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A LENTIVIRAL GENE TRANSFER VECTOR FOR THE TREATMENT OF CYSTIC FIBROSIS AIRWAY DISEASE

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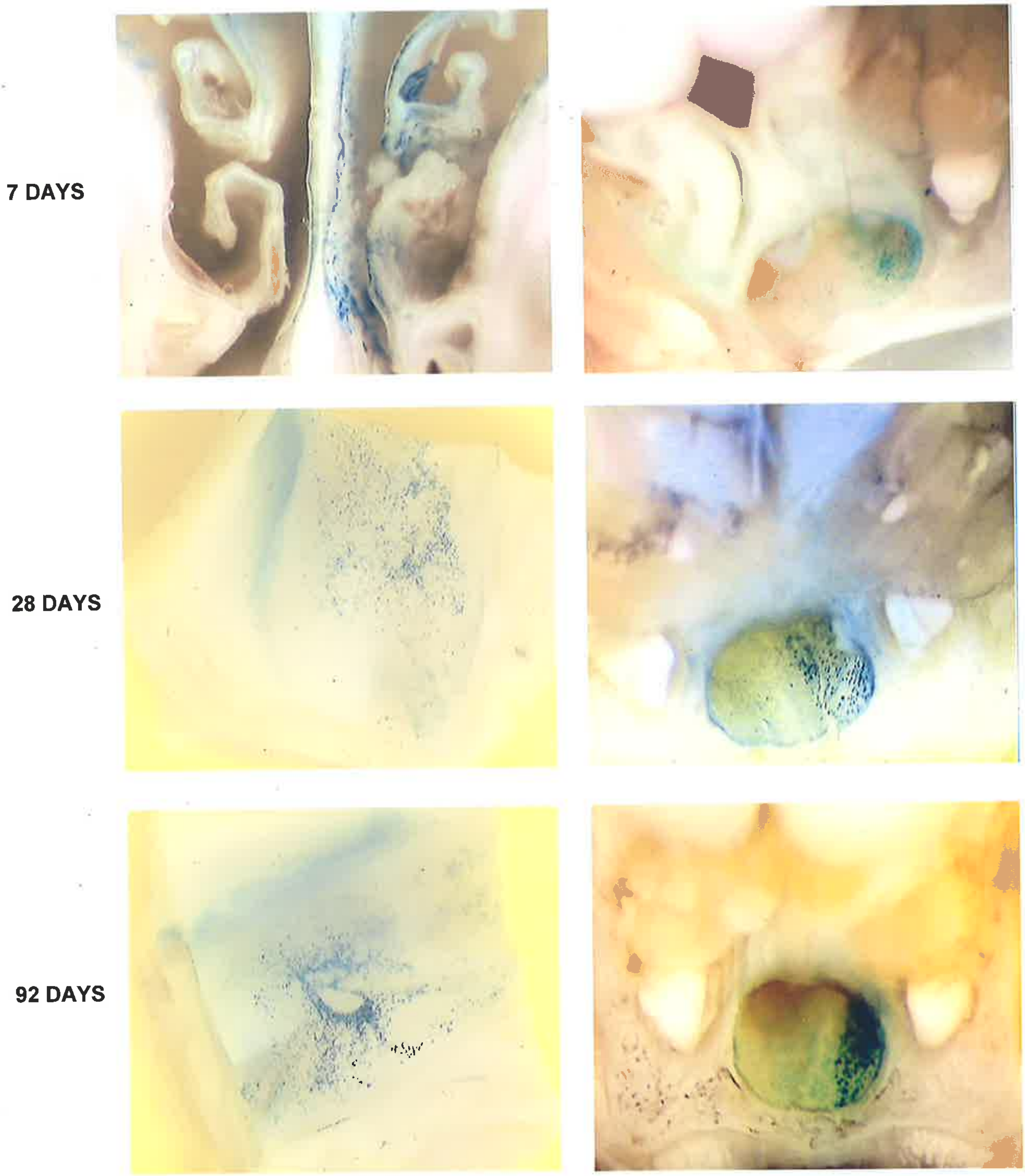
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Example of Lentivirus-Mediated LacZ Gene Transfer (Blue) Persistence in the Ciliated Airway Epithelium. For details refer to section 6.4.

DECLARATION

I declare that this thesis does not incorporate without acknowledgement any material previously accepted or submitted for the award of any other degree or diploma in any university or other tertiary institution, and that to the best of my knowledge and belief, this thesis does not contain any material previously published or written by another person, except where due reference is made in the text.

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"*To laugh often and much; to win the respect of intelligent people and the affection of children; to earn the appreciation of honest critics and endure the betrayal of false friends; to appreciate beauty; to find the best in others; to leave the world a bit better, whether by a healthy child, a garden patch or a redeemed social condition;*

to know even one life has breathed easier because you have lived…

This is to have succeeded".

Ralph Waldo Emerson

PUBLICATIONS AND AWARDS

Publications Arising From This Work

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M. Limberis, D.W. Parsons and D.S. Anson. "A lentiviral vector for gene therapy of airway disease of cystic fibrosis". Presented at the Annual Scientific Meeting, ASMR, Adelaide, Australia. June 2000.

Parsons D. W., Martella T., Hopkins P. J., **Limberis M.**, A. J. Martin. "Lysophosphatidylcholine (LPC) provides greater enhancement of in-vivo adenoviral airway gene transfer than polidocanol. *Respirology,* (1999), **4**, (supplement), A11. Presented at the TSANZ Annual Scientific Meeting, Canberra, Australia. February-March 1999.

M. Limberis, D.W. Parsons and D.S. Anson. "Evaluation of gene therapy for airway disease in the cystic fibrosis knockout mouse". Presented at the Annual Scientific Meeting, ASMR, Adelaide, Australia. June 1998.

SYNOPSIS

With a frequency of 1 in 2,500 live births, cystic fibrosis (CF) is one of the most common life-threatening autosomal recessive disorders affecting the Caucasian population. This disorder results in both severe chronic lung disease, which currently accounts for more than 95% of morbidity and mortality of CF patients, and manageable pancreatic disease, with a current median survival age of approximately 30 years. Following the discovery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene in 1989, attempts to find a cure for CF have included research in the area of gene therapy. To date, the lack of an efficient gene transfer vector system combined with the physical barriers of the airway epithelium limit the successful application of CF gene therapy.

The work described in this thesis has focused on modulating the physical barriers of the airway epithelium with mild detergents, so as to enhance gene transfer by a HIV-1 based lentivirus (LV) vector *in vivo*. Initial work included the establishment of air liquid interface culture systems to model the intact airway epithelium *in vitro*, allowing the evaluation of the action of detergent treatment on the physical barriers of the respiratory epithelium. The results of the *in vitro* gene transfer studies showed that airway pre-treatment with a detergent (polidocanol or lysophosphatidylcholine) prior to the instillation of the LV vector could significantly improve gene transfer.

The efficiency of LV–mediated gene transfer was then evaluated in the nasal airway of C57Bl/6 mice using the LacZ marker gene. Treatment of mouse nasal airway epithelium with the detergent lysophosphatidylcholine prior to instillation of a single dose of a LVLacZ vector produced significant LacZ gene expression for at least 92 days. Transduction of the CFTR gene using the same LV vector system resulted in partial recovery of the electrophysiological function in the *cftr*$^{\mathrm{tm1Unc}}$ mouse nasal airway epithelium for at least 110 days. This first demonstration of lentivirus-mediated *in vivo* recovery of CFTR function in CF airway epithelium illustrates the potential of combining a pre-conditioning of the airway surface with a simple and brief HIV-1–based gene transfer vector exposure to produce therapeutic gene expression in the intact airway.

The findings of this work “Recovery of Airway CFTR Function in CF mice after Single Dose Lentivirus-Mediated Gene Transfer” by authors **Limberis, M.**, Anson, D. S., Fuller, M., and Parsons D. W., have been accepted (without revision) for publication in *Human Gene Therapy* (2002). Nov 1, Vol 13, Issue 16, p 1961-70 (copy on CD).

Furthermore, a patent of the gene transfer protocols that were developed in this project “Respiratory Delivery for Gene Therapy and Lentiviral Delivery Particle” by inventors Parsons D. W., Anson, D. S., **Limberis, M.** and Fuller, M., was lodged with the United States Patent and Trademark Office on the 23rd of August 2002.

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LIST OF ABBREVIATIONS

AAV:	Adeno–Associated virus
AdV:	Adenovirus
ALI:	Airway liquid interface
Amp:	Ampicillin
ANOVA:	Analysis of variance
ASL:	Airway surface liquid
A549–TU:	Transducing units of LV assayed on A549 cells
AU:	Australia
β-gal:	Beta galactosidase
bp:	base pairs
BPE:	Bovine pituitary extract
BSA:	Bovine serum albumin
°C:	Degrees Celsius
Cam:	Chloramphenicol
cAMP:	Adenosine 3', 5'–cyclic monophosphate
CAR:	Coxsackie and adenovirus receptor
cDNA:	copy DNA
CF:	Cystic fibrosis
CFDA–SE:	carboxyfluorescein diacetate–succinimidyl ester
CFTR:	Cystic fibrosis transmembrane conductance regulator
CIP:	Alkaline calf intestine phosphatase
cPPT:	Central polypurine tract
cm:	Centimeter
CMV:	Cytomegalovirus
CT:	Cholera toxin
Cy3:	Carboxymethyl–indocyanine dye
DAPI:	4',6-diamidino-2-phenylindole
ΔF508:	In–frame deletion of phenylalanine at position 508 in exon 10 of the CFTR gene
DMEM:	Dulbecco's Modified Eagle's Medium

DMSO:	Dimethylsulfoxide
ΔPD:	The difference between the TPD value recorded under basal conditions and the TPD value measured under low–chloride conditions
DPX:	Distyrene–tricresyl–phosphate–xylene
EGF:	Epidermal growth factor
EGTA:	Ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid
EYFP:	Enhanced yellow fluorescence protein
FACS:	Fluorescence activated cell sorting
FCS:	Fetal calf serum
F12:	Ham's F12 media
HC:	Hydrocortisone
HIV:	Human immunodeficiency virus
hr:	Hour
H/E:	Haematoxylin/eosin
i. m.:	Intramuscularly
IRT:	Serum immuno–reactive pancreatic trypsinogen
Kb:	Kilo base
l:	Liter
LacZ:	Beta–galactosidase
LB broth:	Luria Bertani broth
lfu:	LacZ forming units
LPC:	Lysophosphatidylcholine
LTR:	Long terminal repeat
LV:	Lentivirus
M:	Molar
MCC:	Mucociliary clearance
MEM:	Modified Eagle's Medium
min:	Minute
mins:	Minutes
ml:	Millilitre
μl:	Microliter
MLV:	Murine leukaemia virus

MOI:	Multiplicity of infection
mV:	Millivolts
MW:	Molecular weight
NBF:	Neutral buffered saline
ng:	Nano grams
nm:	Nanometers
NIH3T3–TU:	Transducing units of LV assayed on NIH3T3 cells
o/n:	Overnight
PBS:	Phosphate buffered saline
PCL:	Periciliary liquid
PCR:	Polymerase chain reaction
PD:	Potential difference
PE:	Phospho–ethanolamine
PFA:	Paraformaldehyde
PFC:	Perfluorochemical liquid
PI:	Pancreatic insufficiency
Polidocanol:	Polyoxyethylene-9-lauryl ether
PS:	Pancreatic sufficiency
RA:	Retinoic acid
RCR:	Replication–competent retrovirus
RT:	Room temperature
RTE:	Rat tracheal epithelial
RT–PCR:	Reverse transcriptase–PCR
SA:	Splice–acceptor
Saf-O:	Safranin–O
SD:	Splice–donor
SEM:	Standard error of mean
SF:	Serum Free
SNK:	Student–Newman–Keuls
SV40:	Simian virus type 40
Tf:	Transferrin
TfB:	Transformation buffer
TJ:	Tight–junction
TPD:	Transepithelial potential difference
TR:	Transepithelial resistance

TU:	Tranducing units
U:	Units
UV:	Ultraviolet
v:	Volume
VSV–G:	Vesicular stomatitis virus glycoprotein G
w:	Weight
WCH:	Adelaide Women's and Children's Hospital
WPI:	World precision instruments
X-gal:	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Ω:	Ohm

CHAPTER 1

INTRODUCTION

Preface

Chapter 1 is divided into three parts. Part A presents a general overview of cystic fibrosis. The topics covered include the history of cystic fibrosis, its epidemiology, the genetics of the disease, its main clinical symptoms and therapies designed to ameliorate symptoms associated with cystic fibrosis. Part B describes the structure of the airway epithelium and also reviews *in vitro* and *in vivo* systems that model the intact human airway epithelium. The impaired ion transport across the cystic fibrosis airways and the hypotheses on the generation of cystic fibrosis airway disease are also examined. Part C reviews the potential of gene therapy as a cure for cystic fibrosis airway disease, the gene transfer vectors currently under development and the methods that have been used to improve the levels of gene transfer to the airway epithelium.

Part A

1.1 Cystic Fibrosis: The Disorder

1.1.1 Cystic Fibrosis: A Brief Historical Perspective

Cystic fibrosis (CF) is the most common life-threatening autosomal recessive disease affecting the Caucasian populations, especially those of Northern European origin (Hodson and Geddes, 1995). Despite the fact that CF was not formally described as a distinct syndrome until the 1930s there are reports of infants and children suffering from pancreatic disease, steatorrhea and lung disease, all typical symptoms of CF, dating from *circa* 1650. There are also many observations in books of folk philosophy like the following from Swiss-German folklore " *Woe to that child, which when kissed on the forehead tastes salty; he is bewitched and soon must die*" (Schmidt, 1729), suggesting that CF was recognised by Europeans long before its classification in the 1930s.

Probably the earliest clinical manifestation of CF is the development of meconium ileus, which occurs in 10 - 15% of CF infants with pancreatic insufficiency (PI) (Park and Grand, 1981). The first description of meconium ileus was an autopsy report of an infant in 1838 by Rokitansky, in which meconium peritonitis and ileal perforation were noted (Busch, 1978). Other early reports of meconium ileus include Landsteiner's case (1905) of a newborn with meconium ileus and PI, and Heubner's case (1906) of a 15-week-old infant with cough and diarrhoea that was found to have bronchiectasis following autopsy.

In 1936 the first paper identifying and naming the syndrome we now know as CF, was published (Fanconi *et al.*, 1936). The disease was named "*cystic pancreas fibromatosis and bronchiectasis*" (Fanconi *et al.*, 1936) and was wrongly linked to coeliac disease, an unrelated disorder that also results in malabsorption and severe malnutrition (Hess and Saphir, 1935). In 1938 Andersen published the first systematic study of CF, reporting on 49 CF patients and renaming the disease as "*cystic fibrosis of the pancreas*". In this study, Andersen reported on neonatal deaths resulting from intestinal obstruction; early infantile deaths resulting from malabsorption; and late childhood deaths resulting from either malabsorption or respiratory complications. Andersen also reported on the pathological changes present in the lungs. In 1945 Farber introduced in Europe another term for CF, mucoviscidosis, as CF severely affects the exocrine glands especially the mucus-producing glands. This term is still widely used in Europe.

As the life expectancy of CF infants improved following the introduction of pancreatic enzyme supplements in the late 1940s, the respiratory manifestations of the disease became more apparent. In 1946 the use of antibiotics to treat CF lung disease was reported by di Sant'Agnese and Andersen. This was followed by a detailed report on the pathophysiology of CF lung disease (Zuelzer and Newton, 1949). Shortly after this report lung function tests were used for the first time in CF patients to monitor their pulmonary status (West *et al.*, 1954). These tests are still used to monitor progression of lung disease in CF patients.

1.1.2 Clinical Presentation of Cystic Fibrosis

CF is generally characterised by (i) viscous mucous secretions in the airways, (ii) PI that is caused by the blockage of the pancreatic ducts with mucus, (iii) excessive salt secretion in the sweat, (iv) male and female infertility and (v) less commonly liver failure (Shale, 1997). The most important clinical manifestations of CF, the lung and pancreatic diseases, are described below.

