *Btk*$^{-}$ males are given. The distribution of IgM^{-}IgD^{-} cells over the compartments III, II, and I in percentages is indicated. Data are shown as 5% probability contour plots representative of the mice examined.

mature IgM^{low}IgD^{high} fraction I cells in spleen and MLN manifested *lacZ* activity (Fig. 3*A*).

When peritoneal cells from *Btk*$^{+/-}$ mice were investigated for *LacZ* activity and surface expression of IgM and CD5, no *lacZ*$^{+}$ cells could be detected in the CD5^{+} B cells nor in the conventional CD5^{-} B cell population (Fig. 3*A*). However, 7% of CD5^{+} B cells and 14% of CD5^{-} B cells expressed *lacZ* in MHCII–h*Btk* transgenic *Btk*$^{+/-}$ females.

Furthermore, we analyzed the surface IgM/IgD profile of the *lacZ*$^{-}$ and the *lacZ*$^{-}$ cells in female spleen and MLN (shown for spleen in Fig. 3*B*). In nontransgenic heterozygous females, the gated *lacZ*$^{-}$ mature B cells exhibited an IgM/IgD surface profile that was identical to the profile of B cells in normal mice, whereas the gated *lacZ*$^{-}$ cells manifested an IgM^{high} phenotype, reminiscent of the B cell population found in *Btk*$^{-}$/Y mice (Fig. 3*B*). In contrast, the gated *lacZ*$^{-}$ cells in MHCII–h*Btk* transgenic heterozygous females, showed an IgM/IgD surface profile that was more similar to the profile in normal mice.

Transgenic MHCII–h*Btk* Expression Corrects Serum Ig Levels, TI-II Responses, and B Cell Proliferation. The serum concentrations of IgM, IgG1, IgG2a, and IgG3 were determined by ELISA. The knock-out mice (*Btk*$^{-}$/Y) had decreased levels of IgM and IgG3, when compared with normal littermates; this defect was corrected by transgenic *Btk* expression, as the MHCII–h*Btk* mice manifested serum Ig levels that were in the normal range (Fig. 4*A*).

To analyze the *in vivo* responsiveness to TD antigens, mice were immunized with TNP-KLH, and primary IgM and secondary IgG1 and IgG2a responses were measured by ELISA. The primary TNP-specific response was low in *Btk*$^{-}$/Y mice, consistent with previous findings in Btk-deficient mice (9, 12) but comparable between normal and transgenic MHCII–h*Btk* knock-out (*Btk*$^{-}$/Y) mice (Fig. 4*B*). The secondary TNP responses were not significantly different between the groups of mice. Because *xid* mice are unresponsive to TI-II antigens (9, 12), we also investigated the IgG3 response to the TI-II antigen DNP-Ficoll. The results showed that the *Btk*$^{-}$/Y

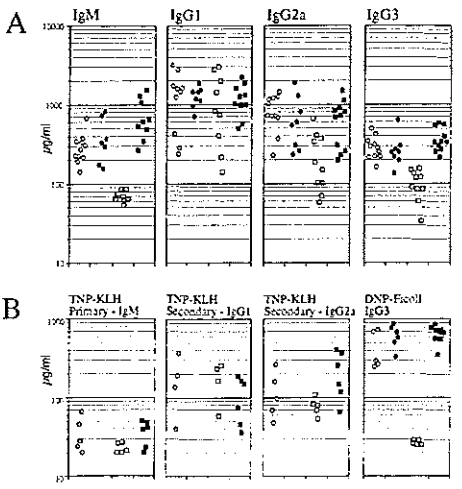

FIG. 4. Correction of serum Ig levels and *in vivo* antigen responses by expression of the MHCII–h*Btk* transgene. (*A*) Serum concentrations of Ig subclasses in unimmunized 4-month-old mice. (*B*) Serum concentrations of DNP/TNP-specific antibodies were determined after primary and secondary immunizations with the TD antigen TNP-KLH, and after immunization with the TI-II antigen DNP-Ficoll in 4- to 6-month-old mice. In transgenic *Btk*$^+$/Y males TD responses were not determined. ○, nontransgenic *Btk*$^-$/Y males; ●, MHCII–h*Btk* transgenic *Btk*$^-$/Y males; □, nontransgenic *Btk*$^-$/Y males; ■, MHCII–h*Btk* transgenic *Btk*$^-$/Y males.

knock-out mice did not respond and that this defect was completely corrected by MHCII–h*Btk* expression (Fig. 4*B*).

To determine the proliferative capacity of B cells, total spleen cell suspensions were stimulated with anti-μ and proliferation was quantified by flow cytometric analyses of the nuclear Ki-67 antigen, which is expressed in all proliferating cells during late G_1, S, M, and G_2 phases of the cell cycle (29). After 3 days of anti-μ stimulation, Ki-67 was expressed in 68 ± 8% of B220$^+$ cells from normal mice and in 35 ± 17% of B220$^-$ cells from *Btk*-deficient animals. In the MHCII–h*Btk* transgenic *Btk*$^+$/Y and *Btk*$^-$/Y mice, the induction of Ki-67 expression was clearly in the normal range (62 ± 11% and 67 ± 8%, respectively, $n = 3$ for each group).

When cells were cultured with 5 μg/ml lipopolysaccharide for 3 days, the Ki-67 antigen was expressed in 70–80% of splenic B220$^+$ cells in all four mice groups. However, the fraction of lymphoblasts in the B220$^+$ cell fraction, as determined by forward scatter characteristics, was low in *Btk*$^-$/Y mice (32 ± 5%, and 45 ± 7% in normal littermates). In the MHCII–h*Btk* transgenic *Btk*$^+$/Y and *Btk*$^-$/Y mice these values were 49 ± 3% and 49 ± 13%, respectively.

DISCUSSION

We have generated transgenic mice in which expression of the h*Btk* gene is driven by the murine class II MHC Eα gene LCR, which was shown to provide position-independent, copy number-dependent expression and could therefore be used to target genes to B lineage cells (25). MHC class II antigens are constitutively expressed on B cells and can be induced on a variety of other cell types, including macrophages and cells of nonhaematopoietic lineage (27). During B cell development, class II genes are first expressed on a major proportion of the pre-B cells (30). Although mature B cells manifest considerable heterogeneity in expression levels, the levels are generally higher than in pre-B cells (27, 30). In plasma cells the expression is down-regulated. Our RT-PCR and Western blot experiments show expression of the MHCII–h*Btk* transgene from the pre-B cell stage onwards.

When the transgenic mice were mated onto a *Btk*$^-$ background, correction of the *xid* B cell defects was observed. In the spleen and MLN of MHCII–h*Btk* transgenic *Btk*$^-$/Y males, B cell numbers and their surface IgM/IgD phenotype were normal; in the peritoneal cavity conventional B cells and CD5$^+$ B cells were present in normal numbers. In addition, expression of the transgene restored the serum IgM and IgG3 concentrations, the responses to TI-II antigen, and the proliferative capacity of B cells to anti-μ and lipopolysaccharide. These results directly show that the presence of Btk from the pre-B cell stage onwards is sufficient for normal development of the conventional and CD5$^+$ mature B cell populations. They also agree with previous findings that in Btk-deficient B lineage cells, defects only become apparent at the transition from small pre-B cells to immature B cells in the bone marrow (13, 22, 31). To date, it is unknown which of the signaling functions of Btk are mainly responsible for the immunodeficient phenotypes of XLA and *xid*. Nevertheless, the outcome of our transgenic rescue experiments are compatible with a role for Btk in signaling both from the Ig-μ H chain—surrogate L chain complex in pre-B cells and from the antigen receptor complex in mature B cells. Such a role for Btk is further supported by recent findings that the B cell defects in Btk-deficient mice resemble those in a mouse mutant that lacks most of the cytoplasmic tail of the B cell receptor-associated signaling molecule Ig-α (32).

Rescue by transgenic MHCII–h*Btk* expression was also observed in heterozygous *Btk*$^{-/-}$ female mice in those B-lineage cells that were *Btk*-deficient as a result of X chromosome inactivation. In MHCII–h*Btk* transgenic females the mature B cells that expressed the *Btk*$^-$*/lacZ* allele did not manifest the aberrant surface IgM^{high} profile, typical for the *xid* phenotype (Fig. 3*B*). However, as the fractions of *lacZ*-expressing cells still did not reach 50%, it can be concluded that mature B cells that only have transgenic hBtk still have a slight selective disadvantage over those cells that have both the endogenous murine and the transgenic human gene. This phenomenon may originate from (*i*) minor insufficiencies in the expression of the transgene, (*ii*) dominant negative effects of the expression product of the targeted *Btk–lacZ* allele, or (*iii*) functional differences between the human and murine Btk protein. Twelve out of 659-amino acid residues are different between murine and hBtk, most of which are conservative substitutions throughout the protein (1, 2). However, three amino acid changes are clustered at positions 207–214 in the proline-rich region, partly located in one of the two 10-amino acids motifs implicated in the interaction of Btk with SH3 domains of Fyn, Lyn, and Hck (33). Hence it remains possible that human and murine Btk show differences in affinity or specificity for SH3 domains, leading to slight selective disadvantages of murine B cells expressing hBtk. Yet, in MHCII–h*Btk* transgenic *Btk*$^{+/-}$ heterozygous females the selection against cells expressing the *Btk*$^-$*/lacZ* allele was not as pronounced as in the nontransgenic females (Fig. 3*A*).

The finding that the *xid* phenotype can be corrected in the mouse by transgenic expression of Btk from the pre-B cell stage onwards has important implications for the design of strategies for gene therapy of XLA. Our findings in the mouse show that the *Btk* gene does not need to be introduced in the very small populations of hematopoietic stem cells or early pro-B cells to restore B cell development in XLA patients. Instead, *Btk* gene transfer to any of the precursor B cell stages up to the small pre-B cell would correct the XLA defect. Thus, ≈10% of total cells or ≈50% of the B-lineage cells (28) in the bone marrow would be suitable targets. In addition to this large target cell population, XLA is also a good candidate for gene

therapy because Btk expression only needs to be transient [Btk is not required in plasma cells (8, 24)].

The observed correction of the B cell defects by transgenic expression of wild-type h*Btk* driven by the MHC class II LCR makes it attractive to perform similar studies with mutated *Btk* transgenes to investigate the function of the various Btk domains.

We thank A. Boonstra, L. Braam, M. de Bruijn, J. Guy, A. Langeveld, A. Maas, S. Philipsen, and M. de Weers for advice, assistance, and materials at various stages of the project. R.W.H. was supported by the Royal Academy of Arts and Sciences. These studies were supported in part by the Dutch Prevention Foundation (G.M.D.).

1. Vetrie, D., Vorechovsky, I., Sideras, P., Holland, J., Davies, A., Flinter, F., Hammerström, L., Kinnon, C., Levinsky, R., Bobrow, M., Smith, C. I. E. & Bentley, D. R. (1993) *Nature (London)* **361**, 226–233.
2. Tsukada, S., Saffran, D. C., Rawlings, D. J., Parolini, O., Allen, R. C., Klisak, I., Sparkes, R. S., Kubagawa, H., Thufuvancheri, M., Quan, S., Belmont, J. W., Cooper, M. D., Conley, M. E. & Witte, O. N. (1993) *Cell* **72**, 279–290.
3. Neet, K. & Hunter, T. (1996) *Genes Cells* **1**, 147–169.
4. Vihinen, M., Iwata, T., Kinnon, C., Kwan, S.-P., Ochs, H. D., Vorechovsky, I. & Smith, C. I. E. (1996) *Nucleic Acids Res.* **24**, 160–165.
5. Mattsson, P. T., Vihinen, M. & Smith, C. I. (1996) *BioEssays* **18**, 825–834.
6. Conley, M. E., Parolini, O., Rohrer, J. & Campana, D. (1994) *Immunol. Rev.* **138**, 5–21.
7. Rawlings, D. J. & Witte, O. N. (1994) *Immunol. Rev.* **138**, 105–119.
8. Sideras, P. & Smith, C. I. E. (1995) *Adv. Immunol.* **59**, 135–223.
9. Scher, I. (1982) *Adv. Immunol.* **33**, 1–71.
10. Rawlings, D. J., Saffran, D. C., Tsukada, S., Largaespada, D. A., Grimaldi, J. C., Cohen, L., Mohr, R. N., Bazan, J. F., Howard, M., Copeland, M. G., Jenkins, N. A. & Witte, O. N. (1993) *Science* **261**, 358–361.
11. Thomas, J. D., Sideras, P., Smith, C. I. E., Vorechovsky, I., Chapman, V. & Paul, W. E. (1993) *Science* **261**, 355–358.
12. Kahn, W. F., Alt, F. W., Gerstein, R. M., Malynn, B. A., Larsson, I., Rathbun, G., Davidson, L., Müller, S., Kantor, A. B., Herzenberg, L. A., Rosen, F. S. & Sideras, P. (1995) *Immunity* **3**, 283–299.
13. Hendriks, R. W., de Bruijn, M. F. T. R., Maas, A., Dingjan, G. M., Karis, A. & Grosveld, F. (1996) *EMBO J.* **15**, 4862–4872.
14. Hardy, R. R., Hayakawa, K., Haaijman, J. & Herzenberg, L. A. (1982) *Nature (London)* **297**, 589–591.
15. Hardy, R. R., Hayakawa, K., Parks, D. R. & Herzenberg, L. A. (1983) *Nature (London)* **306**, 270–272.
16. de Weers, M., Brouns, G. S., Hinshelwood, S., Kinnon, C., Schuurman, R. K. B., Hendriks, R. W. & Borst, J. (1994) *J. Biol. Chem.* **269**, 23857–23860.
17. Saouaf, S. J., Mahajan, S., Rowley, R. B., Kut, S. A., Fargnoli, J., Burkhardt, A. L., Tsukada, S., Witte, O. N. & Bolen, J. B. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 9524–9528.
18. Aoki, Y., Isselbacher, K. J. & Pillai, S. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 10606–10609.
19. Sato, S., Katagiri, T., Takaki, S., Kikuchi, Y., Hitoshi, Y., Yonehara, S., Tsukada, S., Kitamura, D., Watanabe, T., Witte, O. & Takatsu, K. (1994) *J. Exp. Med.* **180**, 2101–2111.
20. Matsuda, T., Takahashi-Tezuka, M., Fukada, T., Okuyama, Y., Fujitani, Y., Tsukada, S., Mano, H., Hirai, H., Witte, O. N. & Hirano, T. (1995) *Blood* **85**, 627–633.
21. Conley, M. E., Brown, P., Pickard, A. R., Buckley, R. H., Miller, D. S., Raskind, W. H., Singer, J. W. & Fialkow, P. J. (1986) *N. Engl. J. Med.* **315**, 564–570.
22. Forrester, L. M., Ansell, J. D. & Micklem, H. S. (1987) *J. Exp. Med.* **165**, 949–958.
23. Nahm, M. H., Paslay, W. J. & Davie, J. M. (1983) *J. Exp. Med.* **158**, 920–931.
24. de Weers, M., Verschuren, M. C. M., Kraakman, M. E. M., Mensink, R. G. J., Schuurman, R. K. B., Van Dongen, J. J. M. & Hendriks, R. W. (1993) *Eur. J. Immunol.* **23**, 3109–3114.
25. Carson, S. & Wiles, M. V. (1993) *Nucleic Acids Res.* **9**, 2065–2072.
26. Van Ommen, R., Vredendaal, A. E. C. M. & Savelkoul, H. F. J. (1994) *Scand. J. Immunol.* **40**, 1–9.
27. Glimcher, L. H. & Kara, C. J. (1992) *Annu. Rev. Immunol.* **10**, 13–49.
28. Hardy, R. R., Carmack, C. E., Shinton, S. A., Kemp, J. D. & Hayakawa, K. (1991) *J. Exp. Med.* **173**, 1213–1225.
29. Gerdes, J., Lemke, H., Baisch, H., Wacker, H. H., Schwab, U. & Stein, H. (1984) *J. Immunol.* **133**, 1710–1715.
30. Tarlinton, D. (1994) *Immunol. Rev.* **137**, 203–229.
31. Reid, G. K. & Osmond, D. G. (1985) *J. Immunol.* **135**, 2299–2302.
32. Torres, R. M., Flaswinkel, H., Reth, M. & Rajewski, K. (1996) *Science* **272**, 1804–1808.
33. Cheng, G., Ye, Z.-S. & Baltimore, D. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 8152–8155.