(a) Cystic Fibrosis Lung Disease

Currently more than 95% of the morbidity and mortality associated with CF can be attributed to the chronic lung disease. This reflects in part the development of effective treatments for the other clinical manifestations of CF described above. Interestingly, siblings with the same genotype have been known to have a different level of lung disease (Burke *et al.*, 1992) suggesting that other factors (i.e. environmental or genetic) significantly influence the status of lung function. Although the respiratory symptoms are not present at birth, some CF patients can develop symptoms within a few days (Armstrong *et al.*, 1995).

Lung disease initially occurs in the small airways and is characterised by airway obstruction, bronchiectasis, suppurative endobronchial infection and impaired alveolar ventilation (Rosenstein and Zeitlin, 1998). The excessive mucus present in the airways provides a supportive environment for the growth of pathogenic organisms such as *Staphylococcus aureus*, *Haemophilus influenzae* and *Pseudomonas aeruginosa* and the enteric organisms *Klebsiella pneumoniae* and *Escherichia coli*. Lung function gradually declines as a result of a vicious cycle of airway infection, inflammation and obstruction, which progressively destroys the lung tissue (Rosenstein and Zeitlin, 1998). Other organisms such as *Burkholderia cepacia*, *Stenotrophomonas (Xanthomonas) maltophilia*, *Aspergillus fumigatus*, although not pathogenic themselves, can also cause serious problems in many CF patients (Rosenstein and Zeitlin, 1998).

(b) Cystic Fibrosis Pancreatic Disease

As discussed earlier (section 1.1.1) the pathological changes of the pancreas in CF were noted as early as 1936 and in 1938 Andersen re-named the disease "*cystic fibrosis of the pancreas*". Andersen used this term based on the observation of progressive deterioration and fibrosis of the exocrine parenchyma of the pancreas, caused by blockage of the pancreatic ducts by inspissated mucus secretions in PI CF patients. Approximately 85 - 90% of CF patients have exocrine PI (Forstner *et al.*, 1980) and as mentioned in section 1.1.1 approximately 10 - 15% of these individuals will also present with meconium ileus at birth (Hodson and Geddes, 1995). CF patients with PI do not secrete sufficient amounts of lipolytic and proteolytic pancreatic enzymes, which cause intestinal malabsorption of fat and protein, respectively. This pancreatic dysfunction leads to severe malnutrition, evidenced as failure of CF infants and young children to thrive. Currently CF pancreatic disease is managed reasonably well by intensive nutritional support, which includes the use of pancreatic enzyme supplements, and is now rarely associated with mortality.

The remaining 10 - 15% of CF patients, which are pancreatic sufficient are characterised by a healthier nutritional status compared to the PI CF patients (Orenstein *et al.*, 2000).

1.1.3 The Cystic Fibrosis Gene

The CF gene was mapped to chromosome 7, position q31.2, by 1985 (Tsui *et al.*, 1985; Wainwright *et al.*, 1985) and using positional cloning the gene was isolated and sequenced in 1989 (Rommens *et al.*, 1989; Riordan *et al.*, 1989; Kerem *et al.*, 1989). The CF gene encodes a membrane protein of ~170 kilo Daltons, which is known as the CF transmembrane conductance regulator (CFTR) (Riordan *et al.*, 1989). The complete cDNA gene sequence spans 6129 base pairs (bp) and contains an open reading frame capable of encoding a polypeptide of 1480 amino acids. The deduced amino acid sequence of the CFTR cDNA indicated that the CFTR protein belongs to the "ATP binding cassette" transporter superfamily of proteins (Riordan *et al.*, 1989). These proteins are mainly involved in the ATP-dependent transport of large molecules across the cell membrane.

The CFTR protein, which is located in the apical membrane of the epithelial cells lining the lungs, the pancreas, the sweat glands and the submucosal glands, functions as a chloride (Cl^-) channel regulated by 3', 5'-cyclic adenosine monophosphate AMP (cAMP) (Boat *et al.*, 1989). In addition to its role as a Cl^- channel, the CFTR also controls the transport of other ions such as sodium (Na^+) (Boucher *et al.*, 1994). In particular, CFTR down-regulates an amiloride sensitive Na^+ channel (ENaC), located in the apical membrane of the epithelial cells (Stutts *et al.*, 1995) and thus regulates the levels of Na^+ in the airway surface liquid layer. In the CF airway epithelium, CFTR is either defective or absent causing impaired ion transport and subsequently resulting in the generation of lung disease.

Details of the ionic transport processes across the normal and the CF airway epithelium will be reviewed in section 1.2.3.

1.1.4 Mutations of the Cystic Fibrosis Gene

Currently, more than 1000 mutations of the CF gene have been identified (Dodge, 2001). The majority of these mutations are missense mutations that give rise to a CFTR molecule that is synthesised and processed normally but is abnormally regulated (Rosenstein and Zeitlin, 1998). In general, CF gene mutations can be classified into five categories based on the alterations of the CFTR protein. These categories are: class I, no synthesis of CFTR; class II, abnormal processing of CFTR; class III, abnormal regulation of CFTR; class IV, altered conductance of Cl^-; and class V, reduced synthesis of CFTR (Rosenstein and Zeitlin, 1998). Examples of each of these categories of CF gene mutations are shown in Table 1.1.

The diagnosis of CF in patients that have either class IV or class V CF gene mutations occurs much later in life as these patients usually present with atypical (generally milder) CF symptomology (Gan *et al.*, 1994). Of the 1000 or so CF gene mutations only a small number of these results in both PI and severe lung disease (Table 1.2).

The most common CF gene mutation, which is found on more than 70% of CF Caucasian chromosomes, is ΔF508 (Rosenstein and Zeitlin, 1998). ΔF508 is a class II CF gene mutation caused by an in-frame deletion of phenylalanine at amino acid residue position 508 in exon 10 (Kerem *et al.*, 1989). This mutation results in the retention of CFTR in the endoplasmic reticulum causing defective cAMP-regulated Cl^- transport across the apical cell surface. Interestingly, the ΔF508 CFTR continues to function as a cAMP-regulated Cl^- channel in the endoplasmic reticulum (Pasyk and Foskett, 1995). This suggests that although the mutated CFTR has undergone a conformational change that affects its processing, its function as a Cl^- channel may be essentially unaltered (Pasyk and

Foskett, 1995). Haplotype analysis of the CF chromosomes carrying the ΔF508 CF gene mutation has shown that this mutation resulted from a single mutational event (i.e. a founder event) rather than a series of multiple events (Kerem *et al.*, 1989). Patients who are homozygous for the ΔF508 CF gene mutation have both PI and severe respiratory symptoms (Shale, 1997).

The prevalence of the ΔF508 CF gene mutation in different Caucasian populations varies widely. The highest prevalence of the ΔF508 CF gene mutation is seen in Denmark where it is found on 82% of CF chromosomes, compared to the lowest incidence of 32% in the Turkish population (Hodson and Geddes, 1995).

Table 1.1: Cystic Fibrosis Gene Mutations Categorised According to the Alterations of the CFTR Protein.

Class I	Class II	Class III	Class IV	Class V
G542X	ΔF508	G551D	R117H	3849 + 10 kb C→T
W1282X	ΔI507	R334W	R347P	A455E
R553X	N1303K		S549R	

Various CF gene mutations categorised according to their role in CFTR production and function. These categories are: class I, no synthesis of CFTR; class II, abnormal processing of CFTR; class III, abnormal regulation of CFTR; class IV, altered conductance of Cl^-; and class V, reduced synthesis of CFTR. *Adapted from Orenstein et al., Cystic Fibrosis Medical Care, Lippincott Williams and Wilkins (2000), p. 8.*

The overall frequency of CF gene mutations other than the ΔF508 is low (Orenstein *et al.*, 2000). The next most common CF gene mutation in the Caucasian population is G542X with a worldwide incidence of ~3.4% (Table 1.2). G542X is a class I CF gene mutation, which results in a truncated CFTR protein that has reduced function (Hamosh *et al.*, 1992).

Some CF gene mutations are found only in certain populations where they occur with a high frequency. For example, W1282X which is a class I CF gene mutation results in both PI and severe respiratory disease and is found on 50 - 60% of CF chromosomes in the Ashkenazi Jewish population (Table 1.2) (Shoshani *et al.*, 1992). This suggests that, similar to the ΔF508 CF gene mutation, a founder effect is responsible for the W1282X CF gene mutation.

Table 1.2: Clinical Symptoms Associated With Some of the More Common Cystic Fibrosis Gene Mutations.

Mutation	Class	Ethnic Incidence	Symptoms
ΔF508	II	70 - 75% North America 82% Denmark 32% Turkey	Severe lung disease & PI
W1282X[1]	I	50 - 60% Ashkenazi Jews (2.0% worldwide)	Severe lung disease & PI
G542X[1]	I	3.4% worldwide	PI
G551D[1]	III	2.4% worldwide	PI
N1303K[1]	II	1.8% worldwide	PI
R553X[1]	I	1.3% worldwide	PI
3849 + 10 kb C→T[1]	V	< 1% worldwide	Variable lung disease & PI

[1] Compound heterozygotes (i.e. one copy of the mutation noted and one copy of another (typically ΔF508)). *Adapted from Orenstein et al., Cystic Fibrosis Medical Care, Lippincott Williams and Wilkins (2000), p. 11.* PI= pancreatic insufficiency.

Heterozygotes for any CF gene mutation (severe or mild) do not exhibit any symptomology typical of CF. That is 50% of the normal level of CFTR gene expression is sufficient to prevent the pancreatic and/or lung disease which is associated with CF (Korst *et al.*, 1995; Dorin *et al.*, 1996). The implication of this for the prevention and treatment of CF airway disease by gene therapy is that $\leq$ 50% of CFTR gene expression should be sufficient to correct the electrophysiological defect present across the CF airways.

1.1.5 Genetics of Cystic Fibrosis

The familial (i.e. genetic) basis for CF was identified in the 1930s (Fanconi *et al.*, 1936; Blackfan and May, 1938; Harper, 1938; Andersen, 1938). Subsequently, Andersen and Hodges (1946) correctly proposed an autosomal recessive inheritance pattern for CF (McKusick, 1978). In Caucasian populations, ~1 in 22 individuals are heterozygote carriers. Males and females are equally at risk and the frequency of CF in Australia is 1 in 2,500 live births. Although CF is less common in mixed-race South-African populations (1 in 12,000), African-American populations (1 in 17,000) and Asian (Chinese, Japanese and Filipino) populations living in Hawaii (1 in 90,000) (Hamosh *et al.*, 1998), a diagnosis of CF ought to be considered in patients of all racial and ethnic backgrounds (Orenstein *et al.*, 2000). Examples of the frequency of CF in Caucasian and non-Caucasian populations are shown in Table 1.3.

Table 1.3: Worldwide Incidence of Cystic Fibrosis.

Ethnic background	Frequency
Ireland[1]	1 in 2,000
Australia[1]	1 in 2,500
United Kingdom[1]	1 in 2,500
United States[1]	1 in 3,200
Sweden[1]	1 in 8,000
African-Americans[2]	1 in 17,000
Asian-Americans[2]	1 in 31,000
Oriental population of Hawaii[2]	1 in 90,000

The birth incidence of CF in countries whose population is of either [1]European Caucasian or of [2]non-European Caucasian origin is shown. *Adapted from Hodson and Geddes (1995), p 3, and Hamosh et al., 1998.*

One of the theories proposed to explain the continual high incidence of CF in Caucasian populations is that CF heterozygosity may provide resistance to a particular disease or diseases. Therefore, despite the high degree of morbidity and mortality associated with CF homozygosity, a heterozygote advantage may provide a selective pressure for the maintenance of the CF gene in the Caucasian populations. A more familiar example of the heterozygote advantage is the high frequency of sickle cell anaemia heterozygosity in the African-American populations, where the heterozygote state provides partial resistance to malaria (Allison, 1954). Likewise it has been suggested that CF heterozygotes have increased resistance to tuberculosis, which was historically endemic in Europe and accounted for high mortality especially amongst children (Crawford, 1972). It has also been proposed that heterozygotes for the ΔF508 CFTR allele may have increased resistance to both typhoid fever (Pier *et al.*, 1998) and cholera (Gabriel *et al.*, 1994).

With the generation of the transgenic CF (*cftr*$^{\mathrm{tm1Unc}}$) mouse (Snouwaert *et al.*, 1992) it became possible to directly assess whether CF heterozygotes were resistant to cholera. Gabriel and colleagues (1994) reported that the low levels of CFTR gene expression in the heterozygote *cftr*$^{\mathrm{tm1Unc}}$ mice provided them with a protective shield against the dehydration (secretory diarrhoea) caused by cholera, which was harmful and in most cases lethal in non-CF mice.

1.1.6 Neonatal Screening for Cystic Fibrosis

In 1951 a high percentage of infants and children that were admitted to the Babies Hospital in New York for heat prostration were reported to have CF (Kessler and Andersen, 1951). This observation led di Sant'Agnese and colleagues (1953) to report on the sweat electrolyte abnormalities typical of most CF patients. This finding in turn led to the development of the CF diagnostic sweat test for the analysis of sweat Cl^-. This test, known as the pilocarpine iontophoresis test, was eventually standardised by Gibson and Cooke in 1959.

Currently, diagnosis of CF is based on carefully defined laboratory and clinical criteria. These are analysis of sweat Cl^- levels coupled with detection for CF gene mutations using the polymerase chain reaction (PCR). An accurate diagnosis of CF can be made when one or more of the typical CF clinical symptoms are present coupled with either a finding of > 60 mmol/liter (l) of Cl^- in sweat using the quantitative pilocarpine iontophoresis test (Rosenstein and Zeitlin, 1998) or the presence of CF gene mutation(s). Approximately 2% of CF patients are characterised by an unusual phenotype in which typical CF symptoms are evident but their sweat Cl^- levels are either borderline (40 - 60 mmol/l) or, more rarely, normal (< 40 mmol/l) (Orenstein *et al.*, 2000). In rare cases where foetal intestinal obstruction is reported upon ultrasonography an *in utero* diagnosis of CF is possible. The foetus is then screened for CF gene mutations by chorionic villus sampling, which is performed at 10 weeks gestation, or by amniocentesis, which is performed at 15 - 18 weeks gestation.

Screening of neonates for CF is increasingly being performed at clinical centres, including the Adelaide Women's and Children's Hospital, and is based on measurements of the serum immuno-reactive pancreatic trypsinogen (IRT) in the neonatal 'Guthrie' blood test. High levels of IRT in the circulating serum reflect an obstruction in pancreatic secretion, which is a typical symptom of CF (Davidson *et al.*, 1984). The Australian infants with an IRT value in the highest 1% of the population are then tested for the five most common CF gene mutations (Mr. E. Ranieri, Department of Chemical Pathology, WCH, personal communication). This testing protocol identifies infants with CF as well as a small number of infants who are asymptomatic carriers of a CF gene mutation. Once there is a positive indicator for CF (i.e. at least one CF gene mutation) the pilocarpine iontophoresis sweat test is performed to confirm the diagnosis.

1.1.7 Current Management of Cystic Fibrosis

At present there is no treatment that can be regarded as curative for CF, with current treatments aimed at ameliorating symptoms as much as possible. Nonetheless, advances in the management of CF have increased the median life expectancy from less than year in 1938 to just over 32 years at present (Tsang, 2001).

In order to minimise respiratory deterioration various therapies are designed for individual CF patients according to the status of their lung function (Rosenstein and Zeitlin, 1998). These therapies include courses of antibiotics, chest physiotherapy for bronchial drainage, anti-inflammatory agents, regular physical exercise, aerosolised recombinant human DNase and treatment of airway reactivity (Rosenstein and Zeitlin, 1998; Korst *et al.*, 1995), which are all aimed toward preventing, delaying or ameliorating CF airway disease. Supplements of oral pancreatic enzymes and vitamins as well as dietary management regimens have proven effective at improving the nutritional status, and ensuring a reasonable quality of life for many PI CF patients (Rosenstein and Zeitlin, 1998).

Although conventional preventive and/or ameliorating treatment has increased life expectancy of PI CF patients to more than 30 years, there is clearly an urgent requirement for novel therapeutic approaches that will directly address the underlying pathogenesis of CF airway disease.

The next section of this chapter (Part B) will provide the reader with an overview of the structure of the airway epithelium and the theories proposed for the mechanisms by which CF airway disease is generated.

Part B

1.2 The Airway Epithelium

1.2.1 Introduction

The lung is comprised of the conducting airways (trachea, bronchi and bronchioles) and the alveoli. The most important function of the airways is to warm and humidify inspired air and to allow the exchange of gases, O_2 and CO_2, across the large area (70 m^2) of the alveoli (Knowles and Boucher, 2002). Since the exchange of these gases occurs deep inside the lung, the airways have evolved an effective defence system to maintain a sterile environment within the lungs. The epithelial cells, which line the airways are encircled by tight-junctions; the latter act as a physical barrier to protect the sub-epithelial vascular spaces from airborne pathogens and other particles that may cause severe respiratory infections (Folkerts and Nijkamp, 1998; Yeaman *et al.*, 1999). In addition, the ciliated epithelial cells, the goblet cells, the submucosal glands, the mucociliary clearance (MCC) mechanisms, as well as the presence of anti-bacterial agents in the thin film of moisture, known as the airway surface liquid layer (see below), are all involved in the capture and removal of inhaled, and potentially pathogenic, foreign particles such as dust, allergens, bacteria and viruses from the airways.

1.2.2 Structure of the Conducting Airways

The pathophysiology associated with the CF lung disease commences in the conducting airways. For this reason gene-based and other therapies to treat CF lung disease, target the epithelial cells that line these airways.

The human proximal conducting airways ("large" airways) are lined with pseudostratified columnar superficial epithelium that consists of non-ciliated columnar cells, ciliated epithelial cells, goblet and basal cells and submucosal glands. The apical surface of the non-ciliated columnar cells is covered with ~300 microvilli that appear, under electron microscopy, as thin finger-like structures of ~1 μm in length (Mygind and Dahl, 1996). The apical surface of the ciliated epithelial cells is covered with ~200 cilia (Satir and Sleigh, 1990) that are ~6 μm in length and 0.3 μm wide. Typical ciliary beat is ~12.5 - 15 beats/second (Seiler *et al.*, 2002) and is effective at clearing foreign particles from the apical surface. Depending on the species this surface is covered with ASL to a depth of 6 - 25 μm (Tarran *et al.*, 2001; Boucher, 1999a). The ASL is divided into a periciliary liquid (PCL) layer and a mucus layer (Boucher, 1994). The PCL layer, which surrounds the cilia on the apical surface, protects the epithelium from the mucus layer and also its low viscosity facilitates rapid cilial beat (Knowles and Boucher, 2002). The overlaying mucus layer is a viscous fluid rich in mucins and can bind and trap practically all inhaled particles for effective MCC, which is co-ordinated by the beating cilia and is assisted by airway reflexes such as coughing and sneezing (Knowles and Boucher, 2002). Effective MCC transport mechanisms, and hence the ability to maintain the underlying epithelial cells in a sterile environment, are highly dependent on the depth and the viscosity of the ASL layer.

The ability of the ASL to destroy microbial pathogens was recognised as early as 1922 by Dr. A. Fleming. The most important antimicrobial constituents of the ASL layer include proteins such as lysozyme, lactoferrin, secretory phospholipase A2, as well as secretory leukocyte protease inhibitors (Travis *et al.*, 1999), antimicrobial peptides such as salt-sensitive defensins (Smith *et al.*, 1996), and salt-insensitive antimicrobial factors (Bals *et al.*, 2001).

1.2.3 Ion Transport Across Human Airway Epithelium

In this section the ionic transport activity across the airway epithelium of both normal and CF individuals is reviewed, to provide the reader with sufficient background against which the necessary effects of corrective CFTR gene transfer can be described in later sections. As mentioned previously, CF is characterised by impaired ion transport across the airway epithelium. Although, many groups have contributed to the understanding of ion transport across the CF airway epithelium (Smith *et al.*, 1996, Goldman *et al.*, 1997a, Boucher, 1999a, Pilewski and Frizzell, 1999, Coakley and Boucher, 2001; Jayaraman *et al.*, 2001, Worlitzsch *et al.*, 2002) for reasons of clarity this overview will focus on two of the major contributors to this field. These are the UNC group (Prof. R. Boucher and colleagues) and the Iowa group (Prof. M. Welsh and colleagues). These groups have come to very different conclusions about the role of CFTR in the normal airway, and the mechanisms by which CFTR deficiency causes the lung pathophysiology associated with CF. In an attempt to provide an understanding of the different mechanisms by which the deficiency of CFTR results in the development of lung disease in CF, the theories proposed by the UNC and Iowa groups on normal and CF airway ion transport are briefly described in this section.

(a) Ion Transport Across Normal Airway Epithelium

The UNC group proposes that although the CFTR is a Cl^- channel (Cl^-_{CFTR}), its main function is to regulate the activity of both the ENaC channel and an alternative (Ca^{2+}-activated) Cl^- channel ($Cl^-_{Ca^{2+}}$) both of which are located in the apical membrane (Figure 1.1; a) (Boucher, 1999a). The Na^+/K^+/ATPase pump located in the basolateral cell membrane (Figure 1.1; a) generates the driving force for the active entry of Na^+ ions through the amiloride-sensitive ENaC channel (Figure 1.1; a). This active transport of Na^+ then facilitates the passive movement of Cl^- (paracellularly) and H_2O (transcellularly). The UNC group proposes that as the airway epithelium is permeable to H_2O, ion transport is isosmotic, which means that the ASL is isotonic under normal conditions (Boucher, 1999a). In brief, the UNC group proposes that the role of ion transport is to regulate the volume/depth of the ASL layer, as an adequate ASL volume is required to maintain MCC mechanisms.

Conversely the Iowa group proposes that the CFTR acts exclusively as a Cl^- channel in the apical membrane of the normal airway epithelium. Cl^- and Na^+ are absorbed transcellularly *via* CFTR and ENaC, respectively (Figure 1.1; a') resulting in low concentrations of NaCl in the ASL layer. The Iowa group suggests that the epithelium is permeable to H_2O and the low concentrations of NaCl in the ASL are maintained by "as yet unidentified passive surface forces", which keep H_2O on the airway surface despite of the high osmotic permeability, leaving the ASL hypotonic under normal conditions (Zabner *et al.*, 1998). In brief, the Iowa group proposes that the role of ion transport is to regulate the ionic composition of the ASL.

(b) Ion Transport Across Cystic Fibrosis Airway Epithelium

The UNC group proposes that as the CFTR is non-functional in CF airways there is no inhibitory control of Na^+ absorption, resulting in increased absorption of Na^+ from the ASL layer, with Cl^- following *via* the paracellular pathway. However, as Na^+ absorption also facilitates the passive movement of H_2O from the ASL, hyperabsorption of Na^+ causes dehydration and lowering of the ASL layer and subsequently results in impaired MCC mechanisms (Figure 1.1; b) (Boucher, 1999a).

In contrast, the Iowa group believes that the absence of a functional CFTR causes reduced Cl^- absorption from the ASL, resulting in an increased ASL NaCl. The elevated NaCl levels subsequently deactivate salt-sensitive antimicrobial proteins and peptides such as defensins present in the ASL layer, thus allowing for bacterial infections to occur (Figure 1.1; b') (Zabner *et al.*, 1998).

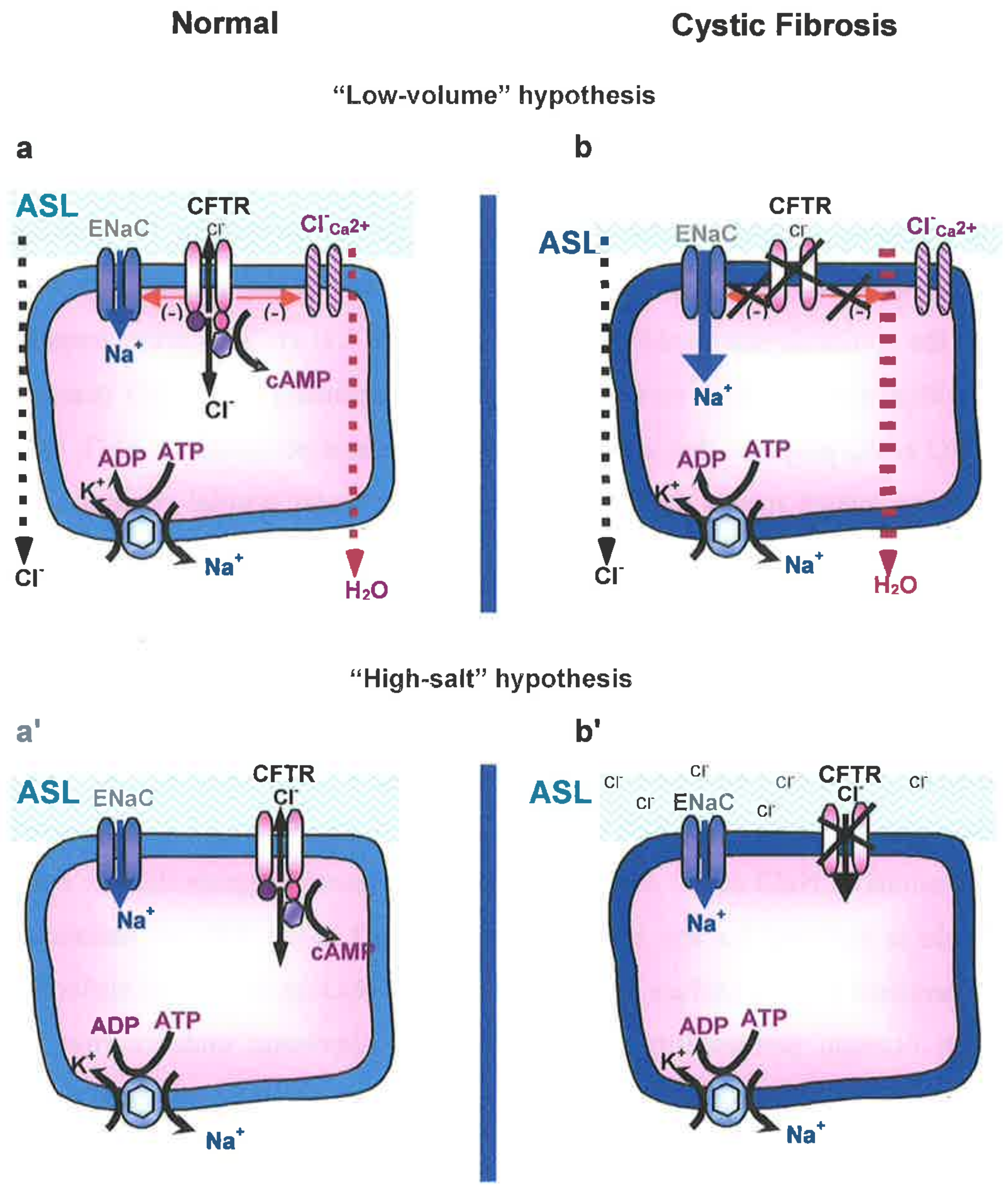
Normal
Cystic Fibrosis
"Low-volume" hypothesis
a
b
ASL
ENaC
CFTR
Cl⁻
Cl⁻Ca2+
(-)
(-)
Na⁺
cAMP
Cl⁻
ADP
ATP
K⁺
Na⁺
Cl⁻
H₂O
"High-salt" hypothesis
a'
b'
ASL
ENaC
CFTR
Cl⁻
Na⁺
cAMP
ADP
ATP
K⁺
Na⁺

Figure 1.1: Ion Transport Across the Human Airway Epithelium.

According to the **UNC group: (a)** in normal airway the CFTR not only functions as a Cl^- channel but it also down-regulates (-) the ENaC and the $Cl^-_{Ca^{2+}}$ channels. The Na^+/K^+/ATPase pump generates the driving force for entry of Na^+ through the ENaC channels. Cl^- follows Na^+ absorption, but paracellularly. The epithelium in this model is H_2O permeable. **(b)** In CF airway the CFTR is defective and thus Cl^- secretion is impaired. As a consequence, Na^+ absorption is increased with Cl^- following paracellularly. Subsequently, H_2O absorption from the ASL layer is increased resulting in a reduced ASL layer depth. According to the **Iowa group (a')** in normal airway Cl^- is absorbed transcellulalry through the CFTR and Na^+ is absorbed transcellularly through the ENaC channel. The epithelium in this model is H_2O impermeable. **(b')** In CF airway Na^+ absorption through the ENaC channel is normal. In contrast as the CFTR Cl^- channel is defective, Cl^- ions are not absorbed from the ASL, resulting in increased Cl^- concentration and subsequently increased Na^+ concentration in the ASL. For clarity the basolateral membrane K^+ channels, as well as the bumetanide sensitive $Na^+/K^+/Cl^-$ co-transporter are omitted from these diagrams.

Understanding the components of ionic transport in the airway epithelium has led to the development of various therapies that are aimed at preventing the decline of lung function in CF patients. An example of these potential therapies, which has been derived from the "low-volume" hypothesis group, is treatment with aerosolised amiloride. Amiloride, a Na^+ channel blocker, can inhibit the hyperabsorption of Na^+ across the CF airway epithelium. This approach has been evaluated in clinical trials and was shown to normalise the depth of the ASL layer, resulting in improved MCC of airway secretions, subsequently inducing mucus expectoration in CF patients (Hoffmann *et al.*, 1997). A combination treatment of nucleotide uridine triphosphate, to increase intracellular Ca^{2+} concentrations to activate the $Cl^-_{Ca^{2+}}$ channel, and amiloride has also been used to produce less viscous secretions and improve MCC in CF patients (Bennett *et al.*, 1996).

1.2.4 Hypotheses on the Generation of Cystic Fibrosis Airway Disease

Since the discovery of the CF gene in 1989, the greater understanding of the biological defect underlying CF airway disease has contributed to the development of therapies designed to improve lung function. However, despite the substantial information gained from molecular and cellular research the exact mechanisms by which CF airway disease is initiated has yet to be fully elucidated. An appreciation of the mechanism(s) that result in CF airway disease is therefore needed to allow the development of rational therapies directed at preventing or halting deterioration of lung function. Currently various hypotheses have been brought forward to help explain the mechanisms by which CFTR mutations cause lung disease. These hypotheses include those that focus (i) on abnormal ASL composition or depth ("high-salt", "low-pH", "low-oxygenation", "low-volume",) or (ii) on reduced fluid secretion from the submucosal glands ("abnormal gland secretion") (Goldman *et al.*, 1997a, Smith *et al.*, 1996; Coakley and Boucher, 2001; Worlitzsch *et al.*, 2002; Boucher, 1999a; Jayaraman *et al.*, 2001). Whilst many research groups have contributed to the area of ion transport and host defence in CF airways this overview will focus on the main two hypotheses that have been proposed to explain how CF airway disease develops. These hypotheses have been briefly outlined below to provide the reader with sufficient information to allow some understanding of the intricate relationship between CF lung disease and its underlying aetiology. It is important to note that all the hypotheses put forward are not mutually exclusive.

The first hypothesis, known as the "low-volume" hypothesis, proposes that CF airway disease results from excessive volume absorption from the ASL (PCL and mucus layer) layer into the airway epithelium, and a low axial clearance movement of the ASL layer, especially from the small airways (Matsui *et al.*, 1998a/b). Effective MCC mechanisms rely heavily on the ability of the cilia to thoroughly extend and perform an effective stroke pattern in an optimal depth of the ASL layer (Matsui *et al.*, 1998b). Therefore, the reduced depth of the ASL layer in CF airways impedes efficient MCC mechanisms allowing for increased residence time of pathogenic bacteria in the ASL layer. The UNC group suggests that potential therapies should focus on adding isotonic liquid (water and NaCl, 120 - 140 mM; Tarran *et al.*, 2001) to the CF airway surfaces to restore the volume of the ASL layer (Tarran *et al.*, 2001), thus facilitating effective cilial action and promoting MCC.