Chapter 4

Murine MHC class II locus control region drives expression of human β-glucocerebrosidase in antigen presenting cells of transgenic mice

J Guy, R Willemsen, A Langeveld, F Grosveld and D Drabek

Gene Therapy 6:498-507, 1999

Murine MHC class II locus control region drives expression of human β-glucocerebrosidase in antigen presenting cells of transgenic mice

J Guy[1,2], R Willemsen[1], A Langeveld[1], F Grosveld[1] and D Drabek[1]
Department of Cell Biology and Genetics, Faculty of Medicine, Medical Genetic Centre, Erasmus University Rotterdam, PO Box 1738, 3000 DR Rotterdam, The Netherlands

Gaucher disease is the most prevalent lysosomal storage disorder in humans, resulting from an inherited deficiency of the enzyme glucocerebrosidase. Although the enzyme is ubiquitously expressed, cells of the reticuloendothelial system are particularly affected since they accumulate the undigested glucosylceramide substrate through their role in scavenging and breaking down cell debris. Gaucher disease is an attractive target for somatic gene therapy. To test the ability to express the enzyme in the affected cell types we have generated transgenic mice expressing human glucocerebrosidase under the control of the murine major histocompatibility complex (MHC) class II Ea^d locus control region (LCR). The four transgenic lines express the human enzyme in a copy number-dependent manner, independent of the integration site of the transgene. Overexpression of the human enzyme in mice did not result in any abnormal phenotype or pathology during the period of observation (>2 years). The enzyme is expressed in B cells, monocytes, dendritic cells, thymic epithelial cells, and macrophages in various tissues: the peritoneal cavity, bone marrow, spleen, kidney, gastrointestinal tract, Kupffer cells in the liver and alveolar macrophages in lungs. Expression in the brain was limited to perivascular macrophages and was not seen in microglial cells. Therefore, the MHC class II LCR could potentially be of use in somatic gene therapy for type 1 Gaucher disease.

Keywords: *glucocerebrosidase; locus control region; transgene; macrophage*

Introduction

Gaucher disease is the most common glycolipid storage disorder, with an incidence of approximately 1:60 000 in the general population and 1:800 in the Ashkenazi Jewish population.[1,2] It results from a deficiency in the activity of β-glucocerebrosidase, which cleaves the β-glucosidic linkage of glucosylceramide to produce ceramide and glucose. This breakdown normally occurs in the lysosomal compartment of the cell. In the absence of β-glucocerebrosidase activity the uncleaved lipid substrate is stored in lysosomes within cells of the macrophage lineage, mostly affecting the spleen, liver, bones and bone marrow. The central nervous system (CNS) may also be involved and depending on the degree of its involvement, Gaucher disease can be divided into three types.[3,4] Type 1 is nonneuronopathic and the most prevalent form of disease seen in adults. Type 2 is an acute neuronopathic form resulting in death in infancy, while type 3 is a subacute neuronopathic form with a variably delayed neuronopathic course that usually results in death between the second and fourth decade of life. Different point mutations, insertional mutations, deletions and splicing mutations in the β-glucocerebrosidase gene have been described, which lead to the heterogeneity of clinical lesions.[5] There is a relationship between the type of mutation and the clinical manifestation of the disease[6,7] but there is also some degree of variability among patients with the same disease genotype.[3]

Current treatment for most Gaucher patients is still supportive and symptomatical, dealing with clinical manifestations (hepatosplenomegaly, anaemia, osseous lesions, fibrosis, cirrhosis etc) rather than with the cause of the disease. Other available options are enzyme replacement therapy,[8,9] allogenic bone marrow transplantation[10,11] and, potentially, somatic gene therapy by gene transfer into haematopoietic stem cells (HSCs).[12–14] It seems that regardless of the approach used, therapy has to start well before neurological symptoms and severe neuronal damage develop and the most promising candidates are type 1 and possibly type 3 Gaucher patients.

Enzyme replacement is based on administration of the missing enzyme in a form that is enriched with terminal mannose moieties to target the enzyme to the high affinity mannose-6-phosphate receptors on macrophages. Although therapeutically effective to some extent (regression of organomegaly, increase in the haemoglobin concentration),[8,9] enzyme replacement is expensive, gives only limited improvement in neuronopathic disease due to the inaccessibility of the CNS[15,16] and the enzyme is not targeted to marrow macrophages.[2] Little activity was found in bone marrow cells, even immediately after marrow infusion.[2,17] The enzyme is also bound by cells other than macrophages via the widely distributed, low affinity mannose receptor.

Correspondence: D Drabek
[2]Present address: Institute of Cell and Molecular Biology, University of Edinburgh, Mayfield Road, Edinburgh EH9 3JR, UK
Received 12 August 1998; accepted 4 November 1998

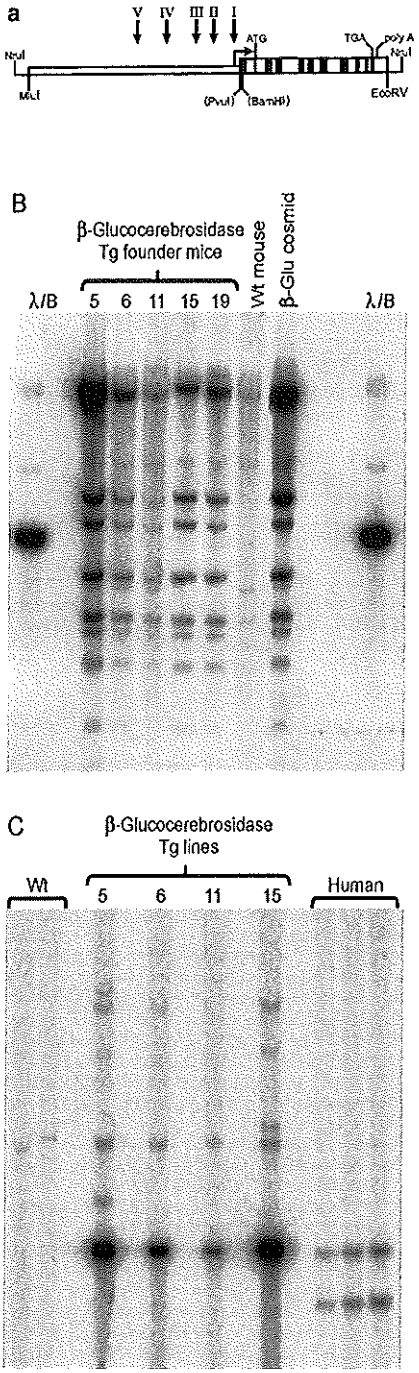

Figure 1 (a) Human β-glucocerebrosidase expression construct. 25 kb of murine MHC class II Ea^d locus with its five DNase I hypersensitive sites, promoter and transcriptional start site was used to drive expression of human β-glucocerebrosidase gene cloned as 10.2 kb BamHI–EcoRV fragment (starting in first exon before the translational start site, including all introns and polyadenilation signals). (b) Southern blot analysis showing integrity of the 35 kb transgene. All DNA samples were BamHI digested and probed with ^{32}P random-primer labelled MHC class II βglu cosmid. Lanes 5, 6, 11, 15 and 19 contain 15 μg of tail DNA from appropriate transgenic founders, lane WT contains the same amount of DNA from a nontransgenic litter mate as a negative control and lane βglu cosmid contains 10 ng of injection fragment diluted in 15 μg of nontransgenic DNA. To prevent cross-hybridisation, sonicated nontransgenic mouse DNA was used as a competitor. In all five founders, all of the expected bands were present. (c) Southern blot performed for the copy number estimation. Tail DNA from nontransgenic (Wt) mice, transgenic mice (heterozygous lines 5, 6 and 11 and homozygous line 15) and human DNA (made from buffy coat of an healthy individual) were BamHI digested. Various amounts of mouse (Wt) and human DNA were loaded for titration purposes. The filter was probed with a human exon 10 probe and the signals quantified using Phosphor Imager, correcting for a background activity, the amounts of DNA used, difference in size between human and mice genome and the fact that transgenic line 15 used in this particular experiment was homozygous. The probe cross-hybridised with the human β-glucocerebrosidase pseudogene (lower band in human samples) and with the mouse gene (band present in Wt lanes and at the same position in all of the lanes with the transgenic mice samples).

Bone marrow transplantation (BMT) has been performed only in patients with severe disease. It requires a human lymphocyte antigen (HLA) identically matched donor and ablation of the patient's own marrow before transplantation. The mortality risk for the procedure is around 10% because of the patient's severely compromised condition. Marrow ablation produces a period of pancytopenia and immunocompromise and there is also the possibility of graft-versus-host disease. Genetic modification of the patient's own HSCs by introducing a correctly expressed copy of the gene, followed by autologous bone marrow transplantation would overcome problem of graft-versus-host disease and shortage of donors.

Here, we describe a construct suitable for gene therapy which utilises the murine MHC class II Ea^d locus control region (LCR)[18] and drives expression of the transgene, in this case human β-glucocerebrosidase, to the cells that constitutively express class II molecules such as B cells, monocytes, macrophages, dendritic cells, myeloid and erythroid precursors and some epithelial cells. Use of the full genomic sequence of the gene in conjunction with the MHC class II LCR, rather than the more commonly used cDNA, increases the likelihood of the enzyme being expressed at the significant levels that would be required for effective gene therapy.

Results

Human β-glucocerebrosidase was expressed in transgenic mice using a construct consisting of a murine MHC class II LCR linked to the human β-glucocerebrosidase gene. Approximately 25 kb of the Balb/c MHC class II I-Ea^d locus was used, comprising all of the 5'DNaseI hypersensitive sites, the promoter and transcriptional start site. The human β-glucocerebrosidase gene was supplied as a genomic fragment starting in the first exon before the translational start site and including all introns and polyadenylation signals.

Five founder mice carrying the construct

MHCclassII/βglu were obtained (Figure 1a) and checked for the integrity of the 35 kb transgene (Figure 1b). All five founders contained the intact locus. Four of the founder mice transmitted the transgene to the F1 generation. The copy numbers of these lines were determined (Figure 1c) and found to be: lines 5 and 15 – 15 copies, line 6 – seven copies and line 11 – two copies.

Expression studies were done using F1 heterozygous mice. The level of expression of the enzyme was determined for all four lines by measuring the activity in the spleen of 2-month-old mice (Figure 2). A high level of expression was expected due to the substantial proportion of MHC class II-positive B cells in this organ. To discriminate between endogenous (mouse) and transgenic (human) enzyme activity, the human enzyme was specifically immunoprecipitated from total spleen extracts using the monoclonal antibody 8E4.[19,20] Immunoprecipitated β-glucocerebrosidase activity was detected using the artificial substrate 4-methylumbelliferyl-β-glu (4-MUβglu). On enzymatic breakdown of this substrate, 4-MU is released and its fluorescence can be measured.

No human activity was detected in the control nontransgenic mice or in the mouse macrophage-like cell line, RAW264. Activity was detected in all four transgenic lines and in the human cell line U937. Enzyme activities for different mice from the same line were of a similar level.

In all cases the level of human β-glucocerebrosidase activity correlated with the number of copies of the transgene. Thus, line 11 (two copies) had the lowest activity, line 6 (seven copies) an intermediate level, and lines 5 and 15 (15 copies each) an equal, high level of expression. The low activity of line 11 compared with that of U937, an immortalised human cell line is explained by the fact that this is the activity in the total spleen (ie a mixture of cells) rather than a homogeneously expressing population as is the case for U937.

FISH analysis using the whole injection fragment on bone marrow cells showed the integration sites of the transgenes to be either telomeric (lines 5 and 6) or centromeric (lines 11 and 15) (Figure 3). In centromeric regions it is likely that the transgenes will be integrated in transcriptionally silent heterochromatin. From this fact

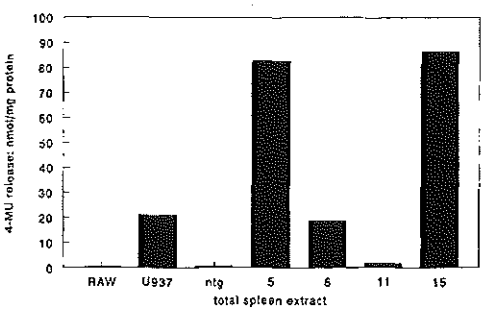

Figure 2 Expression of the human β-glucocerebrosidase in spleen of transgenic mice. Human β-glucocerebrosidase activity was measured in spleen extracts of transgenic mouse lines (5, 6, 11 and 15) and nontransgenic mouse, using 8E4 MoAb for immunoprecipitation. Extract from U937 human cell line was used as a positive and RAW264 mouse line as a negative control.