In contrast, the second hypothesis, known as the "high-salt" hypothesis, proposes that the CF airway epithelium is unable to absorb Cl^- from the ASL resulting in high concentrations of NaCl (defined as > 100 mM; normal levels are considered to be < 50 mM in the ASL layer (Goldman *et al.*, 1997a). These higher than normal levels of NaCl inhibit the activity of the salt-sensitive defensins, which are present in the ASL layer and normally inactivate or kill pathogenic bacteria. As a result, CF patients become prone to chronic bacterial infections (Smith *et al.*, 1996; Wine, 1997; Goldman *et al.*, 1997a; Zabner *et al.*, 1998). The therapeutic intervention for the treatment of CF airway disease consistent with the "high-salt" hypothesis is either removal of the excessive salt present in the ASL layer by the use of osmolytes such as xylitol (Zabner *et al.*, 2000a), or the addition of salt-insensitive antimicrobial factors (Harwig *et al.*, 1996).

In 2001 yet another hypothesis on the mechanism of the generation of CF airway disease, related to the antimicrobial aspect of the "high-salt" hypothesis described above, was proposed by Bals and colleagues (2001). They suggested that CFTR deficiency causes an abnormal composition of the antimicrobial factors present in the ASL layer, and also results in the absence of an unidentified but salt-insensitive antimicrobial factor. The absence of this factor from the ASL layer leads to a reduced ability of the ASL layer to fight bacterial infections.

The knowledge gained from the experiments of both the UNC and Iowa groups has allowed the development of novel experimental techniques for understanding the airway epithelium biology. This has led to the recommendation of various innovative treatments designed to promote clearance, and/or bacterial killing, from the CF lung.

Initially, research findings supported both the "low-volume" and the "high-salt" hypotheses. However, recent evidence from *in vitro* and *in vivo* studies by the UNC group studying the implications and predictions of the "low-volume" hypothesis indicate that the altered airway epithelial ion transport in CF is indeed associated with a reduced ASL layer depth (Tarran *et al.*, 2001; Worlitzsch *et al.*, 2002). It appears that most CF researchers now favour the "low-volume" hypothesis group and its predictions for developing appropriate therapeutical strategies to pursue for the treatment of CF airway disease (Guggino, 2001; Knowles and Boucher, 2002).

1.2.5 Model Systems of the Human Airway Epithelium

The examination of the properties of the human airway epithelium relevant to the study of CF, such as the regulation of the ion transport across the airway epithelium and the antimicrobial activity of the ASL layer, is difficult to perform in the human lung mainly due to the practical and ethical limitations of human experimentation. As a result, several model systems have been employed to study CF both *in vitro* and *in vivo*. In the studies described in this thesis, aspects of one area of CF treatment (gene therapy) were evaluated in two model systems: the *in vitro* air liquid interface cultures of airway epithelial cells and the transgenic CF ($cftr^{tm1Unc}$) mice. These model systems are described here.

(a) Air Liquid Interface Cultures of Airway Epithelium

Air liquid interface (ALI) cell cultures of airway epithelium have found much utility because they (i) model the physical aspects of the airway epithelium (contact to air), (ii) allow for differentiation of the cells and (iii) often retain MCC-like mucus transport mechanisms (Gray *et al.*, 1996). ALI cultures are typically established from epithelial cells isolated from enzyme-dispersed airway tissues such as the trachea or the lung and grown on semi-permeable membranes. This arrangement allows for the apical surface of the airway epithelium layer to be exposed to air while the culture medium remains in contact with the basolateral surface of the culture. Under ALI conditions the airway epithelial cells form TJ's and can differentiate into cultures that mimic the appearance and properties of the pseudo-stratified ciliated epithelium present *in vivo* (Gray *et al.*, 1996).

Successful cultures are characterised by the presence of cilia that act in a co-coordinated fashion to transport the overlaying ASL layer, similar to the intact airway epithelium. ALI cultures of CF airway epithelial cells are now also routinely produced in major CF laboratories, and these have been vital in elucidating the nature of the ion transport impairment across the CF airways.

(b) Cystic Fibrosis Mouse Models

The cloning of the murine CFTR gene (Tata *et al.*, 1991) allowed the generation of animal models of CF by gene targeting. A number of CF knockout mouse models have been generated using this technique (Snouwaert *et al.*, 1992; Ratcliff *et al.*, 1993; O'Neal *et al.*, 1993; Hasty *et al.*, 1995), as well as mice homozygous for either the ΔF508 CF gene mutation (Colledge *et al.*, 1995; Zeiher *et al.*, 1995; VanDoorninck *et al.*, 1995) or the G551D CF gene mutation (Delaney *et al.*, 1996).

Although the CF mice are considered suitable animal models to study CF they do not completely model the CF ion transport impairment underlying the lung disease seen in humans. Specifically, none of the CF mice mentioned above exhibit the spontaneous lung disease seen in the majority of CF patients (Grubb and Boucher, 1999). The reason CF mice do not develop lung disease is not known but this may be due to specific anatomical differences when compared to the human lower airways. In particular, unlike the human CF lungs, the CF mouse lungs lack airway bronchioles and have fewer submucosal glands and goblet cells (Grubb and Boucher, 1999).

Interestingly it is only the nasal airways of the CF mouse model that exhibits the ion transport impairment displayed across the CF human lung (described in section 1.2.3) (Grubb and Boucher, 1999). In addition, the types of epithelial cells that line the CF mouse nasal airway are similar to the CF human lung airways, with respect to the presence of ciliated airway epithelium and MCC mechanisms (Grubb and Boucher, 1999). Therefore it is apparent that the CF mouse nose is an excellent model tissue to assess potential CF therapies (Clarke *et al.*, 1992; Grubb *et al.*, 1994a/b).

In the next section of this chapter (Part C) gene therapy as a potential cure for CF airway disease will be reviewed. The various gene transfer vectors and ways to improve gene transfer to the airway epithelium will also be described.

Part C

1.3 Gene Therapy

1.3.1 Gene Therapy for Cystic Fibrosis Airway Disease

Gene therapy for CF airway disease is based on the premise that if adequate CFTR function can be restored in the defective CF airway epithelial cells then airway epithelial biology and overall lung function would be normalised. Airway infection should then be prevented and the morbidity and mortality associated with CF airway disease averted.

Initial gene transfer studies in cell culture were successful in demonstrating the concept of the restoration of normal Cl^- secretion in CF airway epithelial cells by retrovirus-mediated delivery of the CFTR cDNA (Drumm *et al.*, 1990; Rich *et al.*, 1990). Additional studies suggested that only 6 - 10% of CF airway epithelial cells needed to be corrected in order to restore normal Cl^- secretion (Johnson *et al.*, 1992). However, subsequent studies suggested that in the cell culture system used by Johnson and colleagues (1992) Cl^- could move from non-corrected to gene corrected cells *via* the gap junctions between the epithelial cells. Therefore the increased levels of Cl^- secretion reported by Johnson and colleagues (1992) could have been generated by the non-corrected cells utilising the many CFTR channels that were present on a small population of gene corrected cells (Boucher, 1999b). The number of gap junctions present in the epithelial cells of the intact airway epithelium may be less than those present in cultures of airway epithelial cells (Boucher, 1999b). This suggests that more than 10% of airway epithelial cells would need to be corrected *in vivo* to restore normal Cl^- secretion (Boucher, 1999b). In gene transfer studies using culture systems thought to be more representative of the intact CF airway epithelium it was shown that more than 90% of the epithelial cells would need to be corrected before the Na^+ absorption impairment could return to normal (Johnson *et al.*, 1995).

Many different gene transfer vector systems are currently under development for CF gene therapy, and many have been evaluated in various airway epithelium model systems and in CF subjects in gene therapy clinical trials. These systems can be divided into non-viral and viral-based gene transfer vectors.

To date, the majority of clinical trials for CF gene therapy have been Phase I safety studies in which either the non-viral or the viral gene transfer vectors were delivered topically to the nose, the maxillary sinus or the lower airways either by direct liquid instillation or by nebulisation. These clinical trials have provided valuable insights into the many aspects of the safety concerns associated with the use of gene transfer vectors, which cannot be adequately addressed in non-human model systems.

1.3.2 Non-Viral Gene Transfer Vectors

1.3.2.1 Cationic Lipids

The gene transfer efficiency of most non-viral gene transfer vectors (i.e. cationic lipids), which are employed to deliver genes into the target airway epithelial cells, are dependent on inherent cellular mechanisms that are present in the cells for uptake and intracellular transport of particles (Romano *et al.*, 2000). The possibility that cationic lipids could deliver the CFTR cDNA into the lung airway epithelial cells of CF patients has been the subject of several clinical studies (Bigger and Coutelle, 2001), which are reviewed below.

The efficiency of cationic lipid-mediated gene transfer into the airway epithelium *in vivo* and in ALI cultures of airway epithelial cells has been found to be very low. It appears as though three major barriers are responsible for the poor success rate of the use of cationic lipids as gene transfer vectors. These are (i) the limited ability of the cationic lipid/DNA complexes to bind to the apical membrane of the airway epithelial cells, (ii) the low accumulation of the cationic lipid/DNA complexes within the cell and (iii) the ineffective translocation of the DNA through the nuclear membrane to the nucleus (West and Rodman, 2001).

Initially non-viral-based gene transfer vector systems were thought to have a low toxicity profile *in vivo*. It was later found that when the delivery dose of the cationic lipid/DNA complexes to the mouse lungs was increased to produce an adequate level of transgene expression, this resulted in unacceptably high levels of inflammation and toxicity (McLachlan *et al.*, 2000; Tousignant *et al.*, 2000). Results from earlier studies have suggested that these inflammatory responses may have been due to the high number of CpG motifs present in the bacterial vectors that were used to prepare the DNA (Krieg *et al.*, 1995; Schwartz *et al.*, 1997). In an attempt to minimise immune responses to the CpG motifs the bacterial DNA was methylated, however, lung inflammation was still apparent. This finding suggests that other factors may also be contributing to inflammation and toxicity in response to cationic lipid/DNA administration (McLachlan *et al.*, 2000). The results of gene therapy clinical trials using cationic lipids are briefly reviewed below.

1.3.2.2 Cationic Lipids: Progress in Gene Therapy Clinical Trials

The results from the various Phase I gene therapy clinical trials using cationic lipids to deliver the CFTR cDNA by liquid instillation or nebulisation into the nasal airway epithelium of CF patients have shown that gene transfer is inefficient. In particular, low levels of CFTR mRNA was detected in the nasal tissue, by reverse transcriptase (RT)-PCR analysis, and only resulted in a partial and transient correction of the CF defect measured by the TPD assay (Caplen *et al.*, 1995; Gill *et al.*, 1997; Porteous *et al.*, 1997; Zabner *et al.*, 1997a; Knowles *et al.*, 1998; Alton *et al.*, 1999; Noone *et al.*, 2000).

The results from two recent clinical trials undertaken in the United Kingdom and the USA provide a perspective of the current state of development of these vectors. One clinical trial examined the effectiveness of repeated administration of lipid/DNA complexes. In this double-blinded Phase I clinical study, three doses of lipid/CFTR complexes were instilled into the nasal cavity of ten CF subjects at 4-week intervals. In six subjects, nasal TPD measurements suggested the presence of CFTR gene expression, which correlated with detectable CFTR mRNA, 4 days after each dose. No inflammatory responses evoked toward the lipid/CFTR complexes were observed (Hyde *et al.*, 2000). In the other dose-escalation Phase I clinical study, the cationic lipid Genzyme (GL)-67 was complexed to CFTR cDNA and aerosolised into the lung of eight CF subjects. RT-PCR analysis detected CFTR mRNA in the bronchial scrapings from three of the eight subjects. Within 6 hrs of cationic lipid/DNA vector application, clinical symptoms indicative of inflammation were reported in half the subjects of this study (Ruiz *et al.*, 2001). These inflammatory responses were found to be independent of the un-methylated CpG motifs of the *E.coli* derived DNA (Ruiz *et al.*, 2001), which have been previously shown to elicit immune responses (Krieg *et al.*, 1995).

In conclusion, despite improvements in cationic lipid gene transfer technology the outcomes of gene therapy clinical trials using these non-viral systems to date have been generally disappointing to date.

1.3.3 Viral Vectors for Gene Transfer

In this section the properties of various viral vectors currently under development will be reviewed. Each of these viral gene transfer vector systems (i.e. adenovirus, adeno-associated virus, retrovirus and lentivirus vectors) is characterised by certain properties that render them suitable, in some ways, in the development of gene therapy for CF airway disease.

1.3.3.1 Adenovirus Vectors

Adenoviruses are common pathogens of the respiratory tract and are trophic for respiratory cells. Consequently they were rapidly identified and developed as potential vectors for gene therapy of CF airway disease. Recombinant adenovirus (AdV) vectors are able to transfer transgenes efficiently to many cell types *in vitro* and to some tissues and organs *in vivo* (Qiu *et al.*, 1998; Kay *et al.*, 2001). In addition, they can be produced at high titres (up to 10^{13} vector particles/ml) with no risk of contamination with replication-competent virus (Kay *et al.*, 2001). However, since AdV vectors are non-integrative and non-replicating they can only confer transient gene expression, the duration of which is dependent on the proliferation state of the target cell (Hemmi *et al.*, 1998).

Adenoviruses have four early genes (E1-4), which encode polypeptides responsible for viral and cellular gene expression, viral replication, repression of major histo-compatibility complex responses and inhibition of cellular apoptosis (Kay *et al.*, 2001). In first-generation AdV vectors the early region 1 (E1A/B), containing the transcriptional regulatory genes essential for viral replication, was replaced with an expression cassette for a transgene and the AdV vector propagated in E1-*trans*-complementing cell lines such as 293 (Kay *et al.*, 2001; Graham *et al.*, 1977). First-generation AdV vectors expressing CFTR were studied extensively *in vitro* and *in vivo* and were subsequently tested in CF subjects in gene therapy clinical trials. The administration of first-generation AdV vectors into animals and in subjects in gene therapy clinical trials was found to elicit high inflammatory and immune responses (Kay *et al.*, 2001).

The immune and inflammatory responses mediated by T-cells were found to be directed against both the AdV vector and the transgene product, resulting in the elimination of positively transfected cells and hampering the duration of gene expression (Yang *et al.*, 1994; Zsengeller *et al.*, 1995). These findings suggested that AdV-mediated gene transfer may persist if the immune and inflammatory responses were suppressed. In a subsequent study, Zsengeller and colleagues (1997) demonstrated that AdV vector delivery in immuno-compromised animals resulted in prolonged (~56 days) AdV-mediated gene expression, further supporting the effect of the immune system in attenuating AdV-mediated gene transfer *in vivo*.

The results of a recent AdV dose-escalation gene therapy clinical trial, in which AdV vectors were sprayed locally into the lung of normal subjects, suggested that the immune responses evoked towards the AdV vector in humans may be different to those observed in animals (Harvey *et al.*, 2001). In particular, no signs of an immune/ inflammatory response or an increase in the level of AdV neutralising antibodies was noted in any of the subjects treated. Nonetheless, gene expression was transient suggesting that other host responses may exist in humans that limit AdV-mediated gene transfer (Harvey *et al.*, 2001).

In second-generation AdV vectors, in addition to the E1 region, the E2, E3 or E4 regions are either deleted or disabled. As a consequence, these AdV vectors exhibit lower levels of viral protein expression, which is thought to be responsible for evoking host immune and inflammatory responses (Rasmussen *et al.*, 1999). Also, when these AdV vectors were tested in animals and their gene transfer efficiency compared to that of first-generation AdV vectors there was no significant improvement in the level, or the duration, of gene expression (Rasmussen *et al.*, 1999).

The E3 region of the AdV genome encodes several genes that reduce host immune and inflammatory responses following AdV vector administration (Wold *et al.*, 1994) and thus the deletion of the E3 region from AdV vectors may hamper transgene expression. Therefore, the presence of the E3 region in AdV vectors may be important in maintaining transgene expression *in vivo* (Harrod *et al.*, 1998). It was shown that intracellular expression of the AdV E3-14.7K protein in lung epithelial cells significantly reduced AdV-associated lung inflammation and resulted in increased transgene expression *in vivo* (Harrod *et al.*, 1998).

Since the AdV vector is naturally trophic for the airway epithelial cells it is surprising that the results from most animal studies and clinical Phase I trials (described in section 1.3.3.2) have shown that the efficiency of AdV-mediated gene transfer *in vivo* is poor. The efficiency of infection of airway epithelial cells by AdV gene transfer vectors (serotypes 2 and 5) is dependent on (i) the accessibility of the coxsackie and AdV receptors (CAR) that mediate AdV vector attachment (Pickles *et al.*, 1998), and (ii) subsequent interaction of the penton base protein of the AdV vector with the $\alpha v\beta3/\alpha v\beta5$ integrins, which facilitate AdV vector internalisation (Wickham *et al.*, 1993). The CAR receptors (Bergelson *et al.*, 1997) and the $\alpha v\beta3/\alpha v\beta5$ integrins (Nemerow and Stewart, 1999), which are important for vector attachment and internalisation, are located on the basolateral surface of the airway epithelium (Pickles *et al.*, 1998), which in turn is separated from the apical surface by the TJ's (Yeaman *et al.*, 1999), thus limiting the efficiency of AdV-mediated gene transfer. Transient breaching of the TJ's allows improved AdV vector access to the basolateral cell surface and enhances AdV-mediated gene transfer (Parsons *et al.*, 1998). In section 1.3.6 the modulation of the epithelial TJ integrity as an approach to improve viral-mediated gene transfer will be reviewed.

Finally, it should be noted that not all cells that line the intact airway epithelium are susceptible to AdV-mediated infection. For example, submucosal glands can be effectively transduced by AdV vectors (Pilewski *et al.*, 1995), however, ciliated cells (Zabner *et al.*, 1997b) and columnar cells (Pickles *et al.*, 1996) appear to be resistant to AdV-mediated infection. The results of gene therapy clinical trials using AdV vectors are reviewed below.

1.3.3.2 Adenovirus Vectors: Progress in Gene Therapy Clinical Trials

The first gene therapy clinical trial for CF was initiated by Zabner in 1993. In this non-blinded study an AdVCFTR vector was instilled into the nasal cavity of three CF subjects. Baseline TPD correction was observed in all three subjects for up to 10 days, however, as localised nasal inflammation was present it may have affected these TPD measurements. In another clinical trial, a first generation E1/E3-deleted replication defective AdVCFTR vector was delivered to the nasal cavity and to the bronchi of four CF subjects. CFTR mRNA was detected (by RT-PCR analysis) in the bronchial scrapings of only one of the four subjects, and a transient acute inflammatory response was observed in another subject (Crystal *et al.*, 1994).

In 1995 a double-blinded, dose-escalation (four logarithmically increasing doses) Phase I clinical trial using an E1-deleted AdV5CFTR vector was performed. The AdV vector was instilled intra-nasally in twelve CF subjects. Inflammatory responses were reported in two of the three subjects that received the highest dose (1×10^{10} pfu) of the AdV vector preparation. Although no functional correction of the defective ion transport was evident by the TPD assay, RT-PCR analysis detected CFTR mRNA in the nasal scrapings of five from the six subjects treated with the two highest AdV vector doses ($> 1 \times 10^{8}$ pfu) (Knowles *et al.*, 1995). In a similar, dose-escalation (five increasing doses) Phase I clinical trial, a second-generation E1/E4-deleted AdVCFTR vector was delivered intranasally in six CF subjects approximately every 6 weeks. Partial correction of Cl^- transport by the TPD assay was apparent in two of the subjects that received the highest dose of the AdV vector and humoral immune responses were noted in all six subjects (Zabner *et al.*, 1996a). In a subsequent Phase I clinical trial by Bellon and colleagues (1997) an aerosolised E1/E3-deleted AdVCFTR vector was delivered into the nasal and lung airway epithelium of six CF subjects. No immune or inflammatory responses were noted. RT-PCR analysis detected low levels of CFTR mRNA in the nasal biopsies from all six patients, but CFTR mRNA was detected in only one of the six bronchial specimens (Bellon *et al.*, 1997).

In a dose-escalation Phase I clinical trial by Zuckerman and colleagues (1999) a third-generation E1/E4-deleted AdVCFTR vector was delivered to the conducting airways of eleven CF subjects. RT-PCR analysis detected low levels of CFTR mRNA in bronchial scrapings from all subjects 4 days after the instillation of the AdV vector. No evidence of gene transfer was found at the last gene transfer assessment point (day 43). In addition, most patients showed a specific T-cell response, and influenza-like symptoms were observed in those subjects who received the higher doses (2 x 10^{11} pfu) of the AdV vector preparation (Zuckerman *et al.*, 1999).

In a Phase I clinical trial by Harvey and colleagues (1999) an AdVCFTR vector was aerosolised into the nasal cavity of fourteen CF subjects and gene transfer was assessed by RT-PCR analysis of CFTR mRNA. The highest level of expression was obtained after the administration of the first dose of the higher titre of AdV, but gene expression was transient (72 hrs). Interestingly, the level of gene expression obtained following the second administration of AdV was lower compared to that obtained after the first AdV vector administration, and the third administration of AdV vector resulted in no detectable gene expression (Harvey *et al.*, 1999). Surprisingly, the decrease in gene expression following the second and third AdV vector administrations was not associated with an increase in the level of neutralising AdV antibodies. The findings of this clinical trial demonstrated that although repeated AdV vector administration appeared to be safe, this did not result in persistent transgene expression (Harvey *et al.*, 1999).

In a recent dose-escalation clinical trial an AdV2CFTR vector was delivered to the lungs of thirty-four CF subjects as either an aerosol administration or *via* a bronchoscope (Joseph *et al.*, 2001). The high doses of AdV vector delivered by either method resulted in mild to moderate inflammatory responses (Joseph *et al.*, 2001). Gene transfer to the epithelial cells of the lung was found to be inefficient, regardless of the AdV vector administration method employed (Perricone *et al.*, 2001).

It is worthy of note that in a recent clinical trial for the treatment of a different genetic disease, ornithine transcarbamylase deficiency, one of the subjects that received the higher dose (10^{13} pfu) of a third-generation E1/E4 deleted AdV vector died as a result of a large immune and inflammatory responses (Lehrman, 1999). Although the AdV vector preparation was delivered intravascularly, rather than by liquid instillation or nebulisation, this unfortunate incident has re-focused attention on the safety aspects of the use of AdV vectors for CF gene therapy.

In summary, AdV vectors have long dominated the majority of clinical trials for CF gene therapy due to (i) their natural tropism for the airway epithelium, (ii) their ability to infect non-dividing cells and (iii) the ease with which high titres of AdV vector stocks can be produced. However, the results from animal studies and Phase I gene therapy clinical trials have clearly demonstrated that AdV vectors can only confer transient gene expression and also evoke inflammatory and immune responses that limit the effectiveness of repeated AdV vector administration. For these reasons, other viral vectors able to mediate stable expression of the transgene but which are associated with reduced or no immune/inflammatory responses have been examined.

1.3.3.3 Adeno-Associated Virus Vectors

The adeno-associated virus (AAV) was discovered as a contaminant in an AdV vector stock preparation and was found to require the presence of either an AdV or a Herpes simplex virus to replicate *in vitro* (Kay *et al.*, 2001). To date AAV has not been associated with any known human disease and has therefore been considered safe for use as a gene therapy vector (Curiel *et al.*, 1996). In contrast to wild type AAV, which integrates specifically into the q arm of chromosome 19, recombinant AAV gene transfer vectors integrate randomly. As a consequence, AAV vectors transduce cells either by random integration, raising the possibility of insertional mutagenesis, or remain as stable episomal DNA (Kay *et al.*, 2001).

Although the AAV vector has received much attention as a gene transfer vector for CF airway disease the size of the CFTR cDNA (~4.5 kb) is close to its cloning capacity (<5 kb) preventing the additional cloning of the regulatory sequences necessary to improve CFTR gene expression *in vivo*. Recent molecular advances have allowed for the construction of CFTR mini-genes, which retain CFTR function and most importantly allow for the cloning of promoters, to drive/control CFTR gene expression *in vivo*, within the cloning limits of the AAV vectors (Zhang *et al.*, 1998). Although repeated administration of recombinant AAV vectors can result in increased production of neutralising AAV antibodies, the immune and inflammatory responses evoked in response to the administration of the AAV vectors are minimal compared to those produced in response to the AdV vectors (West and Rodman, 2001).

Currently there are six different AAV serotypes, each of which has different tropism properties (Kay *et al.*, 2001). Most studies have made use of the AAV2 serotype gene transfer vectors (Kay *et al.*, 2001), as these result in long-term transgene expression in several tissues and organs *in vivo* including muscle, photoreceptors, liver and some cell populations of the central nervous system (Kaplitt *et al.*, 1994; Fisher *et al.*, 1996; Xiao *et al.*, 1996). However, the efficiency of gene delivery by AAV2 vectors to the lung is poor and the reasons for this are still not clear. One reason that may account for the ineffectiveness of AAV2-mediated gene transfer to airway epithelial cells is the presence of the glycocalyx structure on the apical cell surface, which consists of glycoproteins, glycolipids and proteolycans (Pickles *et al.*, 2000). The glycocalyx may hamper binding of the AAV vector to the $\alpha v \beta 5$ integrins, thus limiting AAV vector internalisation. Furthermore, the AAV2 receptor known as heparin sulphate proteoglycan (Summerford and Samulski, 1998) is located at the basolateral cell surface (Duan *et al.*, 1998), which is not readily accessible due to of the intercellular TJ integrity barrier. Bals and colleagues (1999) demonstrated that application of AAV2 vectors onto the basolateral surface rather than the apical surface resulted in efficient AAV-mediated gene transfer. Interestingly, treatment of the apical surface with glycerol or trypsin resulted in the opening of the epithelial TJ's vastly improving AAV-mediated gene transfer (Bals *et al.*, 1999).

Despite the fact that the AAV2 vectors have been extensively studied in many gene transfer studies, attention has now shifted toward the use of the AAV5 and AAV6 vectors as these are more effective than AAV2 vectors in transduction of airway epithelial cells *via* the apical surface (Zabner *et al.*, 2000b, Auricchio *et al.*, 2002; Halbert *et al.*, 2001). The use of AAV vectors in gene therapy clinical trials is reviewed below.

1.3.3.4 Adeno-Associated Virus Vectors: Progress in Gene Therapy Clinical Trials

The first gene therapy clinical trial for CF using AAV-based gene transfer vectors was initiated by Wagner and colleagues (1998a/b, 1999). In this non-blinded, randomised, dose-escalation Phase I/II clinical trial a tgAAV2CFTR vector, a replication-defective AAV2-based vector that contains the hCFTR cDNA, was instilled into the maxillary sinuses of ten CF subjects. Sinuses are well-defined 'out-pouchings' within the bone adjacent to the nasal airway and as they are completely lined with ciliated respiratory epithelium they provide a good model of the human conducting airways. Transient correction of the Cl^- transport (monitored by the TPD assay) was apparent for up to 14 days in the subjects that received the highest doses ($> 5 \times 10^4$ AAV replication units) of the AAV vector preparation and no significant immune and inflammatory responses were apparent (Wagner *et al.*, 1998a/b; Wagner *et al.*, 1999).

In another Phase I, single-administration, dose-escalation clinical trial a tgAAV2CF vector was delivered to the lungs of twelve CF subjects as an aerosol. Gene transfer was inefficient and it was also shown that the high doses of AAV vector administration resulted in the production of serum neutralising AAV antibodies (Aitken *et al.*, 2001).

In a recent Phase II double-blind, randomised, placebo-controlled clinical trial by Wagner and colleagues (2002) a tgAAV2CF vector was administered to one of the maxillary sinuses of twenty-three patients (Wagner *et al.*, 2002). Although there was no evidence of successful gene transfer to the maxillary sinus, the administration of the AAV vector was well tolerated and caused no safety issues to the patients (Wagner *et al.*, 2002).

The low gene transfer efficiency apparent in the above clinical trials appears to be due to the poor binding efficiency of the AAV2 vector on the apical surface of the airway epithelial cells as previously discussed (section 1.3.3.3). Therefore, future AAV gene therapy clinical trials should make use of the AAV5 or AAV6 serotypes, which are considered to be more suited for gene transfer to the airway epithelium.

1.3.3.5 Retrovirus Vectors

For situations where stable gene expression is required retroviruses have been favoured, as they stably integrate their genome into the host chromosome (Kalpana, 1999) allowing for the maintenance of the genetic information in dividing cell populations (Kay *et al.*, 2001). Retroviruses, however, integrate randomly into the genome of the host cell and thus the expression of the gene may be subject to gene silencing (Bestor, 2000).

The Murine leukaemia retrovirus (MLV) has been extensively studied and its biology is now well understood. This has allowed for the construction of vector systems in which the genes essential for viral particle formation have been removed from the MLV vector and are provided *in trans*, allowing for up to 8 kb of exogenous cDNA to be inserted (Kay *et al.*, 2001). The combination of developments in MLV vector design and helper plasmid construction have greatly increased the number of recombination events required for the reconstitution of a replication-competent, potentially pathogenic, retrovirus (RCR) and have made MLV gene transfer vectors safer for clinical use.

The potential of MLV vectors has been investigated in numerous gene transfer studies *in vitro* and their effectiveness in humans was recently demonstrated in a successful *ex vivo* gene therapy trial for human severe combined immunodeficiency X1 (SCID-X1). Specifically, MLV-mediated gene transfer resulted in full correction of the SCID-X1 phenotype in the two treated patients (Cavazzana-Calvo *et al.*, 2000).

Despite the apparent advantages of the MLV vectors they are subject to biological limitations, which prevent their successful application to the transduction of airway epithelial cells *in vivo*. The most important limitation is that MLV vectors can only transduce actively dividing cells (Kay *et al.*, 2001). In particular, it has been shown that the nuclear membrane of the target cell has to be disrupted, an event that occurs only during cell mitosis, which then allows the import of the MLV vector into the nucleus for gene transduction (Roe *et al.*, 1993). In normal airway epithelium less than 1% of the cells are actively dividing at any given time (Leigh *et al.*, 1995), thus severely limiting the efficiency of MLV-mediated gene transfer. However, Wang and colleagues (1998) demonstrated that stimulation of airway epithelial cells *in vitro* with a mitogen (a keratinocyte growth-like factor) resulted in increased cell proliferation and improved the level of MLV-mediated gene transfer. A final limitation in the use of the MLV vectors in CF gene therapy is the lack of the MLV receptors (Pit2) on the apical surface of the airway epithelium (Miller and Chen, 1996).

In summary, it is now well recognised that successful gene therapy for CF airway disease requires a gene transfer vector that can transduce dividing and non-dividing cells, provide stable and sustained gene expression and does not induce significant host immune and inflammatory responses. For these reasons lentivirus vectors, such as those derived from the human immunodeficiency virus type-1 (HIV-1), appear well suited as vehicles for the stable introduction of the CFTR gene to the CF airway epithelium.

1.3.3.6 Lentivirus Vectors

Lentiviral (LV) vectors are a type of retroviral vectors that can transduce quiescent cells (Naldini *et al.*, 1996a; Blomer *et al.*, 1997). This is largely due to the karyophilic properties of their pre-integration complex, which allows the import of the LV vector into the nucleus of a cell *via* the nucleopore (Salmon *et al.*, 2000). Considerable progress has been made in the development of LV vectors, especially those based on the HIV-1 (Naldini *et al.*, 1996a), the feline immunodeficiency virus (FIV) (Poeschla *et al.*, 1998; Wang *et al.*, 1999) and the equine infectious anaemia virus (Olsen, 1998). All LV vectors have been disabled (especially important for the HIV-1 based LV vectors) in various ways to render them safe for gene transfer *in vivo* (Buchschacher and Wong-Staal, 2000).