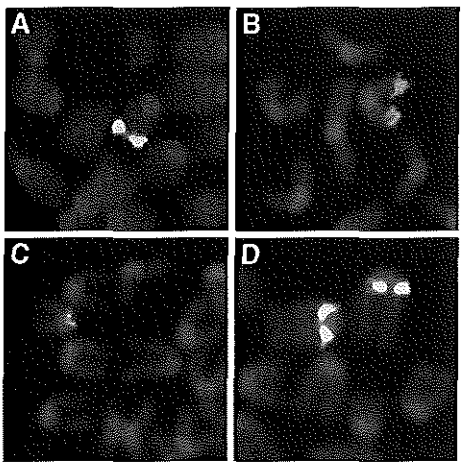

Figure 3 FISH analysis showing the integration site of the transgene. The entire injection fragment (35 kb) was used as a probe detected with FITC. DNA was counterstained with DAPI. (a) Transgenic line 5; (b) transgenic line 6; (c) transgenic line 11; (d) transgenic line 15. In the case of transgenic line 15, a homozygous mouse was used.

and the copy number-dependent expression of the linked gene, it seems that the MHC class II I-Ea^d fragment is able to function fully as an LCR when attached to the heterologous β-glucocerebrosidase gene. This makes it particularly interesting for use in gene therapy protocols where the integration site of the transgene into the host genome still cannot be controlled. Without the use of an LCR-like element integration of the gene into heterochromatin, which makes up a large part of the genome, would result in position effects and low levels of expression.[21,22]

Immunofluorescent staining of spleen single cell suspensions with the rabbit polyclonal antibody R386 against human β-glucocerebrosidase showed that approximately 60% of the cells from transgenic spleens were positive for the human enzyme (Figure 4d) which is the same as the percentage of B cells present in the organ. From double staining with B and T cell markers (data not shown), expressing cells are indeed B220 positive and therefore B but not T cells are responsible for the activity in the spleen. The fact that all B cells were positive shows that integration into the centromeric region did not lead to position effect variegation (PEV).[21,22]

In vitro cultured macrophages from transgenic and nontransgenic mice were stained *in situ* with the polyclonal antibody R386 and with the mature mouse macrophage markers F4/80 and MOMA-2. The F4/80 antigen is found on the membranes of macrophages in most tissues of the body, whereas MOMA-2 is primarily cytoplasmic and is found mainly on macrophages in a lymphatic environment.[23]

Cells were taken from spleen, bone marrow and the peritoneal cavity (after induction of a sterile inflammatory response) and cultured in the presence of L cell-conditioned medium on untreated glass coverslips. After 7 days of culture, most if not all, adherent cells expressed

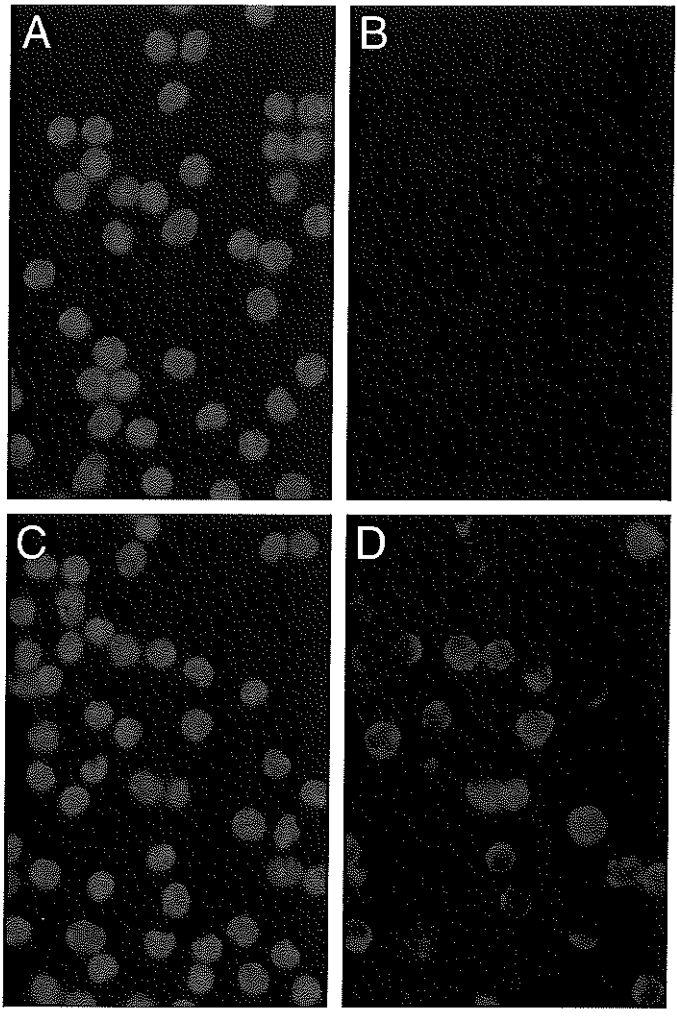

Figure 4 Detection of human β-glucocerebrosidase in a single cell suspension from spleen. (b, d) Indirect immunofluorescence using rabbit polyclonal antibody R386 against human β-glucocerebrosidase with FITC-conjugated goat-anti rabbit Ig as a secondary antibody. (a, c) Nuclear staining of the same fields of cells with 4'-6' diamidino-2-phenylinole (DAPI). (a, b) non-transgenic mouse; (c, d) transgenic mouse.

the F4/80 antigen (Figure 5) demonstrating the purity of the macrophage cultures. MOMA-2 positive cells were also present in significant numbers (approximately 90%).

The cultured cells adopted a macrophage-like morphology, extending long plasma membrane processes and making contact with other cells. Splenic macrophages could be seen to have engulfed smaller cells, probably dead lymphocytes also present in the culture.

Staining of the cultured macrophages for human β-glucocerebrosidase revealed that most but not all F4/80-positive cells from transgenic animals contained the human enzyme, whereas no staining was seen in control cultures.

In order to determine whether other cells belonging to mononuclear phagocyte system (eg Kupffer cells in the liver) express the transgene, RT-PCR was performed, using primers specific for human β-glucocerebrosidase, on different tissues (spleen, liver, kidney, bone marrow, thymus, brain, lungs, testis and ovary). The 581 bp fragment corresponding to the human mRNA could be distinguished from amplification of contaminating DNA in the samples due to the use of primers spanning an intron.

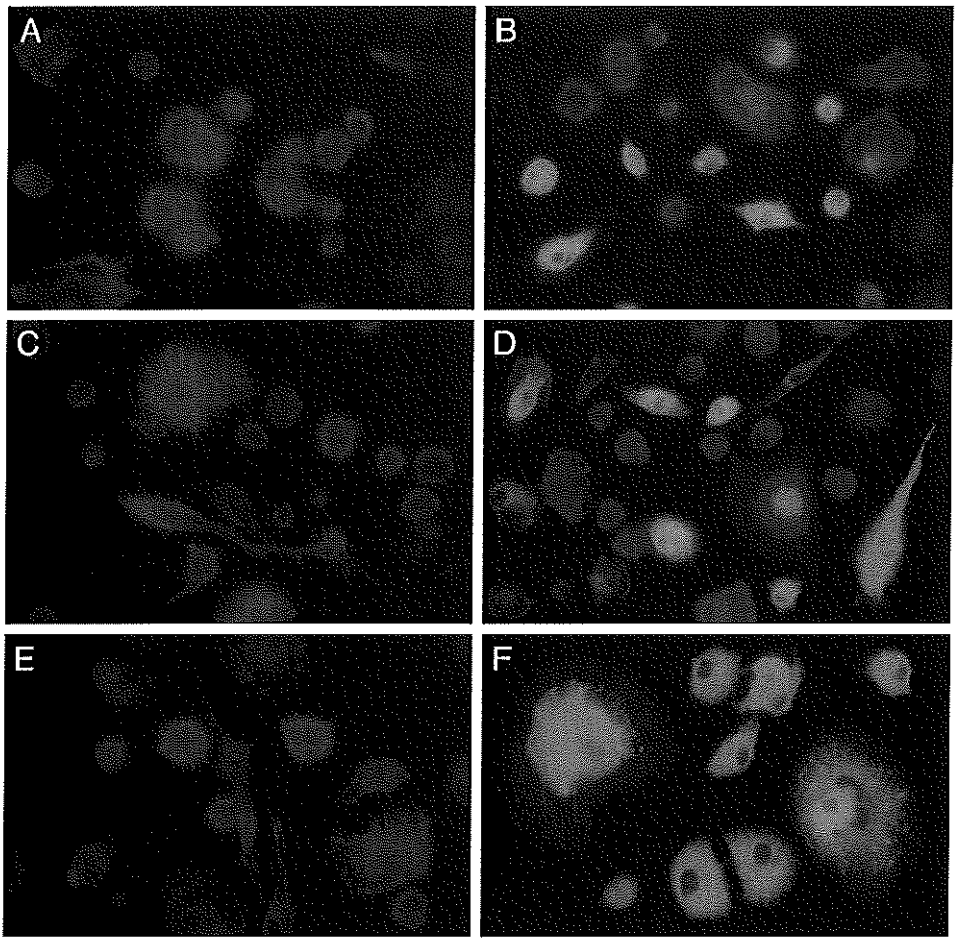

Figure 5 In vitro cultured intraperitoneal, bone marrow and spleen macrophages, double stained with F4/80 macrophage marker (detected by Texas Red) and R386 antibody against human β-glucocerebrosidase (detected by FITC). (a, b) Intraperitoneal macrophages; (c, d) spleen macrophages; (e, f) bone marrow macrophages. (a, c, e) cultures from a nontransgenic mouse; (b, d, e) cultures from a transgenic mouse line 5.

The correct fragment was observed in all tissues tested (Figure 6) from all transgenic lines, and not in nontransgenic controls. RT-PCR on tissues from the lowest copy number line (line 11, two copies) showed that there was low expression in the brain. Considering that the number of microglial cells which are the resident macrophages of the CNS is high (15%), it could be expected that the brain would have a high level of expression if these cells were positive. Thus the PCR result suggests that the MHC class II LCR does not drive high levels of expression in microglial cells. Obviously, the PCR result does not distinguish between cell types and the expression in a number of tissues could be the result of the presence of circulating B cells.

Thus the localisation of the human enzyme was analysed by immunohistochemistry on cryosections of the same tissues which were used for RT-PCR, using the polyclonal antibody R386 alone or in combination with macrophage and dendritic cells markers (F4/80 and N418[24]). Indeed, the resident macrophages were found to be positive for human β-glucocerebrosidase: Kupffer cells in the liver (Figure 7b), alveolar macrophages in the lung (Figure 7c), macrophages of the lamina propria throughout the gastrointestinal tract (Figure 7d), kidney resident macrophages in the renal medullary and cortical interstitium and juxtaglomerular complex (Figure 7e), spleen resident macrophages in both red and white pulp, as well as those in the marginal zone (Figure 7f). In the thymus,

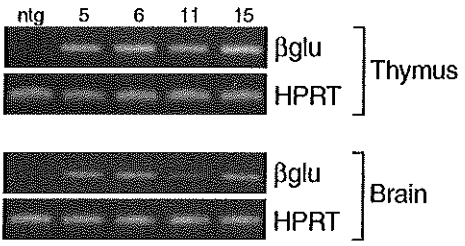

Figure 6 Detection of the human β-glucocerebrosidase by RT-PCR in thymus and brain. The murine hypoxantine phosphoribosyltransferase (HPRT) was used as an internal control. A PCR fragment of the correct size (581 bp) was obtained in all of the transgenic mouse lines but not in nontransgenic wild-type mouse.

not only macrophages and dendritic cells but also thymic epithelial cells were strongly stained (Figure 7g). R386+ cells in brain sections appeared to be perivascular macrophages, based on their morphology and obvious association with the vasculature, and not the extensively arborised parenchymal microglial cells (Figure 7h).

To estimate the activity of the human enzyme in the brain of transgenic animals in comparison with that in the spleen and liver, human-specific immunoprecipitation was again performed using the monoclonal antibody 8E4. Considerably older animals (approximately 2 years) of lines 5 (15 copies) and line 6 (seven copies) were used. As expected from the immunohistochemical data the level of enzyme activity in the brain was extremely low in comparison with levels in the liver and spleen (Figure 8).

Discussion

For effective treatment of Gaucher disease it is necessary to achieve sustained expression of the glucocerebrosidase gene in macrophages, as these are the important cells for the pathogenesis of the disease. Therefore, we have used a murine MHC class II LCR to drive the expression of human β-glucocerebrosidase in the cell type of interest, rather then using other LCRs to overproduce and to secrete the missing enzyme from other cell types. The β globin LCR, for example, was used to overexpress protective protein/cathepsin A in erythroid precursor cells to treat another lysosomal disorder, namely galactosialidosis.[25]

In transgenic mice LCRs confer high level expression on linked genes independent of the position of the transgene in the mouse genome. It is known that an incomplete LCR loses this property when integrated into a heterochromatic region.[21,22] As two of the four transgenic lines that we have generated had the transgene in the centromeric region, and both were expressing human enzyme according to their copy number, we conclude that the fragment of murine MHC class II Ea^d LCR used, with its 5 DNase hypersensitive sites,[18] can fully act as an LCR when linked to genomic sequences of a heterologous gene. The same fragment was previously used linked to the cDNA encoding Bruton tyrosine kinase (BTK).[26] Only two of five transgenic mice lines expressed BTK and rescued the BTK−/− phenotype, most likely due to the use of the cDNA rather than anything missing from the LCR element. Recently, the same LCR fragment has been used linked to a part of the same cDNA followed by large piece of genomic BTK sequence. All transgenic lines obtained expressed.[27] Therefore, we would like to stress the importance of the presence of intervening sequences for any future gene therapy protocols. It seems that the last intervening sequence of multiple intron containing genes is a principal determinant of the efficiency of 3′ end formation and perhaps post-transcriptional regulatory steps in gene expression.[28]

It is well known that class II antigens are expressed on a limited subset of cells in the immune system, including B cells, macrophages, dendritic cells, thymic epithelium and activated T cells in most species, but not the mouse.[29] The level of class II is not static in these cells but can be up- or down-regulated by a large number of external stimuli. However, there is a difference in basal level of MHC class II expression among the cells of the macrophage lineage. Macrophages differ from other haematopoetic cells in that they form a resident population in many tissues of the body.[30] They are generated in the bone marrow and circulate in the blood as monocytes before reaching their target tissues. Once they have entered the tissue, they are known as macrophages, or in some tissues by more specialised names such as Kupffer cells in the liver.

In all the organs of our transgenic mice, resident macrophages expressed human β-glucocerebrosidase (with the exception of the brain). The obvious question that arises is why the microglial cells do not express. The origin and biological functions of microglia are still unclear and debatable. Resident microglia appear in the CNS during late post-natal development.[31] These cells lack many of the surface features and functional properties typically associated with macrophages found in other tissues.[32,33] The brain has been thought of as an immunologically privileged site. It lacks a lymphoid system and there is no evidence for a population of dendritic cells. The expression of class II antigens on cells in the CNS, which might then act as antigen-presenting cells, does not occur in the normal rodent[34] but is variable in studies of the human CNS.[35] The expression of class II antigens has been demonstrated on microglia in a variety of pathological conditions, such as brain injury, Parkinson's disease, Alzheimer's disease and demyelinisation. Recently, it has become possible to clone microglial precursors in agar and expand them in liquid culture. Some of the *in vitro* cultured microglial clones expressed class II MHC, but expression was not sufficient for the processing and presentation of complex protein antigens to T cells.[36] Other reports show that *in vitro* cultured microglia can be induced to express class II MHC antigens upon IFNγ treatment and indicate the absence of expression on unstimulated microglia[37,38] or the normal brain *in vivo*.[39]

In conclusion, most of the monocyte lineage, such as Kupffer cells, Langerhans' cells and the alveolar macrophage, express class II antigens and play a role in initiation of regulation of local immunological events, functioning as antigen-presenting cells or accessory cells. Macrophage-microglia have quite similar, if not the same properties as the cells of monocyte lineage, but class II antigens are detectable on their surface only upon IFNγ treatment, at least in the case of isolated microglia. As the transgene, in this case human β-glucocerebrosidase is

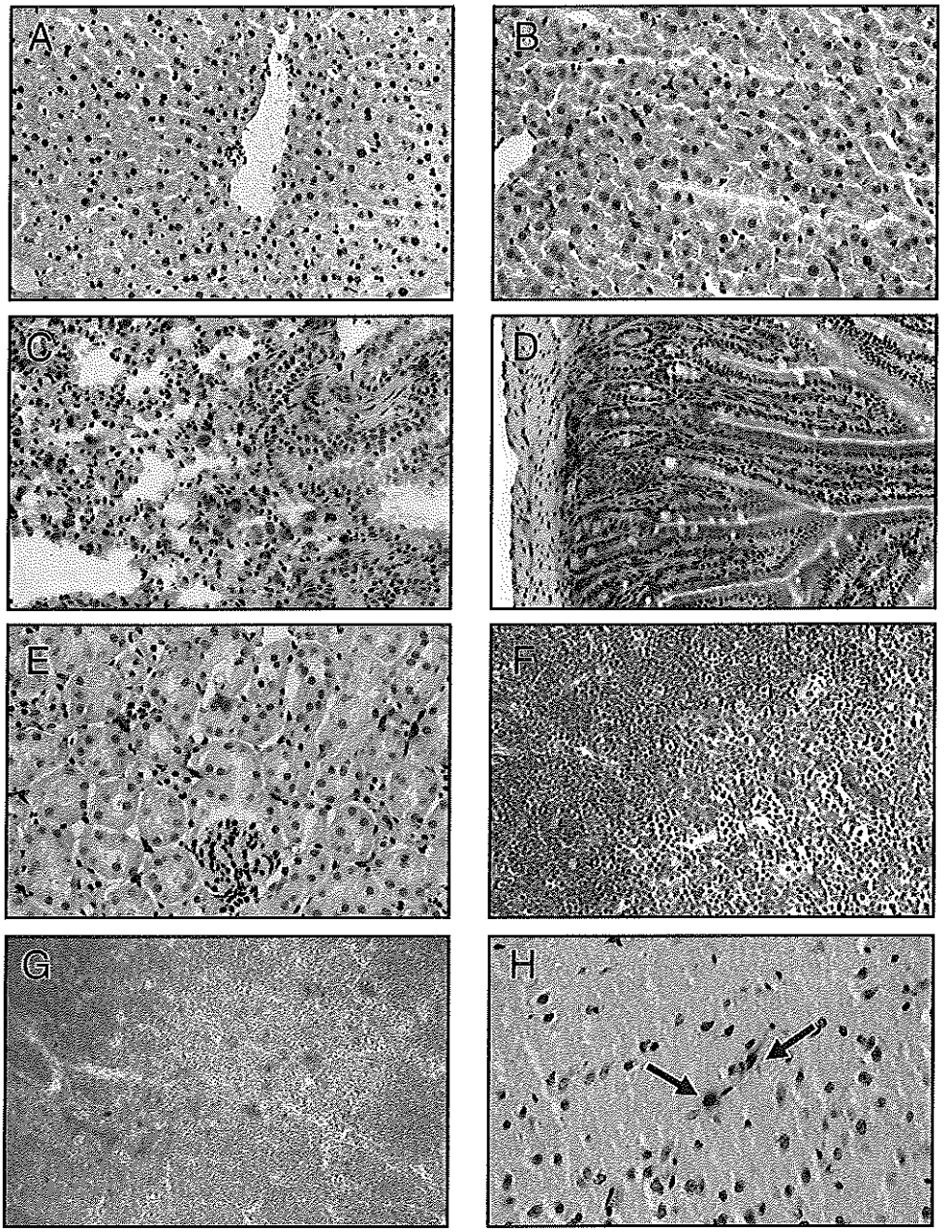

Figure 7 Immunohistochemical staining performed on cryosections using rabbit polyclonal antibody (R386) against human β-glucocerebrosidase. R386⁺ cells are brown, as DAB was used as a substrate for the HRP-conjugated secondary antibody. (a) Nontransgenic liver; (b) transgenic liver – note positive Kupffer cells; (c) transgenic lungs – note positive alveolar macrophages; (d) transgenic intestine; (e) transgenic kidney; (f) transgenic spleen; (g) transgenic thymus; (h) transgenic brain (arrows indicating positive perivascular macrophages). (a–h) were counterstained with hematoxylin.

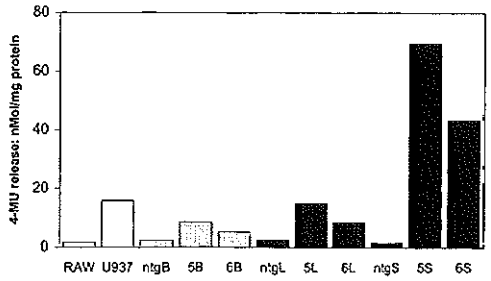

Figure 8 Expression of human β-glucocerebrosidase in different tissues of transgenic mice. Human β-glucocerebrosidase activity was measured in brain, liver and spleen extracts of transgenic lines (5 and 6) and non-transgenic mouse. Extract from U937 human cell line was used as a positive and extract from RAW264 mouse cell line as a negative control (presented in open bars). Immunoprecipitation was performed on all samples with monoclonal antibody 8E4. Activity in brain is presented in grey, activity in liver in dark grey and activity in spleen in black bars.

under the control of MHC class II LCR, its expression follows the expression pattern of class II molecules. This would explain the lack of human β-glucocerebrosidase activity in parenchymal microglial cells of adult transgenic mice. However, using the retroviral vector G2, containing the human glucocerebrosidase cDNA driven by Maloney murine leukemia virus long terminal repeats (LTR), Krall *et al*[13] reported that retrovirally transduced murine BM cells can repopulate up to 20% of the total microglia, but the majority (approximately 90%) of cells appear to be perivascular microglia and only 5–10% parenchymal microglia. This shows that significant numbers of cells are capable of infiltrating the CNS after BMT, but it does not reveal how much microglial expression is required to achieve a therapeutic effect in neuronopathic types of disease.

For many genetic disorders such as sickle cell anaemia and α and β thalassaemias, it is important to correct the defect by providing a functional single copy of the diseased gene. Introduction and expression of more than one copy leads to pathology, for example, precipitation of overexpressed unpaired α or β globin chains, results in red cell lysis and anaemia. It seems that overexpression of human β-glucocerebrosidase (up to 15 copies in our experiment) did not cause any pathology in transgenic mice. There is no reason to suspect that the levels of enzymatic activity measured *in vitro* cannot be directly related to the amount of active enzyme *in vivo*. It is therefore reasonable to assume that overexpression will not cause any harmful effect in patients.

In type 1 Gaucher disease, accumulation of glucocerebroside is mainly restricted to bone marrow-derived macrophages outside of the CNS, and therefore type 1 disease would be a good candidate for somatic gene therapy using this construct. The observed beneficial response after allogenic BMT in patients with type 1 and type 3 Gaucher disease makes the transfer of the normal glucocerebrosidase gene under the control of appropriate LCR elements into HCSs followed by autologous BMT an attractive approach to consider.

Materials and methods

Construct

The gene for human β-glucocerebrosidase was isolated from plasmid PCN 9RI (a generous gift from E Ginns, Bethesda, MD, USA) as a 9.7 kb *Bam*HI–*Eco*RV fragment using RecA-mediated single site cleavage[40] to cut uniquely at the *Bam*HI site in exon 1 of the gene, before the ATG. The sequence of the oligo used to protect the *Bam*HI site was as follows: 5'-CTCTGAAGGATAGAGGATCCACTAAACAAAAACAAGGA-3 (*Bam*HI site underlined). The fragment was subcloned into *Bam*HI–*Eco*RV of pBS II SK$^+$ (Stratagene, La Jolla, CA, USA) resulting in plasmid pBSβglu. The mouse H2-I Ea^d gene was deleted from cosmid 32.1[18] by the removal of a 10 kb *Pvu*I–*Xho*I fragment. The remaining 25 kb fragment containing the 5 DNaseI hypersensitive sites of the mouse MHC class II Ea^d LCR was blunted using Klenow and ligated to the blunted 9.7 kb *Not*I–*Cla*I human β-glucocerebrosidase fragment from pBSβglu resulting in the cosmid MHCclassII/βglu.

Generation of transgenic mice

A 35 kb *Nru*I fragment from cosmid MHCclassII/βglu was purified using a NaCl gradient.[41] The fragment was microinjected into fertilised mouse oocytes (FVB × FVB) at a concentration of 4 ng/μl. Injected eggs were transferred into pseudopregnant mice and transgenic offspring identified by Southern blot analysis of tail DNA.[42] When using the whole cosmid as a probe, the labelled probe was prehybridised with sonicated genomic mouse DNA to prevent cross-hybridisation with repetitive sequences in the genome. For estimation of copy number, equal amounts of *Bam*HI-digested, transgenic mouse and human genomic DNA were Southern blotted and the blot probed with a probe taken from exon 10 of the human β-glucocerebrosidase gene (almost identical to the mouse sequence). The probe was cloned as a 150 bp *Asp*718–*Bsr*FI fragment into *Asp*718/*Xma*I of pBS II SK$^+$ and cut out as a 250 bp *Bss*HII fragment. Quantification was carried out using a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA USA), taking into account the relative genome sizes of mouse and human and the fact that the human and transgenic line 15 samples were homozygous.

In vitro culture of murine macrophages

Two-month-old transgenic and nontransgenic mice were injected intraperitoneally with 0.5 ml of 3% Thiogycolate (Sigma, Zwijndrecht, The Netherlands) in phosphate-buffered saline (PBS). Mice were killed 4 days later. Peritoneal macrophages were collected by flushing the peritoneal cavity several times with PBS, using a Pasteur pipette. For bone marrow-derived macrophages, mouse femurs were cleaned, cut open and the bone marrow removed by injection of PBS through a needle. A single cell suspension was made by pipetting the cells up and down. For spleen-derived macrophages, a single cell suspension was made in PBS by passing the tissue through a 100 μm mesh. All the samples were centrifuged for 5 min at 95 *g* and the cells plated on coverslips in 3 cm dishes in Iscove's modified Dulbecco's medium (Sigma) with 10% FCS and 10% L-cell conditioned medium as a source of M-CSF. L-cell medium was harvested when

cells were almost confluent, the supernatant was centrifuged, filtered and added to macrophage cultures.

Immunocytochemistry

Different organs from nontransgenic and transgenic mice were embedded in Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and frozen in liquid nitrogen. Cryostat sections, 7 μm thick, were air dried and fixed in 3% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer pH 7.4 for 10 min. Cells were permeabilised and endogenous peroxidase activity blocked by incubation in 0.3% H_2O_2 in methanol for 20 min. Subsequently, slides were washed in PBS⁺ (PBS/0.5% BSA/0.02 M glycine). Slides were incubated at 4°C overnight with rabbit polyclonal anti-human β-glucocerebrosidase antibody R386 (1:500 dilution), washed in PBS⁺ solution for 10 min and incubated for 30 min at room temperature with horseradish peroxidase (HRP)-conjugated swine anti-rabbit Ig (1:100) (DAKO, Glostrup, Denmark). Slides were washed in PBS⁺ for 10 min and exposed for 5–10 min to Peroxidase Substrate (3,3'-Diaminobenzidine Tetrahydrochloride (DAB) tablets; Sigma). After washing in PBS slides were counterstained with hematoxylin, washed again and mounted in modified Aquamount mountant (BDH, Poole, UK).

For staining of cultured macrophages, coverslips with adherent cells attached were taken from culture dishes after 7 days of culture and washed once in PBS and once in PBS⁺. Cells were fixed in 3% PFA in 0.1 M Na phosphate buffer pH 7.4 for 10 min and directly transferred into 100% methanol at room temperature for 20 min. The coverslips were washed twice in PBS⁺ before incubating with the antibodies for 45 min. R386 was used at 1:500 dilution. The rat monoclonal antibodies MOMA-2 and F4/80 (a kind gift from Peter Leenen, Erasmus University, Rotterdam) were used as undiluted cultured supernatants. The samples were washed three times in PBS⁺ and incubated for 30 min with goat anti-rabbit IgG-FITC antibody (1:100) (Sigma) and/or donkey anti-rat IgG-cy3 (1:100). After washing in PBS, coverslips were mounted in Vectashield mount (Vector Laboratories, Burlingame, CA, USA) containing 0.75 μg/ml DAPI and analysed under the fluorescence microscope (Leitz, Wetzlar, Germany).