LV vectors have a cloning capacity of ~9 kb and like all retroviruses can stably integrate their transgene into the genome of the host cell. Although integration is random, LV vectors appear to produce persistent transgene expression, which is not subject to gene silencing mechanisms, in a variety of tissues including rodent brain (Naldini *et al.*, 1996b; Bosch *et al.*, 2000), muscle, liver (Kafri *et al.*, 1997; Park *et al.*, 2000) and eye (Takahashi *et al.*, 1999; Galileo *et al.*, 1999). In contrast to the MLV vectors the genome of the LV vectors, derived from HIV-1, is considerably more complex. In addition to the *gag*, *pol* and *env* genes found in all retroviruses HIV-1 also encodes two regulatory genes, *tat* and *rev*, which are essential for viral replication, and four accessory genes, *vif*, *vpu*, *vpr* and *nef*, which are important for pathogenesis (Kay *et al.*, 2001). The complex nature of the HIV-1 genome has been extensively exploited to allow for the design of LV vector packaging constructs with novel safety features.

Although LV vectors hold promise as gene transfer vectors their origin, especially of those LV vectors based on HIV-1, has naturally raised safety concerns. To address these concerns LV vectors have been generated by expression of the essential viral genes from separate genetic elements (split-function) in order to improve their safety. However, this still does not overcome the specific concern that genetic recombination between the different components of the LV vector system (LV vector and helper plasmids) could lead to the emergence of an RCR potentially sharing the pathogenic nature of the parental virus. Other possible consequences of such recombinations include mutagenesis and/or oncogene activation, and the genetic alteration of the germ line.

For these reasons great care has been taken to develop LV vector systems designed with safety features to prevent the emergence of RCR. For example, in a LV vector system recently reported by Wu and colleagues (2000) the accessory genes (*vif*, *vpu* and *nef*) and the regulatory genes (*tat* and *rev*) were eliminated or separated from the packaging construct. As the genes critical for viral replication and pathogenesis were removed, the risk of emergence of an RCR sharing the pathogenic features of the parental HIV-1 virus theoretically became negligible (Wu *et al.*, 2000). The biosafety of the LV vector system has been further improved by the construction of self-inactivating (SIN) LV vectors. The SIN LV vectors contain a deletion in the long terminal repeat (LTR), which inactivates its transcriptional activity, thus eliminating any chance of vector recombination following cellular transduction (Zufferey *et al.*, 1998; Miyoshi *et al.*, 1998). The novel safety features of the LV vector system developed and used in this thesis project will be described in more detail in Chapter 5 (section 5.1).

In addition to the safety issues mentioned above, another potential limitation in the *in vivo* use of the LV vectors is the possibility of host immune and inflammatory responses to the high number of LV vector particles that would most likely be required in gene therapy clinical trials. Unlike AdV vectors, LV vectors do not transfer any virus coding sequences and thus would be expected to be less immunogenic. As a result, positive LV-transduced cells should theoretically not be destroyed by T-cell-mediated immune responses.

The potential of LV vectors for CF gene therapy was demonstrated by Goldman and colleagues (1997b) soon after the publication of the first paper describing recombinant LV vectors based on HIV-1 (Naldini *et al.*, 1996a). They showed that a LV vector carrying the CFTR cDNA could only transduce undifferentiated airway epithelial cells in cultures of human CF bronchial xenografts. Johnson and colleagues (2000) reported the first *in vivo* study of LV-mediated gene transfer into mouse and rat nasal airway epithelium. They demonstrated that the efficiency of LV-mediated gene transfer was significantly improved following treatment of the airway epithelium with sulphur dioxide (SO_2), which modulated the epithelial TJ integrity. Recently, Kobinger and colleagues (2001) investigated the use of envelope pseudotypes, other than the VSV-G pseudotype, to improve targeting of the LV vectors to airway epithelial cells *in vivo*. Specifically, they demonstrated efficient LacZ gene transfer into airway epithelium using an Ebola Zaire virus pseudotyped LV vector.

In summary, the various gene transfer vectors currently available display different strengths and weaknesses. Non-viral based gene transfer systems suffer from low efficiency, transience of gene expression and evoke immune responses when administered at high doses. At present, these limitations cannot be easily overcome and attention has been shifting steadily to viral-based gene transfer systems.

Although the AdV vector has received much attention in CF gene therapy development, there are many limitations to its application *in vivo*. This non-integrating vector elicits immune and inflammatory responses, which limits the efficiency of the obligatory AdV re-administration required to maintain gene expression. Although AAV vectors are not as effective as AdV vectors in infecting airway epithelial cells they appear promising as gene transfer vectors for CF, as they can stably integrate into the genome of the host cell and are associated with minimal immune and inflammatory responses. Most retrovirus-based gene transfer vectors, such as the MLV vectors, cannot be effectively used for CF gene therapy as the airway epithelium consists mainly of quièscent cells, which are refractory to transduction by these vectors. In contrast, the LV vectors ought to be able to integrate their genome into quiescent cells with no associated signs of immune and inflammatory responses. Although LV vectors do appear promising as gene transfer vectors for CF gene therapy their origin, especially of LV vectors derived from HIV-1, has raised significant safety concerns that need to be addressed very carefully.

Despite the continuing improvements reported in the area of non-viral and viral-based gene transfer vector development, the results from pre-clinical studies and Phase I gene therapy clinical trials suggest that airway barriers, that apply to all vector systems, may also hinder efficient gene transfer.

1.3.4 Barriers to Viral-Mediated Gene Transfer to the Airway Epithelium

While the conceptual basis of gene therapy for CF airway disease with viral gene transfer is both elegant and simple and is easily demonstrated *in vitro* (Johnson *et al.*, 1992), practical barriers to its application *in vivo* have become apparent (Koehler *et al.*, 2001; Davies *et al.*, 2001). The highly effective airway defences that have evolved to protect the mammalian airway epithelium against allergens, irritants, dust, viruses and microbial pathogens (Bevins *et al.*, 1999) also apply to gene transfer vectors. Therefore, one way to improve gene transfer is to increase our understanding of the biological barriers of the airway and how these limit gene transfer, with the aim of seeking methods to temporarily circumvent some or all of these barriers during gene delivery.

(a) Barriers of the Normal Airway Epithelium

The difficulties associated with efficient vector delivery to airway epithelial cells have helped reveal some of the complexity of the surface barriers present in normal airway, which that have evolved to protect the underlying tissue against foreign particles (Bevins *et al.*, 1999). A gene transfer vector is also a foreign particle and therefore will also be subjected to these physical barriers.

One important barrier is the presence of the ASL layer on the apical cell surface (Figure 1.2) that continually captures inhaled or introduced particles for removal by MCC mechanisms. At the cell surface the glycocalyx (Pickles *et al.*, 2000), which has been shown to bind most vector types, further hinders vector particle entry *via* the apical cell surface (Figure 1.2). Finally, the epithelial TJ's (Figure 1.2) present another physical barrier to the delivery of gene transfer vectors to their receptors, which are located predominantly on the basolateral cell surface below the TJ's. This is especially true of the CAR receptors required for binding of AdV vectors serotypes 2 or 5 (Bergelson *et al.*, 1997), the heparin sulphate proteoglycan receptors required for AAV2 vector internalisation (Duan *et al.*, 1998) and the VSV-G receptors, a commonly used retrovirus pseudotype (Johnson *et al.*, 2000), all of which are predominantly expressed on the basolateral cell surface.

(b) Barriers of the Cystic Fibrosis Airway Epithelium

In addition to the physical barriers reviewed above other barriers also exist in the CF airways, such as the established inflammatory milieu throughout the ASL layer (Otake *et al.*, 1998). The mucus layer that overlays the PCL layer in CF conducting airways is viscous, relatively static and in most cases infected with pathogenic micro-organisms. In cultures of airway epithelial cells, the presence of viscous CF mucus significantly decreased the efficiency of AdV-mediated gene transfer (Stern *et al.*, 1998; Kitson *et al.*, 1999). When the CF sputum was treated with DNase, which reduces the viscosity of the sputum, AdV-mediated gene transfer was significantly improved (Stern *et al.*, 1998).

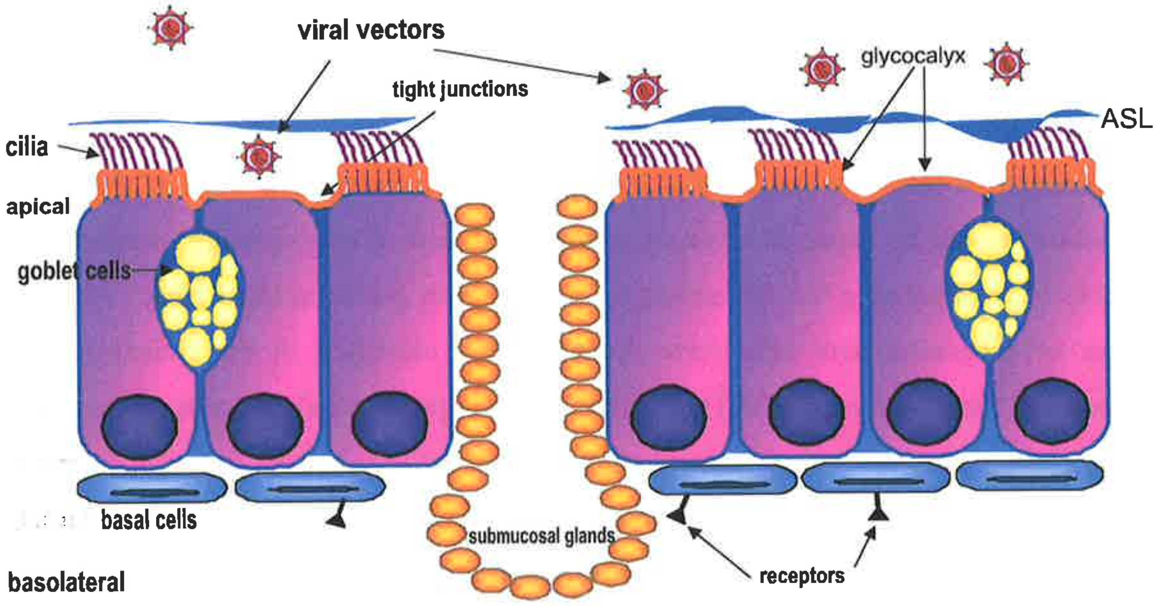

Figure 1.2: Schematic Diagram of the Barriers to Viral Vector-Mediated Gene Transfer to the Airway Epithelium.

The superficial ASL layer continually captures inhaled or introduced particles for constant removal by MCC mechanisms. Closer to the cell surface the glycocalyx prevents vector particle entry *via* the apical surface. Finally, the TJ's between the epithelial cells form yet another barrier to efficient gene transfer vector delivery.

Using negatively charged polystyrene nanospheres as surrogate viral vector particles, it was demonstrated that the CF sputum is also a size-dependent barrier. Specifically, it was shown that smaller nanospheres (124 nm) of similar size to the AdV vectors (100 nm) were able to diffuse through the CF sputum (Sanders *et al.*, 2000). It has been reported that the aqueous non-viscous fraction of the CF sputum inhibits AdV-mediated gene transfer, most likely due to the presence of AdV neutralising antibodies (Perricone *et al.*, 2000). The CF bronchoalveolar lavage fluid has also been shown to inhibit AdV- and AAV- mediated gene transfer *in vitro* possibly due to the presence of viral infectivity inhibitory factors rather than the presence of neutralising AdV or AAV antibodies, respectively (Bastian and Bewig, 1999; Virella-Lowell *et al.*, 2000).

Of particular interest were the observations made in mouse lungs infected with *P. aeruginosa*, a pathogenic organism found in most CF patients. The level of AdV-mediated gene transfer was considerably lower, compared to that of non-infected mouse lungs, suggesting that bronchorespiratory inflammation also inhibits AdV-mediated gene transfer (Van Heeckeren *et al.*, 1998; Parsons *et al.*, 1998).

1.3.5 Progenitor/Stem Cells of the Human Airway Epithelium

The ideal target cell for gene therapy of CF airway disease is the airway epithelial progenitor/stem cell. Successful transduction of the progenitor/stem cells of the airway epithelium would be expected to result in the generation of an expanding progeny pool capable of re-populating the CF airway epithelium with gene-corrected cells. Although niches of progenitor/stem cells have recently been identified in the rodent tracheal epithelium (Borthwick *et al.*, 2001), the identity of the progenitor/stem cells of the human ciliated lung airway epithelium remains controversial.

Therefore, it is obvious that identifying the airway progenitor/stem cells that need to be corrected to normalise the electrophysiological defect across the CF airways is of major interest in terms of establishing long-lasting gene therapy for CF airway disease.

1.3.6 Methods for Improving Gene Transfer to the Airway Epithelium

In order to improve gene transfer to the airway, it is imperative to facilitate deposition, transfer and uptake of the viral vector particles. A number of approaches for improving the efficiency of viral-mediated gene transfer have been explored and some of these are reviewed here.

(a) Increasing the Residence Time of Viral Vectors on the Surface of the Airway Epithelium

Increasing the residence time of the AdV vector on the apical surface of the airway epithelium has been shown to significantly improve the level of gene transfer both *in vitro* and *in vivo* (Johnson *et al.*, 1996; Zabner *et al.*, 1996b; Jiang *et al.*, 1997). Specifically, AdV-mediated gene transfer to airway epithelial cells *in vitro* and to mouse lung *in vivo* has been improved by co-precipitation of the AdV vector with calcium phosphate (Fasbender *et al.*, 1998; Lee *et al.*, 1999; Lee and Welsh, 1999; Walters and Welsh, 1999). Calcium phosphate acts by neutralising the negatively charged AdV vector particles, which would otherwise be repelled by the negatively charged apical cell surface. Cationic polymers such as polybrene or protamine have also been used to improve AdV-mediated gene transfer (Arcasoy *et al.*, 1997). These positively charged polymers are thought to act by binding to the negatively charged apical cell surface to improve AdV entry and/or by encapsulating the AdV vector particles to protect them from the damaging effects of the neutralising AdV antibodies. When these polymers were administered prior to, or during, AdV vector administration gene transfer to the airway epithelial cells *in vitro* was enhanced (Arcasoy *et al.*, 1997). Co-formulation of AdV vectors with poly-L-lysine also prevents antibody-mediated neutralisation of the AdV vector particles and significantly improves gene transfer *in vitro* (Matthews *et al.*, 1999). AdV vectors coated with GL-67 or polyethylene glycol have also been shown to be partially protected from the detrimental effects of the AdV neutralising antibodies (Chillon *et al.*, 1998).

Recently, a different approach was used to increase the residence time of the AdV vector on the apical cell surface of the mouse lung epithelium. Specifically, co-formulation of the AdV vector with a thixotropic solution improved AdV-mediated gene transfer (Seiler *et al.*, 2002). This gel-like solution reversibly liquefies when shaken vigorously and has been reported to inhibit MCC mechanisms, thus leading to minimal removal of the AdV vector particles. When the AdV vector was delivered with the thixotropic solution the level of gene transfer was significantly improved compared to that produced by a PBS-AdV vector formulation (Seiler *et al.*, 2002).

The different approaches taken to improve the residence time of the viral vector particles on the apical surface of the airway epithelium were not considered for this thesis project. This is because the aim of this project was to increase the entry of the viral vectors to the basolateral regions, where their relevant receptors are located, rather than improving their residence time on the apical surface which is devoid of their specific receptors.

(b) Improving the Distribution of Viral Vectors on the Surface of the Airway Epithelium

The co-formulation of AdV vectors with bovine surfactant has been shown to improve the distribution of viral-mediated gene expression in the lungs of rats (Katkin *et al.*, 1997). Bovine surfactant, used to treat respiratory distress in premature infants (Jobe, 1993), distributes rapidly and uniformly throughout the lung and may provide one reason for the improved gene transfer observed in Katkin's study (1997). Co-formulation of AdV vectors with perfluorochemical (PFC) liquids has also been found to improve the level of AdV-mediated gene transfer to the lung airways (Lisby *et al.*, 1997; Weiss *et al.*, 1999a;

Weiss *et al.*, 2001). PFC liquids are fluorinated organic compounds that are colourless, odourless and both chemically and biologically inert (Lisby *et al.*, 1997). As they have a very high solubility for O_2 and CO_2 they have been used successfully for liquid ventilation in animals with recovery to gas breathing (Wolfson *et al.*, 1992). It has also been reported that during liquid ventilation the PFC liquid distributes evenly throughout the lung (Wolfson *et al.*, 1996). This property of PFC liquids may improve the distribution of viral gene transfer vector particles in the airways, consequently improving gene transfer.