β-glucocerebrosidase enzymatic activity assay

Enzyme activity was assayed in the spleen, liver and brain of transgenic and nontransgenic mice. Spleens were made into single cell suspensions and liver and brain were homogenised. The spleen cells were washed three times in phosphate-buffered saline (PBS) and the pellet dissolved in 200 μl PBS. All samples were then sonicated for 3 s. Triton X-100 was added to a final concentration of 0.1%. Cell debris was removed by centrifugation for 10 min at 16 000 *g* at 4°C. Supernatants were transferred into new tubes and the protein concentration was measured using Coomassie Plus protein assay reagent (Pierce, Rockford, IL USA). Immunoprecipitation was performed using the monoclonal antibody 8E4 MoAb[19,20] (diluted 1:2500. 100 μg of protein extract and 30 μl of Protein G Sepharose (Pharmacia Biotech, Uppsala, Sweden) were incubated with the antibody in PBS/0.5% BSA, in a total volume of 500 μl at 4°C overnight on a rotating wheel. The beads were washed six times in PBS/BSA (10 s spin at 16 000 *g*). After the last wash the beads were carefully drained and resuspended in 40 μl of assay medium containing 5 mM 4-methylumbelliferyl-β-D-glucopyranoside (Sigma) in substrate buffer (0.2 M sodium phosphate-citric acid buffer pH 5.8, 0.15% Triton X-100, 0.3% synthetic sodium taurocholate (Sigma)). The beads were incubated at 37°C for 1 h. The reaction was terminated by adding 200 μl of sodium carbonate buffer pH 10.7. Samples were centrifuged and 200 μl of supernatant was used for measurement of 4-methylumbelliferone in a fluorimeter. As a standard 750 pmol 4-methylumbelliferone (Sigma) was used.

DNA FISH analysis

Extracted bone marrow was cultured for 24 h in RPMI medium (GIBCO BRL, Breda, The Netherlands). Chromosome preparations were made according to standard procedures. FISH was carried out as described by Mulder *et al.*[43] The entire 35 kb *NruI* injection fragment was used as a probe. The probe was labelled with dioxygenin and immunochemically detected with FITC. The DNA was counterstained with DAPI.

RT-PCR

For RT-PCR analysis, RNA was isolated from different tissues using the Ultraspec RNA isolation system (Biotecx Laboratories, Houston, TX, USA). cDNA was synthesised in a 50 μl reaction using reverse transcriptase (Super RT; HT Biotechnology, Cambridge, UK) and an oligo(dT) primer. PCR reactions were performed in 50 μl PCR buffer (Life Technologies, Paisley, UK) with 1.5 mM $MgCl_2$, 100 ng of each primer, 1 μl RT reaction, 0.2 mM each dNTPs and 2.5 U *Taq* DNA polymerase (Pharmacia). Thirty cycles of amplification were performed with denaturation at 94°C for 45 s, annealing at 60°C for 30 s and extension at 72°C for 45 s. PCR products were visualised by electrophoresis on a 2% agarose gel. Primers specific for the human β-glucocerebrosidase transgene were used – forward primer: 5'-GCCTTTGAGTAGGGTA AGCA-3' and reverse primer: 5'-AGGAGTGAAA CGGGACGCCT-3'. Primers specific for murine hypoxanthine phosphoribosyltransferase (HPRT) were 5'-CACAGGACTAGAACACCTGC-3' and 5'-GCTGGTGA AAAGGACCTCT-3'.

Acknowledgements

We would like to thank Dr E Ginns for genomic β-glucocerebrosidase clone PCN 9 RI, Dr P Leenen for useful suggestions and provision of monoclonal antibodies (MOMA-2, F4/80, N418) and U937 cell line and M Kuit and R Koppenol for photography. JG and DD were supported by Therexsys Ltd (UK).

References

1 Rosnes JS, Sharkey MF, Veille JC, Mueller-Heubach E. Gaucher's disease in Pregnancy. *Obstet Gynecol Sur* 1996; 51: 549–558.
2 Beutler E, Kuhl W, Vaugham LM. Failure of algucerase infused into Gaucher disease patient to localize in marrow macrophages. *Mol Med* 1995; 1: 320–324.
3 Barranger JA, Ginns EI. Glucosylceramide lipidosis: Gaucher disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds). *Metabolic Basis of Inherited Disease*, 6th edn. McGraw Hill: New York, 1989, pp 1677–1698.
4 Frenkel EP. Gaucher disease: a heterogeneous clinical complex

for which effective enzyme replacement has come of age. *Am J Med Sci* 1993; 305: 331–344.
5 Beutler E. Gaucher disease as a paradigm of current issues regarding single gene mutations of humans. *Proc Natl Acad Sci USA* 1993; 90: 5384–5390.
6 Zimran A *et al.* Prediction of severity of Gaucher's disease by identification of mutations at DNA level. *Lancet* 1989; 2: 349–352.
7 Meivar-Levy I, Horowitz M, Futerman H. Analysis of glucocerebrosidase activity using N-(1-[^{14}C]hexanoyl)-D-erythroglucosylsphingosine demonstrates a correlation between levels of residual enzyme activity and the type of Gaucher disease. *Biochem J* 1994; 303: 377–382.
8 Barton NW *et al.* Replacement therapy for inherited enzyme deficiency macrophage-targeted glucocerebrosidase for Gaucher's disease. *New Engl J Med* 1991; 324: 1464–1470.
9 Beutler E *et al.* Enzyme replacement therapy for Gaucher disease. *Blood* 1991; 78: 1183–1189.
10 Rappeport JM, Ginns EI. Bone marrow transplantation in severe Gaucher's disease. *New Engl J Med* 1984; 311: 84–88.
11 Ringden O *et al.* Long-term follow-up of the first successful bone marrow transplantation in Gaucher disease. *Transplantation* 1988; 46: 66–70.
12 Xu LC *et al.* Long-term *in vivo* expression of the human glucocerebrosidase gene in nonhuman primates after CD34$^+$ hematopoietic cell transduction with cell-free retroviral vector preparations. *Proc Natl Acad Sci USA* 1995; 92: 4372–4376.
13 Krall WJ *et al.* Cells expressing human glucocerebrosidase from a retroviral vector repopulate macrophages and central nervous system microglia after murine bone marrow transplantation. *Blood* 1994; 83: 2737–2748.
14 Migita M *et al.* Selection of transduced CD34$^+$ progenitors and enzymatic correction of cells from Gaucher patients, with bicistronic vectors. *Proc Natl Acad Sci USA* 1995; 92: 12075–1207.
15 Fallet S *et al.* Enzyme augmentation in moderate to life-threatening Gaucher disease. *Pediatr Res* 1992; 31: 496–502.
16 Barranger JA, Rapoport SI, Brady RO. Access of enzymes to brain following osmotic alteration of the blood–brain barrier. *Birth Defects* 1980; 16: 195–205.
17 Beutler E. Gaucher disease: new molecular approaches to diagnosis and treatment. *Science* 1992; 256: 794–799.
18 Carson S, Wiles MV. Far upstream regions of class II MHC Ea are necessary for position-independent, copy-dependent expression of Ea transgene. *Nucleic Acids Res* 1993; 21: 2065–2072.
19 Barneveld RA *et al.* Monoclonal antibodies against human β-glucocerebrosidase. *FEBS Lett* 1983; 134: 585–589.
20 Barneveld RA *et al.* Assignment of the gene coding for human β-glucocerebrosidase to the region q21-q31 of chromosome 1 using monoclonal antibodies. *Hum Genet* 1983; 64: 227–231.
21 Festenstein R *et al.* Locus control region function and heterochromatin-induced position effect variegation. *Science* 1996; 271: 1123–1125.
22 Milot E *et al.* Heterochromatin effects on the frequency and duration of LCR-mediated gene transcription. *Cell* 1996; 87: 105–114.
23 Leenen PJM *et al.* Markers of mouse macrophage development detected by monoclonal antibodies. *J Immunol Meth* 1994; 174: 5–19.
24 Agger R, Crowley MT, Witmer-Pack MD. The surface of dendritic cells in the mouse as studed with monoclonal antibodies. *Int Rev Immunol* 1990; 6: 89–101.
25 Zhou XY *et al.* Mouse model for the lysosomal disorder galactosialidosis and correction of the phenotype with overexpressing erythroid precursor cells. *Genes Dev* 1995; 9: 2623–2634.
26 Drabek D *et al.* Correction of the X-linked immunodeficiency phenotype by transgenic expression of human Bruton tyrosine kinase under the contol of the class II major histocompatibility complex Ea locus control region. *Proc Natl Acad Sci USA* 1997; 94: 610–615.
27 Dingjan GM *et al.* Severe B cell deficiency and disrupted splenic architecture in transgenic mice expressing the E41K mutated form of Bruton's tyrosine kinase. *EMBO J* 1998; 17: 5309–5320.
28 Antoniou M, Geraghty F, Hurst J, Grosveld F. Efficient 3' end formation of human beta-globin mRNA *in vivo* requires sequences within the last intron but occurs independently of the splicing reaction. *Nucleic Acids Res* 1998; 23: 721–729.
29 Glimcher LH, Kara CJ. Sequences and factors: a guide to MHC class-II transcription. *Annu Rev Immunol* 1992; 10: 13–49.
30 Gordon S. Biology of the macrophage. *J Cell Sci* 1986; 4: 267–286.
31 Ling EA. The origin and nature of microglia. In: Fedoroff S, Hertz L (eds). *Advances in Cellular Neurobiology* Academic Press: New York, 1981, pp 35–82.
32 Wood GW *et al.* The failure of microglia in normal brain to exhibit mononuclear phagocyte markers. *J Neuropathol Exp Neurol* 1979; 38: 369–379.
33 Oemichen M. Inflamatory cells in central nervous system. *Prog Neuropathol* 1983; 5: 273–325.
34 Villiams KA, Hart DN, Fabre JW, Morris PJ. Distribution and quantitation of HLA-ABC and DR (Ia) antigens on human kidney and other tissues. *Transplantation* 1980; 29: 274–279.
35 Perry VH, Gordon S. Macrophages and microglia in the nervous system. *TINS* 1988; 11: 273–277.
36 Walker WS *et al.* Mouse microglial cell lines differing in constitutive and interferon-γ-inducible antigen-presenting activities for naïve and memory CD4$^+$ and CD8$^+$ T cells. *J Neuroimmunol* 1995; 63: 163–174.
37 Suzumura A, Mezitis SGE, Gonatas NK, Silberberg DH. MHC antigen expression on bulk isolated macrophage-macroglia from new born mouse brain: induction of Ia antigen expression by γ-interferon. *J Neuroimmunol* 1987; 15: 263–278.
38 Suzumura A, Sawada M, Itoh Y, Marunouchi T. Interleukin-4 induces proliferation and activation of microglia but suppresses their induction of class II major histocompatibility complex antigen expression. *J Neuroimmunol* 1994; 53: 209–218.
39 Imamura K *et al.* Generation and characterisation of monoclonal antibodies against microglia and ontogenic distribution of positive cells. *Lab Invest* 1990; 63: 853–861.
40 Koob M, Burkiewicz A, Kur J, Szybalski W. Rec A-AC: single site cleavage of plasmids and chromosomes at any predetermined restriction site. *Nucleic Acids Res* 1992; 21: 5831–5836.
41 Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: a Laboratory Manual.* Cold Spring Harbor Laboratory Press: New York, 1989, pp 2.88–2.89.
42 Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 1975; 98: 503–517.
43 Mulder MP *et al.* Positional mapping of loci in the Di George critical region at chromosome 22q11 using a new marker (D22S183). *Hum Genet* 1995; 96: 1133–141.

Chapter 5

Microinjection of Cre recombinase RNA induces site-specific recombination of a transgene in mouse oocytes

Ton de Wit, Dubravka Drabek and Frank Grosveld

Nucleic Acids Research 26:676-678, 1998

Microinjection of Cre recombinase RNA induces site-specific recombination of a transgene in mouse oocytes

Ton de Wit, Dubravka Drabek and Frank Grosveld*

Erasmus University Rotterdam, MGC-Department of Cell Biology, PO Box 1738, 3000 DR Rotterdam, The Netherlands

Received October 30, 1997; Revised and Accepted November 25, 1997

ABSTRACT

We have developed a strategy for producing single copy transgenic mouse lines using *Cre-loxP* site specific recombination. The method is based on transient expression of the recombinase after injection of *in vitro* transcribed mRNA into the cytoplasm of fertilised eggs containing multiple copies of the transgene. The success rate of the recombination event is 100% (15 out of 15).

The Cre-*loxP* recombination system of Bacteriophage P1 is a powerful tool for targeting and excising DNA sequences from the genome of higher eukaryotic organisms both *in vitro* (1–3) and *in vivo* (4–6). Cre recombinase is a 38 kDa protein that mediates site specific intramolecular and intermolecular recombination between 34 bp repeats termed *loxP* sites, in the absence of additional cofactors. The process is reversible, but the integration is less efficient due to ongoing excision. In the case of intramolecular recombination, DNA flanked by two directly orientated *loxP* sites is efficiently excised leaving a single *loxP* site in the genome. If the *loxP* sites are in opposite orientation the segment is inverted.

In transgenic mice, the expression level of the transgene depends on the regulatory elements in the construct, it's integration site in the genome and the number of integrated transgenes. However, in many cases it is necessary to compare the expression level between single copy transgenic mice (such as the testing of gene therapy constructs which would be present in the host genome as single copies) (7). Thus, it has become common practice to introduce a *loxP* site into the DNA construct that is microinjected to obtain transgenic mice. Multiple copy transgenic lines are usually reduced in copy number by injecting a plasmid expressing Cre (8) into their fertilised eggs. The disadvantage of this method is the possibility that the plasmid sequences may be present for many generations and integrate into the mouse genome. In addition, and in contrast to published results, in our hands it does not have a 100% efficiency. Finally, the recombination event has to take place as soon as possible to avoid mosaicism. We therefore tested the injection of Cre RNA as an alternative.

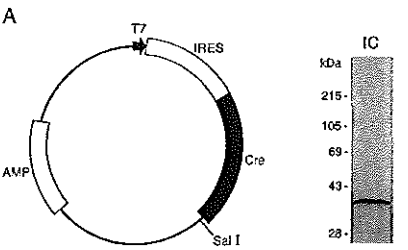

Figure 1. (A) Map of T7-IC used for the *in vitro* transcription of IRES-Cre RNA. The Cre cDNA was amplified via PCR and cloned in the vector pGEM-T (Promega). The IRES was derived from IRES-β-geo (17) and introduced 5′ of the Cre gene. (B) Analysis of translation product of IRES containing mRNA. RNA template was translated in a rabbit reticulocyte lysate and the translation product was analysed on a 12.5% SDS-polyacrylamide gel. The migration of proteins of known molecular weight is indicated in kDa.

It has been shown previously that picorna virus mRNAs have a specialised 5′ non-translated region called the internal ribosomal entry site (IRES) that allows cap-independent translation (9–12). *In vitro* studies have been done using the picorna virus 5′NTR and heterologous protein-coding sequences under the control of a bacteriophage T7 or T3 promoter (13,14). The EMCV 5′ NTR IRES was shown to function in mouse embryos as well as in embryonic stem cells (15). Here, we present a method for efficient removal of transgenic sequences from fertilised mouse oocytes by site specific recombination occurring after cytoplasmatic microinjection of an *in vitro* transcribed IRES-Cre mRNA.

Cre cDNA was PCR amplified using pMC-Cre as a template (16; gift from K.Rajewsky) and subcloned into pGEM-T vector (Promega, Madison, WI) resulting in the plasmid pGEM-T-Cre. From the plasmid IRES-βgeo (17; gift from P.Mountford) the IRES was cut out as a 600 bp *Not*I-blunted *Nco*I fragment and subcloned into *Sph*I-blunted *Nco*I site of pGEM-T-Cre, resulting in the plasmid T7-IRES-Cre (T7IC) (Fig. 1A).

T7IC DNA was *Sal*I linearized and transcribed *in vitro* with T7 RNA polymerase as described (18). The DNA template was

*To whom correspondence should be addressed. Tel: +31 10 408 7169; Fax: +31 10 436 0225; Email: grosveld@chl.fgg.eur.nl

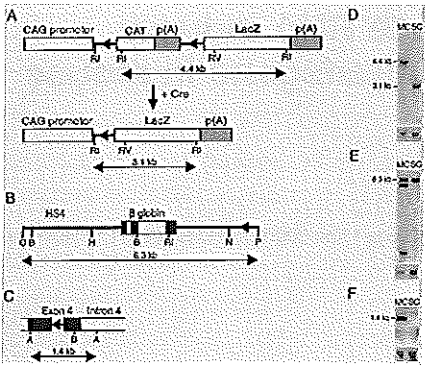

Figure 2. (A) CAG-CAT-Z construct (8) containing the chicken β-actin (CAG) promoter separated from the lacZ gene by the CAT gene, before and after recombination. *Eco*RI (RI) and *Eco*RV (RV) sites are indicated. Arrow heads represent the *loxP* sites. (B) HS4-β-*loxP* construct shown as a 6.3 kb *ClaI–PvuII* fragment, containing hypersensitive site four of the human β-globin LCR and the β-globin gene with ~800 promoter. *ClaI* (C), *Bam*HI (B), *Hin*dIII (H), *Eco*RI (RI), *Not*I (N) and *PvuII* (P) sites are indicated. (C) Thy-*loxP* construct shown as a 1.4 kb *ApaI* fragment, containing parts of exon 4 and intron 4 of the mouse Thy 1.2 gene. *ApaI* (A) and *Bam*HI (B) sites are indicated. (D) Southern blot analysis of *Eco*RI digested tail DNA from multi copy (mc) and single copy (sc) animals of line CAG-CAT-Z16. Before recombination the 4.4 kb band corresponds to an *Eco*RI fragment of the unrecombined transgene and after recombination the 3.1 kb *Eco*RI band indicates that the CAT gene was recombined out. A 2.0 kb *Eco*RV–*Eco*RI LacZ fragment was used as a probe. The Southern blot was rehybridized with a Thy 1.2 probe as an internal control for loading (lower panel). Please note that lane sc contains twice the amount of DNA as lane mc. (E) Southern blot analysis of *Eco*RI digested tail DNA from mc and sc animals of line HS4-β-*loxP*. After hybridisation with a 900 bp *Bam*HI–*Eco*RI fragment of the human β-globin gene, a 6.3 kb band that represents the head-to-tail repeats of the transgene (approximately two copies) and two smaller fragments are seen. After recombination only the 6.3 kb band is seen, with an intensity compatible with a single copy. Lower panel shows loading control as in (D). (F) Southern blot analysis of *Bam*HI digested tail DNA from mc and sc animals of lineThy-*loxP*. Before recombination, a 1.4 kb band is seen representing the head-to-tail arranged repeats of the transgene (~10 copies) after hybridisation with the complete transgene. After recombination only the two end-fragments are left. Lower panel shows loading control as in D.

removed by treatment with 10 U RNase-free DNase for 15 min at 37°C and the reaction was terminated by adding stop mix (10 mM EDTA and 0.4% SDS final concentration). The transcript was purified by sequential phenol/chloroform extraction and ethanol precipitated twice with NH_4Ac at a final concentration of 0.4 M. To confirm that the transcription product encoded a protein of the expected molecular mass, the RNA was translated in a reticulocyte lysate system (Promega). A band of ~38 kDa was detected correlating with the correct size of the Cre protein (Fig. 1B).

Multiple copy transgenic lines were established by injecting three different DNA constructs. A 7 kb *SalI–PstI* fragment of pCAG-CAT-Z (Fig. 2A) (8; gift from P.Vassalli) resulted in three lines (#12, 16 and 18) having three, two and three copies respectively in a head to tail arrangement. One HS4-β-*loxP* transgenic mouse line (having two copies of the transgene in a head to tail arrangement), was generated using a 6.3 kb

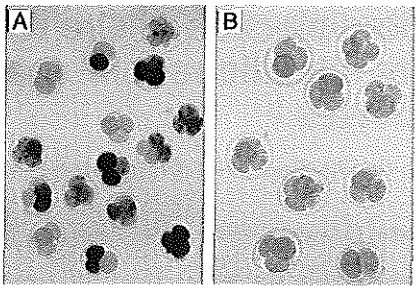

Figure 3. X-Gal staining of two- and four-cell stage embryos of CAG-CAT-Z16, recombined or not recombined. Fertilised eggs were collected from FVB females mated with heterozygote CAG-CAT-Z16 males and IC mRNA was injected into the cytoplasm at a concentration of 5 ng/μl. After incubation (5% CO_2, 37°C) for 24 or 48 h, two- and four-cell stage embryos were stained. (A) Injected transgenic eggs (12) were positive and injected non-transgenic eggs (3) as well as (B) uninjected transgenic eggs (9) were negative for X-gal staining.

ClaI–PvuII fragment containing hypersensitive site 4 of the human β-globin LCR, the β-globin gene with ~800 promoter followed by a *loxP* site downstream of the gene (Fig. 2B). One Thy-*loxP* transgenic mouse line (having ~10 copies of the transgene in a head to tail arrangement), was generated using a 1.4 kb *ApaI* fragment containing parts of exon 4 and intron 4 of the mouse Thy 1.2 gene with a *loxP* site cloned in the *StuI* site (Fig. 2C). All fragments were injected as described (7).

Non-transgenic FVB females, 3 weeks old, were superovulated and mated with heterozygous multi copy transgenic (CAG-CAT-Z, HS4-β-*loxP* or Thy-*loxP*) males. Fertilised oocytes were collected and IRES-Cre (IC) mRNA was microinjected into the cytoplasm at a concentration of 5 ng/μl. During microinjection the RNA was kept on ice and the needle was changed every 25–30 eggs. Injected and non-injected eggs from CAG-CAT-Z lines, were incubated in M16 medium for 24 h (two-cell stage) or 48 h (four-cell stage) at 37°C in 5% CO_2 (50% survival rate). Recombination results in removal of the CAT gene between the *loxP* sites and β-galactosidase expression (8) (Fig. 2A). From seven independent injection experiments in the three different CAG-CAT-Z transgenic lines, 45% of the eggs injected with IC mRNA developed to two- or four-cell stage and stained for β-galactosidase as expected from Mendelian distribution (Fig. 3A). Non-transgenic eggs (Fig. 3A) or non-injected transgenic eggs (Fig. 3B) were negative for β-galactosidase. These results show that by injecting IC mRNA into murine oocytes, Cre recombinase is expressed at a sufficient level to perform site-specific recombination. In experiments with all three lines, we have seen that not all the cells of the dividing embryo were β-galactosidase positive, as previously reported (8). This was seen in both two- and four-cell stage embryos. When transferred into pseudopregnant BCBA foster mothers and stained for β-galactosidase at day 16 p.c. embryos showed a ubiquitous staining pattern (data not shown). This suggests that the recombination event is an early process although not instantaneous.

IC mRNA injected eggs were also transferred into the oviducts of pseudopregnant female BCBA mice. Tail DNA was examined

by Southern blotting. Complete recombination occurred in all the lines (15/15) and resulted in single copy transgenic mice. The IC sequences were not integrated into the genomes of any of these transgenic mouse lines.

We have found that injecting IC mRNA is more efficient than injecting the plasmid pCAGGS-Cre as previously described (8). Using the 'plasmid method' and injecting DNA at a concentration of 5 ng/μl we have seen complete recombination but also a significant number of partial recombination events (3 out of 11) using the same multicopy transgenic lines.

Using RNA (by injection or otherwise) to deliver genetic material to cells, rather then DNA, would also be of great interest for gene therapy protocols. In this way, the necessity of delivering DNA to the nucleus, currently one of the main obstacles in non-viral therapy protocols to achieve successful gene expression, could be avoided.

ACKNOWLEDGEMENTS

We thank P.Vassalli for pCAGGSCre and pCAG-CAT-Z, P. Mountford for IRES-βgeo, M.Wijgerde, N.Gillemans, J.Guy, L.Braam and M.Kuit for materials and assistance at various stages of the project. T.d.W. and D.D. were supported by Therexsys, UK. The work is supported by the EUR, NWO (NL) and Therexsys (UK).

REFERENCES

1 Sauer, B. and Henderson, N. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 5166–5170.
2 Sauer, B. and Henderson, N. (1990) *The New Biologist*, **2**, 441–449.
3 Baubonis,W. and Sauer,B. (1993) *Nucleic Acid Res.*, **21**, 2025–2029.
4 Lakso, M., Sauer, B., Mosinger, Jr., B., Lee, E.J., Manning, R.W., Yu, S.-H., Mulder, K.W. and Westphal, H. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 6232–6236.
5 Orban, P.C., Chui, C. and Marth, J.D. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 6861–6865.
6 Gu, H., Marth, J.D., Orban, P.C., Mossmann, H. and Rajewsky, K. (1994) *Science*, **265**, 103–106.
7 Ellis, J., Pasceri, P., Tan-Un, K., Wu, X., Harper, A., Fraser, P. and Grosveld, F. (1997) *Nucleic Acid Res.*, **6**, 1296–1302.
8 Araki, K., Araki, M., Miyazaki, J-I. and Vassalli, P. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 160–164.
9 Jang, S.K., Kräusslich, H., Nicklin, M.J.H., Duke, G.M., Palmenberg, A.C. and Wimmer, E. (1988) *J. Virol.*, **62**, 2636–2643.
10 Pelletier, J. and Sonenberg, N. (1988) *Nature*, **334**, 320–325.
11 Jackson, R.J., Howell, M.T. and Kaminski, A. (1990) *Trends Biochem. Sci.*, **15**, 477–483.
12 Macejak, D.G. and Sarnow, P. (1991) *Nature*, **353**, 90–95.
13 Elroy-Stein, O., Fuerst, T.R. and Moss, B. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 6126–6130.
14 Zhou, Y., Giordano, T.J., Durbin, R.K. and McAllister, W.T. (1990) *Mol. Cell. Biol.*, **10**, 4529–4537.
15 Kim, D.G., Kang, H.M., Jang, S.K. and Shin, H.-S. (1992) *Mol. Cell. Biol.*, **12**, 3636–3643.
16 Gu, H., Zou, Y-R. and Rajewsky, K. (1993) *Cell*, **73**, 1155–1164.
17 Mountford, P., Zevnik, B., Düwel, A., Nichols, J., Li, M., Dani, C., Robertson, M., Chambers, I. and Smith, A. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 4303–4307.
18 Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984) *Nucleic Acid Res.*, **12**, 7035–7056.

Chapter 6

Concluding remarks and future prospects

Concluding remarks and future prospects

The data described in Chapters 2-5 has already been discussed separately. In this section I will give an overview of the work presented in this thesis and point out conclusions and lines of research emerging from it.

The projects appear very different in nature but they are based on a common theme, namely the improvement and exploitation of transcriptional studies, in particular LCR based expression. Thus investigating the potential of a new enzyme/prodrug system *in vivo* for future anticancer therapies (see Chapter 2); testing possible gene therapy constructs in transgenic mouse models for two different inherited genetic disorders in men, namely XLA immunodeficiency (see Chapter 3) and Gaucher lysosomal storage disease (see Chapter 4); and using a new methodology for producing single copy transgenic mice for the purposes of therapeutic constructs comparison (see Chapter 5) all have one common goal, which is to test and eventually to use LCR elements in gene therapy.