Perflubron is a form of a PFC liquid that is radioopaque (Lisby *et al.*, 1997) and has been used to improve gene transfer *in vivo*. In particular, perflubron transiently displaced the airway surfactant and protein materials in abnormal alveoli, therefore allowing for improved access of the gene transfer vector particles to the epithelial cell binding sites (Weiss *et al.*, 1999b; Weiss *et al.*, 2002).

PFC liquids were not considered for use in the mouse nasal airways in this project. Since the nasal airways are easily accessible to solution instillation and are much smaller than the lung airways, the improvement of viral vector distribution was not deemed necessary.

(c) Modulating the Epithelial Tight Junction Integrity

Modulating the airway epithelial TJ integrity to facilitate gene transfer vector particle access to receptors located on the basolateral cell surface is an approach to improving *in vivo* gene transfer that has only recently received attention (Parsons *et al.*, 1998; Wang *et al.*, 1999; Johnson *et al.*, 2000, Wang *et al.*, 2000; Coyne *et al.*, 2000; Chu *et al.*, 2001). Parsons and colleagues (1998, 1999) have shown that the use of low doses of surface-active detergents on the apical surface of the airway epithelium results in modulation of the TJ integrity and as a consequence improves AdV-mediated gene transfer. In particular, when polidocanol (Parsons *et al.*, 1998) and lysophosphatidylcholine (Parsons *et al.*, 1999) were used as pre-treatment agents the level of AdV-mediated gene transfer *in vivo* was significantly increased. Furthermore, they demonstrated partial correction of Cl^- transport, monitored by the TPD assay, in the polidocanol pre-treated CF mouse nasal airway epithelium following a single dose of an AdVCFTR vector (Parsons *et al.*, 1998).

Treatment of the airway epithelium with ethylene glycol-bis (β-aminoethylether)-*N, N, N', N'*-tetraacetic acid (EGTA), a Ca^{2+} chelator, in a hypotonic buffer was shown to permeabilise the epithelial TJ integrity and improve the efficiency of FIV LV- (Wang *et al.*, 1999), AdV- (Wang *et al.*, 2000; Chu *et al.*, 2001) and retrovirus- (Wang *et al.*, 2000) mediated gene transfer *in vivo*. In contrast to the relatively slow action of EGTA, ~1 hr to achieve opening of the TJ's, apical application of sodium caprate on airway epithelial cells *in vitro* resulted in a rapid opening of the TJ's and also improved the level of AdV-mediated gene transfer (Coyne *et al.*, 2000).

In addition to the reagents mentioned above, the toxic gas SO_2 has also been effective in improving gene transfer *in vivo*. In a study by Johnson and colleagues (1998) mice were firstly exposed to SO_2 and then transduced with a VSV-G pseudotyped MLVLacZ vector. Interestingly in this study, although the age of the mice was influential on the level of gene transfer, i.e. the efficiency of gene transfer decreased in older mice, it was the injury induced by SO_2 across the airway epithelium that allowed for gene transfer to occur. LacZ gene transfer was absent in mice that were not exposed to SO_2 prior to the MLVLacZ vector administration (Johnson *et al.*, 1998). In another study by Johnson and colleagues (2000) exposure of mice to SO_2 prior to transduction with a VSV-G pseudotyped LVLacZ vector improved the efficiency of LV-mediated gene transfer.

The use of SO_2 or high concentrations of EGTA to permeabilise the TJ integrity of the airway epithelium was not considered in this project, as both would be impracticable in a clinical setting, due to their obvious toxic profiles. In order to provide an alternate but safe approach to modulating the epithelial TJ integrity the use of detergents to condition the airway was investigated in this project.

1.3.7 Detergent Modulation of the Airway Tight Junction Integrity

As mentioned previously detergents have been shown to improve viral-mediated gene transfer to the airway epithelium *in vivo*. Exactly how detergents act on the airway epithelium to improve gene transfer is not entirely known. In general, the effects of detergents on cell membranes *in vitro* are characterised by their surface-active properties, which allow them to disrupt the structure of cellular surfaces that are held together by hydrophobic interactions between the various lipid structures that constitute the membrane (Helenius and Simons, 1975). When applied to intact tissue such as the airway epithelium, a number of other actions of the detergents may also apply. As a consequence, the dose-response relationships between the detergent concentration, the extracellular effects of the detergent and eventual effects on the cells themselves must be carefully investigated.

In this section the properties of two detergents, polidocanol and LPC will be reviewed in terms of their ability to permeabilise the airway epithelium TJ integrity and consequently improve the efficiency of viral-mediated gene transfer *in vitro* and *in vivo*.

(a) Polidocanol

Polidocanol has a large body of literature describing its role in biological systems and this literature can provide substantial information on its biological activity, which may potentially lead to its development in improving gene transfer *in vivo*.

Polidocanol (Figure 1.3; a) is a non-ionic, amphiphilic synthetic compound that is thought to act on the apical cell surface by transiently opening the airway epithelial TJ's (Parsons *et al.*, 1998) and/or by inhibiting MCC transport (Gizurarson *et al.*, 1990). Polidocanol has been used in animal studies to facilitate absorption of drugs such as recombinant methionyl-human growth hormone (Daugherty *et al.*, 1988) and insulin (Chandler *et al.*, 1991) in rats, and recombinant human interferon-α in sheep (Baglioni and Phipps, 1990), through the nasal mucosa. Polidocanol has also been used in a clinical trial to deliver insulin to the nasal mucosa of Type I diabetes patients (Salzman *et al.*, 1985).

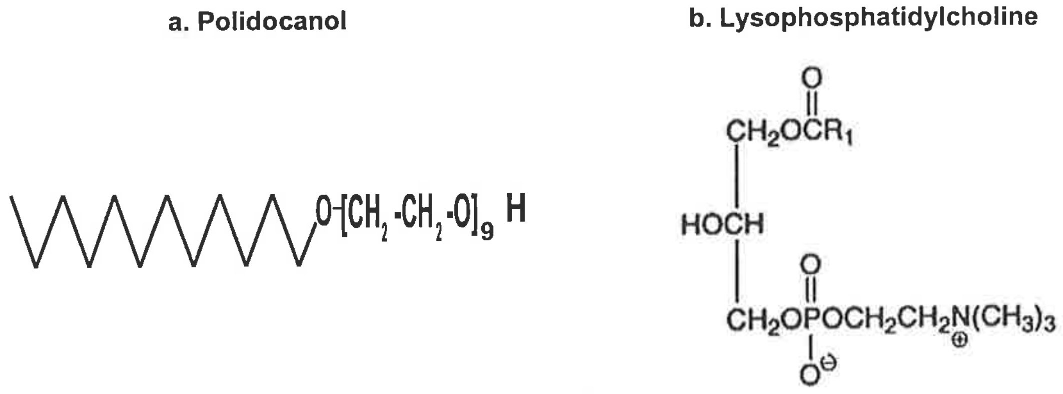

Figure 1.3: Molecular Structures of Polidocanol and Lysophosphatidylcholine.
(a) Polidocanol (polyethylene-glycol-monododecyl-ether) is produced by the reaction of ethylene oxide and dodecyl alcohol. **(b)** LPC (1-acyl-sn-glycero-3-phosphocholine) is generated by the hydrolysis of an acyl group of phosphatidylcholine by phospholipase A2.

(b) Lysophosphatidylcholine

Lysophosphatidylcholine (LPC) (Figure 1.3; b) is a naturally occurring molecule with detergent properties. It is a phosphoglyceride produced from the hydrolysis of an acyl group of phosphatidylcholine, the principal lung phospholipid and the ubiquitous structural molecule of cell membranes, by phospholipase A2. As the detergent component of airway surfactant, comprising 2 - 5% of total phospholipids (Niewoehner *et al.*, 1989; Weltzien *et al.*, 1979), endogenous LPC is rapidly converted in cell systems (Besterman and Domanico, 1992) and lung alveoli (Seidner *et al.*, 1988) to the ubiquitous and non-toxic dipalmitoyl-phosphatidylcholine (DPPC), a primary component of biological membranes. LPC has also been used as an absorption enhancer for drugs such as insulin in rat nasal mucosa *in vivo* (Illum *et al.*, 1989; Chandler *et al.*, 1991).

Like the synthetic polidocanol (Parsons *et al.*, 1998), LPC has been shown to permeabilise the airway epithelial TJ's (Parsons *et al.*, 1999), presumably providing access of the viral vectors to the basolateral surface. However, it is still not known how LPC modulates the various airway barriers. Mucolytic properties of LPC could act to solubilise airway mucus by reducing its viscosity and elasticity (Martin *et al.*, 1978). LPC also potentially reduces MCC since it can reduce cilial beat (Merkus *et al.*, 1993). It has also been reported that LPC improves surrogate vector particle deposition onto the airway epithelium *in vivo* (Parsons *et al.*, 2000a).

While there are likely to be other biological effects of LPC (Prokazova *et al.*, 1998), the direct airway surface effects described above would each be expected to contribute to enhanced gene transfer *in vivo,* by improving retention and also delivery of viral vector particles to the apical and basolateral surfaces, respectively.

In this project the effects of polidocanol and LPC were studied in terms of their efficiency in improving AdV- and LV-mediated gene transfer *in vitro* (Chapters 4 and 5, respectively). These approaches were then adapted and applied to studies of LV-mediated gene transfer *in vivo* (Chapter 6).

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial Media and Bacterial Strains

Media:

LB broth	1% (weight (w)/volume (v)) Bacto-tryptone, 0.5% (w/v) Bacto-yeast extract, 0.5% (w/v) NaCl in deionised H_2O (d. H_2O)
LB-Agar	1.2% (w/v) Bacto-agar in LB broth
LB-Agar-amp	1.2% (w/v) Bacto-agar in LB broth, 100 μg/ml ampicillin
LB-Agar-cam	1.2% (w/v) Bacto-agar in LB broth, 20 μg/ml chloramphenicol

Selective antibiotics:

Ampicillin (amp)	Boehringer Mannheim, Germany, Cat. no. 835 269
Chloramphenicol (cam)	Sigma® Chemicals Co., USA, C-0378

Bacto-tryptone, Bacto-yeast extract and Bacto-agar were purchased from Difco, Michigan, USA.

All solutions were autoclaved to sterilise. When preparing agar plates, the agar solution was heated in a microwave to boiling point and cooled to 50 °C before adding selective antibiotics (amp or cam). The agar plates were stored at 4 °C until use.

***E. coli* strains:**

DH10B ElectroMAX	F^- *mcr*A Δ(*mrr-hsdRMS-mcrBC*) φ80d*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ^- *rps*L *nup*G GIBCO-BRL, USA
MC1061	F^- *araD139 Δ (ara-leu)7697 galE15 galK16 Δ (lac) X74 rpsL* (Str^r) *hsdR2* $(r_k^- m_k^+)$*mcrA mcrB1* Dr. D. S. Anson, Department of Chemical Pathology, Women's and Children's Hospital (WCH)

2.1.2 Enzymes

Alkaline phosphatase, calf intestine (CIP)	Boehringer Mannheim, Germany, Cat. no. 713 023
Restriction enzymes	New England Biolabs, USA; Boehringer Mannheim, Germany
Klenow fragment *E.coli* DNA polymerase I	New England Biolabs, USA
Taq DNA polymerase	QIAGEN, Germany, Cat. no. 201 205
T4 polynucleotide kinase	New England Biolabs, USA
T4 DNA ligase	Boehringer Mannheim, Germany, Cat. no. 709 557
DNase I, RNase-free	Boehringer Mannheim, Germany, Cat. no. 776 785

2.1.3 Electrophoresis

Agarose, DNA grade	Boehringer Mannheim, Germany, Cat. no. 1 816 594
Ethidium Bromide	Boehringer Mannheim, Germany, Cat. no. 200271
Formamide	BDH Laboratory supplies
10 x loading buffer	50% (v/v) glycerol, 1% (w/v) SDS, 100 mM EDTA, pH 8.0, 0.1% (w/v) Bromophenol Blue
Molecular size markers	SPP1/*EcoR*I and pUC19/*Hpa*II, BRESATEC, Australia (AU)
TBE buffer	89 mM Tris-base, 89 mM boric acid, 2 mM EDTA, pH 8.3

2.1.4 Preparation of Plasmid DNA

Qiagen 'Plasmid Midi-Kit' (100)	QIAGEN, Germany, Cat. no. 12143
LiCl-Triton X-100 lysis buffer	2.5 M LiCl, 50 mM Tris-HCl, pH 8.0, 62.5 mM EDTA, 4% (v/v) Triton X-100
TE buffer	10 mM Tris-HCl, pH 7.5, 1 mM EDTA

2.1.5 Tissue Culture Solutions and Materials

DMEM	Dulbecco's Modified Eagle's Medium, GIBCO-BRL, Cat. no. 12100-046

F12	Ham's F12 media, GIBCO-BRL, Cat. no. 21700-075
MEM	Modified Eagle's Medium, GIBCO-BRL, Cat. no. 61100-061
Medium-199	GIBCO-BRL, Cat. no. 31100-035

All media were reconstituted in Baxter H_2O for injection, adjusted to pH ~7.3 and filtered through a 0.2 µm filter to sterilise.

Fetal Calf Serum (FCS)	CSL Limited, AU
L-glutamine solution	CSL Limited, AU
Phosphate Buffered Saline (PBS) (without Ca^{2+} and Mg^{2+})	CSL Limited, AU
Trypsin-Versene solution	CSL Limited, AU
Penicillin-G/Streptomycin sulphate solution	CSL Limited, AU
Tissue culture flasks and dishes	CELLSTAR®, Greiner Bio-one GmbH, Germany; CORNING®, USA.

2.1.6 Cell Lines

The cell lines used in this project were obtained from the American Type Culture Collection (ATCC), Rockville, MD, USA unless otherwise stated.