In transgenic studies, LCRs provide position independent, copy number dependent expression of the linked genes at levels equivalent to the appropriate endogenous gene(s). Most of the functional data and research into mechanisms of LCR action arise from studies of the β-globin LCR and the CD2 LCR (see Chapter 1). Extensive deletion analysis was performed and transcription and expression monitored at the single cell level. It was concluded that only a complete LCR is capable of opening the locus into a fully active chromatin configuration in all of the targeted cells (e.g. all erythroid precusor cells when using β-globin LCR or $CD2^+$ cells when using CD2 LCR). An incomplete LCR loses this property when integrated into heterochromatic regions, resulting either in classical PEV and expression in a clonal subpopulation of cells, or in a cell timing effect where the gene is transcribed in all of the cells but not at all times (Festenstein *et al.* 1996; Milot *et al.* 1996). One of the future goals for gene therapy is to have a therapeutic gene correctly expressed regardless of the chromosomal integration site. As yet transgene integration cannot be controlled. Therefore, with considerations for PEV and cell timing effects on LCR mediated gene expression for gene therapy, it is necessary to define what constitutes a complete LCR in each particular case. The MHC class II Ea gene LCR, described in the Intoduction and in this thesis research, has not been extensively characterised for this effects (Carson and

Wiles 1993). In the studies presented here thesis this LCR was tested in the context of two different heterologous genes, expression of which was followed in detail. As the appropriate elements are defined for each LCR, it may even be desirable to target the transgenes into heterochromatic regions in order to avoid possible problems with insertions into transcriptionally active euchromatin. Until now, attempts to improve illegitimate recombination, (ie non-homologous DNA integration) by using repetitive DNA (namely mouse minor satellite) sequences added to both sides of transgenic constructs have not given positive results (Drabek and de Wit, unpublished data).

In order to obtain efficient expression of an exogenous gene, it is not only necessary that the gene is transcribed efficiently, but also that it produces stable transcripts. If the complete transcription unit of a gene is used (see Chapter 5 where the β-glucocerebrosidase gene was used), this will not be a problem. However, many genes have a very large transcription unit and it will often be necessary to use a "minigene" constructed from a cDNA (see Chapter 3 where the Btk cDNA was used) in such a way that it produces a stable transcript. The question is what are the requirements for the stability? It is known that the majority of eukaryotic mRNAs need the addition of a poly–A tail at the 3' end of the mRNA for transcript stability and export from the nucleus (reviewed in Proudfoot 1991).

The other major processing event required for the conversion from primary transcript to mRNA is the removal of introns. There is a substantial amount of data supporting the role of introns in transcript stability (Palmiter *et al.* 1991; Jonsson *et al.* 1992; Jonsson *et al.* 1994; Nesic *et al.* 1993; Nesic and Maquat 1994; Antoniou *et al.* 1998). Therefore it is advisable to include at least one intron in a gene that is to be expressed in human tissues.

Which intron is best for gene constructs for therapeutic purposes? Although heterologous introns can enhance expression of transgenes in mice (Palmiter *et al.* 1991), there is no ideal universal intron as such. The best choice would be to use natural introns if possible. Even then, there may be a requirement for specific introns. In contrast to reports that the first intron of rat growth hormone is essential for high level growth hormone expression (Palmiter *et al.* 1991), the human β-globin gene, for example, fails to produce stable transcripts in erythroid cells unless the second intron from the very same gene is included in the transcriptional unit (Collis *et al.* 1990). The requirement cannot be substituted by the first intron or by introns from other genes, implying that the second (and last)

intron of the β-globin gene possesses unique structural features (in the terminal 60 nucleotides of βIVS II) which allow it to enhance the efficiency of 3'-end formation (Antoniou *et al.* 1998). There are also other examples that support the importance of the last intervening sequence of multiple intron containing genes for efficiency of 3' end formation (ie. the last intron of the 7 exon triosephosphate isomerase gene) (Nesic *et al.* 1993; Nesic and Maquat 1994). It should also be taken into account that some introns could improve expression of transgenes by other mechanisms. They may contain enhancers or other cis-acting elements which bind proteins that influence transcription (reviewed in Mitchell and Tjian 1989; Jonsson *et al.* 1994) or contain the sequences that facilitate opening of chromosomal domains, perhaps by affecting nucleosome composition, position, or higher-order packaging (reviewed in Svaren and Chalkley 1990).

The choice of the expression cassette containing the heterologous βIVS-II in combination with Btk cDNA (Chapter 3) was probably not ideal, since not all of the transgenic mice expressed, although when expressed, Btk was restricted to the appropriate cell type (B-cells, thymic epithelial cells, macrophages). When the same LCR elements and promoter were linked to genomic sequences of the β-glucocerebrosidase gene (Chapter 4) all transgenic mice expressed the gene. These observations led to the employment of a new strategy applied to transgenic constructs used for further investigation of the E41K mutated form of Btk (Dingjan *et al.* 1998). The new constructs contained the first three exons of human BTK as cDNA sequence followed by a genomic DNA fragment encompassing exons 13-19. In this way 16 out of 18 intron segments together with 3'UTR were included, resulting in high level Btk expression. It seems likely that the modified transgene contained endogenous regulatory elements of the Btk gene, since Btk intron sequences contain multiple clusters of extensive conservation between mouse and man, one of which (a 229 bp region in the fifth intron), showed co-localisation with a Dnase I hypersensitive site present in B but not T cells (Dingjan *et al.* 1998). In conclusion, the importance of 5'and 3' controlling sequences, intronic sequences and the existence of distant locus-specific regions all favour the use of large DNA constructs for the maintenance of normal control of gene expression.

In Chapter 2 a new enzyme/prodrug system is tested for a possible "suicide gene" approach to cancer therapy. Since the NTR/CB1954 system was reported to function in mammalian cells *in vitro* (Bridgewater *et al.* 1995), our main goal was to test its specificity and a possible bystander

effect *in vivo*. For this purpose we needed an animal model where nitroreductase is expressed only in one specific tissue, the fate of which we can easily monitor after the systemic application of CB1954. Most of the data on the CD2 LCR (Greaves *et al.* 1989; Lang *et al.* 1991; Festensein *et al.* 1996; Kaptein *et al.* 1998) has come from studies on expression of eukaryotic genes (CD2, β-globin, ADA), thus a bacterial gene such as NTR under its control was novel. After introducing the optimal sequence for initiation by eukaryotic ribosomes (Kozak 1986), the bacterial gene was placed in the CD2 LCR expression cassette (for more details see Chapter 2). Transgene expression, when detected, was confined to the expected cell types: T cells and thymocytes. Enzyme expression did not affect cells in the absence of the prodrug. After its administration, cells expressing the NTR enzyme were able to convert the non-toxic prodrug to a cytotoxic drug, resulting in highly specific killing of T cells and thymocytes. Although, in our cell culture experiments a bystander effect was not observed (probably due to the very small percentage of cells expressing the enzyme and lack of intimate cellular contact between expressing and non-expressing cells), results from *in vivo* studies suggest its occurrence in spleen, where not only T but also B cells were affected. NTR-mediated ablation by CB1954 involves apoptosis.

Another selective cell ablation in transgenic mice expressing *E. coli* nitroreductase was reported (Clark *et al.* 1997). The gene was expressed in luminal cells of the mammary gland of transgenic mice using the tissue specific β-lactoglobulin promoter. Only one transgenic mouse line expressed NTR at sufficient levels. The expression pattern was mosaic (variegated), but killing of the targeted cell type was nonetheless achieved. In cell-lineage ablation therapies the aim would be to minimise the bystander effect whereas in therapies for tumour destruction the aim would be to maximise the response. It is obvious that further research in this field will be directed towards: investigating the requirements for a bystander effect *in vivo*, development of tumour models where the efficacy of the system can be evaluated (ie. McNeish *et al.* 1998), investigation of alternative and maybe more effective prodrugs for use with *E.coli* nitroreductase (Bailey *et al.* 1996) and development of appropriate gene delivery methods (both viral and non-viral) for solid tumours. At this time, even anaerobic bacteria are being considered as a potential gene delivery system (Lemmon *et al.* 1997).

In Chapters 3 and 4, the role of gene therapy in correction of hereditary diseases is studied. XLA is one of the most frequently occuring inherited

immunodeficiencies and Gaucher disease is the most frequently occuring lysosomal storage disease in man. XLA, caused by Btk deficiency, affects only the B cell lineage (see Chapter 1). The results of Hendriks *et al.* (that the first selective disadvantage of Btk^- B cells in mice occurs at the transition from pre-B to B cells) suggested that correction of the *xid* phenotype might be possible by expression of Btk from the pre-B cell stage onwards. Indeed, transgenic expression of human BTK corrected the *xid* defects, as indicated by the appearance of normal proportions of mature IgM^{low} IgD^{high} circulating B-cells, $CD5^+$ cells in the peritoneum, restored serum IgM and IgG3 levels and B-cell responses to TI-II and TD antigens *in vivo* or LPS *in vitro*. The fact that transgenic human Btk can compensate for the absence of murine Btk indicates that the essential sites for Btk interaction with other signal transduction components are conserved between the two species. Due to the use of the murine MHC class II LCR, expression of the transgene is confined to class II positive cells in a developmentally regulated manner. BTK is expressed only from the pre-B cell stage onwards. It is also expressed in the macrophage lineage. Our results suggest that the BTK gene does not need to be introduced into the very small population of haematopoietic stem cells (see Chapter 1) to restore B cell development in XLA patients. If gene could be transferred to any of the B lymphoid precursors up to the small pre-B cell stage, the possible target cell population would increase approximately 1000 times. There are other advantages that make XLA an attractive target for gene therapy. Bone marrow cells are easily accessible for *ex vivo* manipulation (Chapter 1) and there is an obvious selective advantage for B cells that have been corrected. In addition Btk expression only needs to be transient, since Btk is not required in plasma cells (De Weers *et al.* 1993).

In Chapter 4, the same murine MHC class II LCR was used to drive the expression of human β-glucocerebrosidase in B-cells, dendritic cells, thymic epithelial cells and macrophages in various tissues. In Gaucher disease, macrophages are particularly affected. In the absence of functionally active β-glucocerebrosidase they accumulate the undigested glucosylceramide substrate. Gene therapy offers an alternative treatment in which improvement of the disease symptoms will require at least the partial replacement of the defective macrophages by gene corrected cells, in conjunction with sustained expression of the gene. This approach is promising for lysosomal storage diseases with slower onset but probably not applicable to type 2 Gaucher disease (acute neuronopathic form) due to the severity of neuronal impairment during fetal development. There is

another problem to take into account and that is the repopulation capacity of bone marrow derived macrophages after successful *ex vivo* genetic manipulation. Retrovirally transduced murine BM cells can repopulate macrophages and central nervous system microglia 3-4 months after bone marrow transplantation (Krall *et al.* 1994). Although up to 20 % of the total number of microglia are repopulated, the majority (approximately 90 %) of cells are perivascular and only 5-10 % parenchymal microglia. The question that remains to be answered is how much microglial expression is required to achieve a beneficial effect in neuronopathic types of disease. The transgenic mice described in Chapter 4 do not express the human enzyme in parenchymal microglia, but they do express it in perivascular macrophages. Crossing of our transgenic mice with the murine β-glucocerebrosidase deficient mouse (Tybulewicz *et al.* 1992) does not reveal to what extent the Gaucher phenotype could be "rescued" (unpublished data). Differences in skin biology between mice and humans are the limiting factor (see Chapter 1). The mice still die soon after birth, most probably as a consequence of a still compromised epidermal permeability barrier, since our transgene is not designed to rescue epidermal GC activity.

In Chapter 5 we describe a new method of injecting Cre RNA into mouse oocytes for the purposes of single copy transgenic line generation. Possible applications of this method have already been discussed (see Chapters 1 and 5). The same approach could be applied to the Flp recombinase although the recombination process is less effective, probably due to a specific temperature requirement when not using a termostable Flp mutant (Drabek and de Wit, unpublished data). The results obtained with different RNAs prompted investigation of the possible use of RNA in gene therapy (in cases where transient expression of a gene would be sufficient). However, there are many questions that remain to be answered (i.e. RNA stability, how much protein is needed for a therapeutic effect to be seen, what is the best way of introducing the RNA into cells etc).

References

- Antoniou M, Geraghty F, Hurst J and Grosveld F. Efficient 3'-end formation of human β-globin mRNA in vivo requires sequences within the last intron but occurs independently of the splicing reaction. *Nucleic Acids Res.* 1998; **26**:721-729.
- Bailey SM *et al.* Investigation of alternative prodrugs for use with E. coli nitroreductase in 'suicide gene' approaches to cancer therapy. *Gene Ther.* 1996; **3**:1143-1150.
- Bridgewater JA *et al.* Expression of the Bacterial Nitroreductase Enzyme in Mammalian Cells Renders Them Selectively Sensitive to Killing by the Prodrug CB1954. *Europian Journal of Cancer* 1995; **31**:2362-2370.
- Carson S and Wiles MV. Far upstream regions of class II MHC Ea are necessary for position-independent, copy-dependent expression of Ea transgene. *Nucleic Acids Res.* 1993; **21**:2065-2072.
- Clark AJ *et al.* Selective cell ablation in transgenic mice expressing E. coli nitroreductase. *Gene Ther.* 1997; **4**:101-110.
- Collis P, Antoniou M and Grosveld F. Definition of minimal requirements within the human beta-globin gene and the dominant control region for high level expression. *EMBO J.* 1990; **9**:233-240.
- De Weers M *et al.* The Bruton's tyrosine kinase gene is expressed throughout B cell differentiation, from early precusor B cell stages preceding immunoglobulin gene rearrangement up to mature b cell stages. *Eur. J. Immunol.*1993; **23**:3109-3114.
- Dingjan GM *et al.* Severe B cell deficiency and disrupted splenic architecture in transgenic mice expressing the E41K mutated form of Bruton's tyrosine kinase. *EMBO J.* 1998; **17**:5309-5320.
- Festenstein R *et al.* Locus Control region function and heterochromatin-Induced Position Effect Variegation. *Science* 1996; **271**:1123-1125.
- Hendriks R *et al.* Inactivation of BTK by insertion of LacZ reveals defect in B cell development only past the pre-B cell stage. *EMBO J.* 1996; **15**:4862-4872.
- Jonsson JJ, Converse A and McIvor RS. An enhancer in the first intron of the human purine nucleoside phosphorylase-encoding gene. *Gene* 1994; **140**:187-193.
- Jonsson JJ, Foresman MD, Wilson N and McIvor RS. Intron requrement for expression of the human purine nucloside phosphorylase gene. *Nucleic Acids Res.* 1992; **20**:3191-3198.
- Kozak M. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* 1986; **44**:283-292.
- Krall WJ *et al.* Cells expressing human glucocerebrosidase from a retroviral vector repopulate macrophages and central nervous system microglia after murine bone marrow transplantation. *Blood* 1994; **83**:2737-2748.
- Lemmon MJ *et al.* Anaerobic bacteria as a gene delivery system that is controlled by the tumor microinvironment. *Gene Ther.* 1997; **4**:791-796.
- McNeish IA *et al.* Virus directed enzyme prodrug therapy for ovarian and pancreatic cancer using retrovirally delivered E. coli nitroreductase and CB1954. *Gene Ther.* 1998; **5**:1061-1069.

- Milot E. *et al.* Heterochromatin Effects on the Frequency and Duration of LCR-Mediated Gene Transcription. *Cell* 1996; **87**:105-114.
- Mitchell PJ and Tjian R. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* 1989; **245**:371-378.
- Nesic D and Maquat LE. Upstream introns influence the efficiency of final intron removal and RNA 3'-end formation. *Genes Dev.* 1994; **8**:363-375.
- Nesic D, Cheng J and Maquat LE. Lack of an effect of the efficiency of RNA 3'-end formation on the efficiency of removal of either the final or the penultimate intron in intact cells. *Mol. Cell. Biol.* 1993; **13**:3359-3369.
- Palmiter RD, Sadgren EP, Avarbock MR, Allen DD and Brinster RL. Heterologous introns can enhance expression of transgenes in mice. *Proc. Natl. Acad. Sci. USA* 1991; **85**:478-482.
- Proudfoot N. Poly (A) Signals. *Cell* 1991; **64**:671-674.
- Svaren J and Chalkley R. The structure and assembly of active chromatin. *Trends Genet.* 1990; **6**:52-56.
- Tybulewicz WLJ *et al.* Animal model of Gaucher's disease from targeted disruption of the mouse glucocerebrosidase gene. *Nature* 1992; **361**: 226-233.

Summary

Gene therapy offers new opportunities to treat human diseases, either by restoring gene functions that have been lost through mutations or by introducing genes that can inhibit the replication of infectious agents, render cells resistant to cytotoxic drugs, or cause the elimination of aberrant cells. Although the concept may appear to be elegantly straightforward and a direct application of recombinant DNA technology, research has indicated that successful implementation of gene transfer in the clinic will require the co-ordinated development of a variety of new technologies and the establishment of unique interactions between investigators from divergent medical and basic science disciplines.

In addition to problems related to the effectiveness and safety of gene delivery, a new set of problems emerged when expression of integrated DNA from various promoters was studied in transgenic animals or in model gene therapy experiments using viral vectors in animals. Promoter-enhancer combinations that worked well in transient expression or even in stable transfection experiments in cultured cells, often work poorly or not at all in whole–animal experiments. Individual mouse lines with multicopy transgenes exhibit expression levels that have no relationship to the number of integrated copies, indicating that the site of integration played an important role. This implied negative effects imposed by most chromosomal locations and/or insufficiency of cis-acting elements to activate transcription irrespective of integration site. The identification of regulatory elements, termed locus control regions (LCRs) that were able to overcome such position effects was a major discovery. LCRs can direct high levels of tissue-specific and developmentally controlled expression of a transgene irrespective of the chromosomal site of integration. The work described in this thesis utilises these properties of LCRs to establish animal models to test possible gene therapy constructs.

Transgenic mice expressing nitroreductase (see Chapter 2) express the enzyme in a tissue specific manner only in T-cells due to the use of the human CD2 LCR. In this way we generated a mouse model where the new enzyme/prodrug system could be tested for the first time *in vivo*, and its specificity, toxicity and possible bystander effect evaluated. Our results showed that system works efficiently *in vivo* and that there is a potentially useful bystander effect. They showed that more extensive toxicity studies have to be performed to establish therapeutic window.

Transgenic mice expressing human Btk (see Chapter 3) or human beta-glucocerebrosidase (see Chapter 4) express the enzymes in a tissue specific and developmentally controlled manner. Due to the use of a murine MHC class II LCR, transgenes are expressed in all of the MHC class II positive cells (B-cells, macrophages, dendritic cell, thymic epithelial cells). Rescue experiments showed that the Btk^- phenotype in the mouse is corrected by expression of human Btk from the pre-B cell stage onwards. This finding might have some implication for future gene therapy designs for X-linked agammaglobulinaemia in humans. However, the murine class II LCR does not result in expression in microglial cells, which could be a major obstacle when considering therapy for a lysosomal storage disease that involves the central nervous system ie. Gaucher type 2 disease. Taken together, both animal models are valuable. They demonstrate that by using suitable constructs, gene augmentation is feasible as a curative treatment.

Samenvatting

Gentherapie biedt nieuwe mogelijkheden om ziektes te behandelen. Een verloren gen functie als gevolg van DNA mutaties kan worden hersteld, de vermenigvuldiging van pathogenen kan worden geremd of cellen kunnen resistent gemaakt worden tegen cytostatica of schadelijke cellen kunnen worden gedood. Alhoewel het principe een elegante en eenvoudige directe toepassing lijkt van DNA recombinant technologie, is uit onderzoek gebleken dat de succesvolle toepassing van gen overdracht in de kliniek de gecoördineerde ontwikkeling vereist van een aantal technologieën en unieke interacties tussen onderzoekers met een verschillende klinische en/of wetenschappelijke achtergrond.

Naast de problemen voor wat betreft de effectiviteit en veiligheid van gen overdracht, zijn een aantal nieuwe problemen naar voren gekomen toen de expressie van geïntegreerd DNA werd bestudeerd in transgene muizen of in model gentherapie experimenten met virale vectoren in proefdieren. Combinaties van promotoren en enhancers, die goed tot expressie komen in transiente of zelfs stabiele transfectie experimenten, functioneren vaak slecht of helemaal niet in dierproeven. Muizen die meerdere kopieën van een transgen bevatten, vertoonden expressieniveaus, die niet correleerden met het aantal geïntegreerde kopieën. Dit gaf aan dat de plaats van integratie in belangrijke mate het expressieniveau beïnvloedt. Dit impliceerde tevens dat er negatieve effecten worden veroorzaakt door de meeste chromosomale posities van integratie en dat de gebruikte cis elementen niet in staat waren om deze negatieve effecten te overwinnen.

De ontdekking van Locus Control Regions (LCR's), die in staat zijn om deze negatieve effecten wel te overwinnen, was in dit verband belangrijk. Het gebruik van LCR's resulteert in hoge expressieniveaus tijdens het gewenste stadium van de ontwikkeling onafhankelijk van de plaats van integratie in het genoom van de gastheer. Het werk dat in dit proefschrift beschreven is, gebruikt de eigenschappen van LCR's om diermodellen te genereren, die geschikt zijn voor het testen van gentherapie constructen.

Eerst worden transgene muizen beschreven die een *in vivo* model zijn voor de behandeling van tumoren door de toediening van een "prodrug", die door het ingebrachte nitroreductase gen omgezet wordt tot een agens dat DNA alkyleert met de celdood tot gevolg. Het nitroreductase

gen wordt door het gebruik van de LCR van het humane CD2 gen specifiek tot expressie gebracht in T cellen. De resultaten laten zien dat het systeem effectief werkt en dat er een potentieel nuttig "bystander" effect aanwezig is *in vivo*. De resultaten toonden echter ook aan dat uitgebreider onderzoek naar toxiciteit noodzakelijk is om het juiste therapeutische effect te bereiken.

Dit proefschrift beschrijft verfoolgens ook muismodellen voor de behandeling van twee erfelijke ziektes, namelijk X-gebonden agammaglobulinemia en ziekte van Gaucher. Het humane btk gen en het humane glucocerebrosidase gen respectievelijk zijn op weefsel specifieke wijze op de juiste tijd van de ontwikkeling tot expressie gebracht d.m.v. het gebruik van de muizen MHC klasse II LCR. De transgen constructen komen in deze muizen in alle MHC klasse II positieve cellen (B cellen, macrofagen, dendritische cellen, thymus epitheel cellen) tot expressie. Kruisingen van de muizen met het humane btk gen met btk negatieve muizen laten zien dat het defect in B cellen gecorrigeerd wordt vanaf het pre B cel stadium. Deze bevinding zou toegepast kunnen worden voor de behandeling van agammaglobulinaemia patiënten waarin het btk gen gemuteerd is. De correctie van de lysosomale stapelingsziekte van Gaucher die veroorzaakt wordt door een defect in het glucocerebrosidase gen, is echter met dit constuct waarschijnlijk moeilijker te verwezenlijken. Het MHC klasse II construct komt namelijk niet tot expressie in microglia cellen hetgeen een belangrijk obstakel zou kunnen vormen voor de behandeling van patiënten met Gaucher type 2, waarbij het centrale zenuwstelsel wordt aangetast. Deze experimenten tonen daarmee het belang van diermodellen aan, omdat zij laten zien wanner een therapeutische behandeling door het introduceren een bepaald gen construct wel of niet mogelijk is.

Curriculum vitae

Name: Dubravka Drabek
Date of birth: 22-8-1965
Place of birth: Dubrovnik
Education:
1979-1983 XI th Belgrade's gymnasium, natural science direction, winner of Vuk's diploma (top marks in all subjects through all school years), diploma work in microbiology-laboratory project on Pseudomonas aeruginosa under supervision of Dr. Z. Sokolovski (Clinical Centre: " Bezanijska Kosa"-Zemun).
1983-1989 medical student at Medical Faculty, University of Belgrade;
Summer 1987-clinical practice in internal medicine, Nephrology Department, University Clinic Freiburg, Germany, under supervision of Dr. C. Wanner
Graduated from Medical School (17-5-1989) with GPA 9.28 (out of 10);
June 1989-Jun 1990 preregistration year
June 1990 state examination for medical doctors passed with distinction.
1989-1991 Serbian Academy of Science fellowship for Mr Sc degree in Immunology, Medical Faculty, University of Belgrade, project : Phenotypical characterisation of thymic macrophages in culture, under supervision of Dr. M.Colic -VMA, Belgrade.
1991-1993 working in Gene Structure and Expression Department, National Institute for Medical Reaserch (NIMR), London, UK under supervision of Dr. F. Grosveld on gene therapy related projects.
Since August 1993 working in Cell Biology and Genetics Department, Medical Faculty, Erasmus University Rotterdam, Netherlands.
June 1998 animal course (Art 9) at EUR

List of publications

- Colic M, Popovic Lj, Gasic S, **Drabek D** and Dujic A. Primary culture of rat thymic non-lymphoid cells: influence of culture time on the expression of macrophage differentiation antigens defined by monoclonal antibodies. *Thymus* 1991; **18**:243-256.
- Colic M and **Drabek D**. Expression and function of intercellular adhesion molecule 1 (ICAM-1) on rat thymic macrophages in culture. *Immunol. Lett.* 1991; **28**:251-257.
- **Drabek D** and Colic M. Expression of Interleukin-2R on Rat Thymic Macrophages in Culture. In: Lymphatic Tissues and In Vivo Immune Responses 1991, edited by Imhof BA.Berrich-Aknin S and Ezine S; Marcel Dekker, Inc. pp. 803-808.
- Colic M and **Drabek D.** Phenotype and adhesion characteristics of rat thymic macrophages cultivated in serum-free medium. *Thymus* 1993; **21**:43-60.
- Ellis J, Philipsen S, Strouboulis J, Pruzina S, Tewari R, Gillemans N, **Drabek D**, Harper A, Grosveld F and Fraser P. The regulation of human β-globin gene expression; the roles of 5'HS1-4. In: Volume 1 of the Proceedings of the 9th Conference on Hemoglobin Switching held at Orcas Island, Washington, USA, 1994; edited by Stamatoyannopoulis G; Inercept Ltd,Andover, Hampshire, UK, pp 29-38.
- Guy J, **Drabek D** and Antoniou M. Delivery of DNA into mammalian cells by receptor-mediated endocytosis and gene therapy. *Mol. Biotechnol.* 1995; **3**:237-248.
- Grosveld F, Antoniou M, Berry M, Dillon N, **Drabek D**, Ellis J, Fraser P, Haley J, Philipsen S, Pruzina S, Raguz-Bolognesi S, Trimborn T and Wijgerde M. Drug discovery and the transcriptional contol of the human beta globin gene locus. In: Genomes, Molecular biology and drug discovery 1996; pp. 117-127.
- Tewari R, Gillemans N, Harper A, Wijgerde M, Zafarana G, **Drabek D**, Grosveld F and Philipsen S. The human beta-globin locus control region confers an early embryonic erythroid-specific expression pattern to basic promoter driving the bacterial LacZ gene. *Development* 1996; **122**:3991-3999.

- **Drabek D**, Raguz S, De Wit TP, Dingjan GM, Savelkoul HF, Grosveld F and Hendriks WR. Correction of the X-linked immunodeficiency phenotype by transgenic expression of human Bruton tyrosine kinase under the control of the class II major histocompatibility complex Ea locus control region. *Proc. Natl. Acad. Sci.* USA 1997; **94**:610-615.
- **Drabek D**, Guy J, Craig R and Grosveld F. The expression of bacterial nitroreductase in transgenic mice results in specific cell killing by the prodrug CB1954. *Gene Ther.* 1997; **4**:93-100.
- de Wit T, **Drabek D** and Grosveld F. Microinjection of cre recombinase RNA induces site –specific recombination of a transgene in mouse oocytes. *Nucleic Acids Res.* 1998; **26**:676-678.
- Guy J, Willemsen R, Langeveld A, Grosveld F and **Drabek D**. Murine MHC class II locus control region drives expression of human β-glucocerebrosidase in antigen presenting cells of transgenic mice. *Gene Ther.* 1999; **6**:488-507.

Acknowledgements

I would like to thank all those who were involved in the realisation of this thesis. Frank Grosveld supervised all the projects and Therexsys Ltd. (UK) has been the main source of funding. I am grateful to Frank for giving me the opportunity to work in his lab and participate in many interesting projects. Thanks for all the letters you wrote for me to whom it may concern (embassies, police, even the phone company) saying that I am a normal person. Maybe you should be more careful with such statements?

Special thanks to my excellent co-workers and friends Jacky Guy and Ton de Wit. Jacky, I hope I spelled this correctly and no extra articles were needed.

I am grateful to Frans, Joost, Mirko, Mieneke, Stefania and Marike for technical and secretarial assistance.

I would like to thank my old lab 706 (Rudi, Gemma, Nikos, Rick and Alex) for the good working atmosphere, free exchange of ideas and useful discussions.

Finally, thanks to all my friends for understanding and having patience in dealing with my difficult personality and my often sarcastic comments especially through my battles with endless bureaucracy.