A549	CCL-185
CFPAC-1	CRL-1918
MDCK	CCL-34
NIH3T3	CRL-1658
293	CRL-1573
293T	SD-3515
293T(MJ)	Provided from Prof. M. Jones, Michigan State University
CFBE41o$^-$	Dr. D. C. Gruenert, UCSF, San Francisco (provided with permission from Dr K. Foskett, Flinders Medical Centre, Adelaide, AU)
1HAE	"
16HBE	"

2.1.7 Air Liquid Interface Culture Media and Additives

Bovine Pituitaries	Richmond Pacific, Hastings, New Zealand
Bovine Pituitary Extract (BPE)	Sigma® Chemicals Co., USA, P-1167
Bovine Serum Albumin (BSA)	Sigma® Chemicals Co., USA, A-7638
Cholera toxin (CT)	Sigma® Chemicals Co., USA, C-3012
DNase	Sigma® Chemicals Co., USA, DN-25
Epidermal Growth Factor (EGF) (mouse)	Collaborative Research, USA, Cat. no. 40001
Ethanolamine	Sigma® Chemicals Co., USA, E-0135
EVOM™ epithelial ohmmeter	World Precision Instruments, Inc., Sarasota, Florida
High-protein medium formulation	DMEM:F12 (1:1), 10 µg/ml insulin, 0.1 µg/ml hydrocortisone, 0.1 µg/ml CT, 5 µg/ml transferrin, 50 µM phospho-ethanolamine, 80 µM ethanolamine, 25 ng/ml EGF, 1% (v/v) BPE, 30 mM HEPES, 0.1 mg/ml penicillin, 0.1 mg/ml streptomycin, 3 mg/ml BSA, 50 mM retinoic acid. The medium was adjusted to pH ~7.3
Actrapid® Human Insulin	Novo Nordisk®, Denmark
Collagen (rat tail)	Boehringer Mannheim, Germany, Cat no. 1 179 179
Hydrocortisone (HC)	Sigma® Chemicals Co., USA, H-0888
Low-protein medium formulation	DMEM:F12 (1:1), 10 µg/ml insulin, 0.1 µg/ml hydrocortisone, 0.1 µg/ml CT, 5 µg/ml transferrin, 50 µM phospho-ethanolamine, 80 µM ethanolamine, 25 ng/ml EGF, 1% (v/v) BPE, 30 mM HEPES, 0.1 mg/ml penicillin, 0.1 mg/ml streptomycin, 0.5 mg/ml BSA, 50 mM retinoic acid. The medium was adjusted to pH ~7.3
Phospho-ethanolamine (PE)	Sigma® Chemicals Co., USA, P-0503
Pronase	Boehringer Mannheim, Germany, Cat. no. 165 921
Protease XIV	Sigma® Chemicals Co., USA, P-5147
Retinoic acid (RA)	Sigma® Chemicals Co., USA, R-2625

Septum-medium formulation (Modified from Fannuchi *et al.*, 1999)	F12 media, 10 µg/ml insulin, 0.1 µg/ml HC, 5 µg/ml Transferrin, 25 ng/ml EGF, 1% (v/v) BPE, 30 mM HEPES, 0.1 mg/ml penicillin, 0.1 mg/ml streptomycin, 28 ng/ml RA. The medium was adjusted to pH ~7.3
Human Transferrin (Tf)	Collaborative Research, USA, Cat. no. 40204
12-well COL- and 12-, 24- well polycarbonate Transwell™ supports	CORNING®, USA

2.1.8 Transfection/Transduction of Mammalian Cell Cultures

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)	Molecular Probes, B-1690 (dissolved in N, N, dimethylformamide)
Conjugation buffer	0.1 M sodium carbonate buffer, pH ~9.3
DOTAP	Boehringer Mannheim, Germany, Cat. no. 1 811 177
pEYFP vector	CLONTECH Laboratories, Inc., USA, Cat. no. 6004-1
FluoroLink™Cy3™	Amersham Life Sciences, USA, Cat. no. PA23000
Fugene-6	Boehringer Mannheim, Germany, Cat. no. 1 815 091
HBS buffer	20 mM HEPES, 150 mM NaCl, pH ~7.4
HEPES	Sigma® Chemicals Co., USA, H-3375
2x HEPES-buffered saline solution (2x HeBS)	0.28 M NaCl, 0.05 M HEPES, 1.5 mM Na_2HPO_4, pH ~7.05
Magnesium Chloride hexahydrate	Sigma® Chemicals Co., USA, Cat. no. M-2670
N, N, dimethylformamide	Sigma® Chemicals Co., USA, Cat. no. D-8654
Lipofectamine	Life™ Technologies, USA
α-L-lysophosphatidylcholine Type I from egg yolk	Sigma® Chemicals Co., USA, Cat. no. L-4129
Polyoxyethylene-9-lauryl ether (polidocanol)	Sigma® Chemicals Co., USA, Cat. no. P-9641
Polybrene	Sigma® Chemicals Co., USA, Cat. no. P-4515
Potassium Ferricyanide ($K_3Fe(CN)_6$)	Sigma® Chemicals Co., USA, Cat. no. P-3667

Potassium Ferrocyanide ($K_4Fe(CN)_6$)	Sigma® Chemicals Co., USA, Cat. no. P-9387
Propidium iodide	Sigma® Chemicals Co., USA, Cat. no. P-4170
1% SDS Buffer	0.01 M Tris-HCl (pH 7.5), 0.01 M EDTA, 1% SDS
Trypan Blue	Sigma® Cell Culture Reagents, USA, T-6146
'Vybrant™ CFDA-SE Cell Tracer Kit'	Molecular Probes, USA, Cat. no. C-12883
'HIV-1 p24 ELISA Kit'	NEN™ Life Sciences, USA, NEK 050A

2.1.9 DNA Plasmids

pBluescript® II SK	Stratagene, USA
pBCKS	Stratagene, USA
pcDNA3.1 expression vector	Invitrogen, USA
pCMVCFTR-936C	Dr. R. J. Gregory, Genzyme Corporation
pCMVΔRnr	Prof I. Verma, Salk Institute (provided with permission from Dr. J. Fleming, New Children's Hospital, Sydney, AU)
pCMV-*rev*	NIH AIDS research and reference reagent program (cat. no. 1443)
pHCMV-G	Drs. T. Friedmann and J. K. Yee (University of California, San Diego)
pHR'CMVLacZ	Prof I. Verma, Salk Institute (provided with permission from Dr. J. Fleming, New Children's Hospital, Sydney, AU)

2.1.10 Lentivirus Vector Stock Production and Concentration

DIAFLO®Ultrafiltration ZM500 Membranes	Amicon, Inc., USA, Cat. no. 14522
Polyallomer centrifuge tubes (for the SW-60 rotor)	Beckman, USA, Cat. no. 342630
Ultrafiltration stirred cell apparatus	Amicon, Inc., USA

2.1.11 Adenoviral Vectors

AdV5CMVLacZ (Lot. no. 11333)	University of North Carolina (UNC) at Chapel Hill. Gene Therapy Vector Core Lab (a gift from Prof. R. C. Boucher)

2.1.12 Mice

C57Bl/6	Laboratory Animal Services, University of Adelaide
CF (*cftr*^{tm1Unc}) mice	Transgenic mice bred from stock purchased from the Jackson Labs (USA) in 1993. The CF colony was housed in the PC-2 facility of the WCH Animal House.

2.1.13 Fixing and Processing of Mouse Heads

DeCal Buffer	7% (v/v) HCl in 2% (w/w) EDTA in d. H_2O
Drawing ink, Design™ Higgins®	Eberhard Faber, Inc, USA
35% Formalin	Fronine, Australia
Glutaraldehyde, grade II (25%)	Sigma® Chemicals Co., USA, Cat. no. G-6257
Gurr Certistain® Eosin	Crown, AU
10% (v/v) Neutral Buffered Formalin (NBF)	10% (v/v) Formalin, 0.22 M NaH_2PO_4, 0.45 M Na_2HPO_4 in d. H_2O
Paraformaldehyde	Sigma® Chemicals Co., USA, Cat. no. P-6148
Surgipath® Haematoxylin	Surgipath Medical Industries, Inc, Canada
Histopathology cassettes Tissue-Tek® II (code: green)	Bayer Diagnostics, AU
Embedding wax (Tissue -Tek® wax III)	Bayer Diagnostics, AU
Carnoy's solution	6:3:1 absolute ethanol:chloroform:glacial acetic acid
DPX	distyrene-tricresyl-phosphate-xylene

2.1.14 Measurement of Mouse Nasal Potential Difference

Agar Bridges	A 3% (w/v) agarose (Type C) in 0.9% (w/v) NaCl solution was used to fill the bridge tubing, the gel loading tips and the 23g needles (used as reference electrodes)

Agarose, Type C	CALBIOCHEM®, USA
6-inch arterial pressure tubing	Sorenson Research, USA
Basal solution (Modified from Grubb *et al.*, 1994a to include HEPES buffer)	135 mM NaCl, 2.4 mM KH_2PO_4, 0.6 mM K_2HPO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES in boiled H_2O, pH 7.4 with NaOH. The solution was adjusted to pH ~7, then 0.2 μm filtered, and stored in collapsible drip bags to minimise ambient gas uptake and reduce bubble formation in the fine perfusion tubing
INDOPLAS™Luer lock extension tube	Maersk Indoplas Pty Ltd, AU
Hamilton syringes (1 ml)	Hamilton Instruments, NV, USA
Ilium xylazil-20 (xylamine)	Troy Laboratories Pty Ltd, AU
Low-chloride solution (Modified from Grubb *et al.*, 1994a to include HEPES buffer)	135 mM Na-gluconate, 2.4 mM KH_2PO_4, 0.6 mM K_2HPO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES in boiled H_2O, pH 7.4 with NaOH. The solution was adjusted to pH ~7, then 0.2 μm filtered, and stored in collapsible drip bags to minimise ambient gas uptake and reduce bubble formation in the fine perfusion tubing
Microinfusion pump (modified to accept 2 x 1 ml Hamilton glass syringes)	IVAC, Syringe Pump Model 770, IVAC Corporation
WPI Isomillivoltmeter	World Precision Instruments, Inc., Sarasota, Florida
Parnell Ketamine	Parnell Labs Pty Ltd, AU
Polyethylene TPD cannula	PE10, Intramedic, Becton-Dickinson (heat drawn)
0.9% Sodium Chloride solution for irrigation	Baxter, AU

2.1.15 PCR Primers

MCF-1:	**5'**	ACATTGGGAGACTTGTGATTGG	**3'**	GIBCO-BRL
MCF-2:	**5'**	CTGTAGTTGGCAAGCTTTGAC	**3'**	“
MCF-3:	**5'**	ACACTGCTCGAGGGCTAGCCTCTT	**3'**	“
Ext 4F:	**5'**	GGGTGCGAGAGCGTCAGTATTAG	**3'**	“
Ext 4R:	**5'**	CTTCTCTAAAGCTTCCTTGGTGTC	**3'**	“
LacZa:	**5'**	GGTAGCGAAAGCCATTTTTTGA	**3'**	“

LacZb:	5'	CGGTCGCGTTCGGTTGCACTAC	3'	"
LacZc:	5'	GGCAGTTCAGGCCAATCCGCGC	3'	"
MSD236:	5'	AGGCGAGGGGCGGCGACTGG	3'	GIBCO-BRL

Oligonucleotides were prepared at a 50 nmol scale and were desalted.

2.1.16 General Materials

BARASTOC mouse breeder cubes	Ridley AGRIPRODUCTS, AU
'BigDYE Terminator Cycle Sequencing Ready Reaction Kit'	PE Applied Biosystems
Chloroform	Ajax Finechem, AU
ColonLYTELY PEG balanced electrolyte gastrointestinal lavage solution	DENDY pharmaceuticals, AU
Colony-PlaqueScreen™ filters	DuPont NEN, Research Products, USA
Denhardts solution (1x)	0.02% (w/v) BSA fraction Type V, 0.02% (w/v) Ficoll Type 400, 0.02% (w/v) polyvinyl-pyrrolidone
di-sodium hydrogen orthophosphate (Na_2HPO_4)	Ajax Chemicals, AU
'DNeasy™ Tissue Kit'	Qiagen, Germany
Ethanol (EtOH)	Ajax Finechem, AU
'1st Strand cDNA Synthesis Kit' (for RT-PCR)	Boehringer Mannheim, Germany, Cat. no. 143 188
Formamide loading buffer	95% (v/v) formamide, 20 mM EDTA, pH 8.0, 0.1% (w/v) Bromophenol Blue
Formaldehyde	Ajax Finechem, AU
'Geneclean™ Kit'	BIO 101 Inc., USA, Cat. no. 1001-200
GeneScreen *Plus*™ Nylon Filters	DuPont NEN, Research Products, USA
Glycerol	Ajax Finechem, AU
Herring Sperm DNA	Boehringer Mannheim, Germany, Cat. no. 223 646
High Prime	Boehringer Mannheim, Germany, Cat. no. 1 585 592

Isopropanol	Ajax Finechem, AU, No. 1219
Isolute lipophilic column C18, 100 mg-1ml	Isolute, AU, Cat no. IST-220-0010-A
NA45 elution buffer	1 M NaCl, 0.05 M arginine (free-base)
NA45 membrane (DEAE)	Schleicher and Schuell, Germany
PCR nucleotide mix (dNTPs)	Boehringer Mannheim, Germany, Cat. no. 1 814 362
Phenol	Wako Chemicals Co., Japan
Polaroid land film 667 (ASA 300)	Kodak, AU
3-0 DYSILK suture	DYNEK Pty Ltd, South Australia
Slide-A-Lyzer®, 10K, Cassette	Pierce, USA, No. 66380
Sodium di-hydrogen orthophosphate ($NaH_2PO_42H_2O$)	Ajax Chemicals, AU
Titanium dioxide	Tronox® Kerr-McGee Pigments Ltd, Western AU
'QuickChange™Site-Directed Mutagenesis Kit'	Stratagene, USA, Cat. no. 200518
'RNAqueous™Phenol-Free Total RNA Isolation Kit'	Ambion®, USA, Cat. no. AM-1912
20 x SSC buffer	3 M NaCl, 0.3 M tri-sodium citrate
TfB buffer	50 mM $CaCl_2$ in PBS/10% (v/v) glycerol
'UltraClean™PCR Clean-Up Kit'	MoBio Laboratories, USA, Cat. no. 12500-100

2.2 Methods

2.2.1 Blunt Ending DNA Fragments

5' overhangs produced by restriction endonuclease digestion were made blunt by the addition of 1 mM dNTPs and 1 U of Klenow fragment of DNA polymerase I and incubation of the reaction at room temperature (RT) for 20 mins. The reaction was typically performed in the endonuclease digestion buffer after digestion was completed.

2.2.2 De-phosphorylation of DNA Ends

Vector constructs were de-phosphorylated after digestion to avoid re-circulisation during the ligation process. In the 100 µl restriction reaction, 5 U of alkaline phosphatase (CIP) were added and incubated for 1 hr at 37 °C. The vector constructs were then phenol/chloroform extracted and precipitated.

2.2.3 Agarose Gel Electrophoresis of DNA

DNA analysis was performed in 1 - 3% (w/v) agarose, 1 x TBE gels. At the completion of electrophoresis the gels were stained with ethidium bromide (0.5 µg/ml) for visualisation. The DNA was visualised by direct illumination with ultraviolet (UV) light and a positive photograph then taken using Polaroid land film 667.

2.2.4 Isolation of DNA from Agarose Gels

(a) Isolation of DNA Using the Geneclean™ Kit

The gel was visualized under UV light and the band of interest excised. The piece of gel containing the DNA was then transferred into a sterile eppendorf tube and the DNA extracted using the 'Geneclean™ Kit' (BIO 101 Inc., USA) according to the manufacturer's instructions.

(b) Electrophoresis onto the NA45 Membrane

This method was used for smaller DNA fragments (< 2 kb). After electrophoresis the gel was visualised and a piece of NA45 membrane inserted at the end of the fragment of interest. The gel was then rotated by 90 degrees and was subject to further electrophoresis, such that the fragment of interest migrated onto the membrane paper. The membrane was then removed from the gel and incubated in 100 µl of elution buffer at

70 °C for 30 mins. At the end of the incubation the elution buffer was transferred to a clean eppendorf tube, mixed with 2 volumes of TE buffer and precipitated with an equal volume of isopropanol.

2.2.5 DNA Ligations

All ligations were performed with 1 x ligation buffer (Boehringer Mannheim, Germany) and 1 μl T4 DNA ligase in a total reaction volume of 20 μl. Typically 10 - 30 ng of vector and 10 - 100 ng of insert were used (the amounts were quantified by minigel electrophoresis). Three ligation reactions were usually set up: a control (no insert), one with a 1:1 and one with a 1:3 ratio of vector to insert. The reactions were then incubated overnight (o/n) at RT (for blunt ends) or at 16 °C (for sticky ends).

2.2.6 Preparation of MC1061 Competent Cells

A single colony of MC1061 cells was inoculated into 100 ml of LB broth and grown o/n at 37 °C with shaking. One ml of the o/n culture was then used to inoculate 100 ml of fresh LB broth and incubated with agitation at 37 °C until an absorbance reading of ~0.2 at OD_{550} was obtained. The cells were then recovered by centrifugation at 2,500 x g for 10 mins at 4 °C and the pellet resuspended in 1 x volume of ice-cold TfB buffer and incubated on ice for 30 mins. The cells were then recovered by centrifugation and resuspended in one-tenth volume of ice-cold TfB buffer, aliquoted, snap-frozen in liquid N_2 and stored at minus 70 °C.

2.2.7 Transformation of Competent MC1061 Cells

One hundred μl of competent MC1061 cells (stored at minus 70 °C) was thawed on ice and mixed with 5 μl of the ligation reaction and incubated on ice for 10 mins. The cells were then heat shocked at 37 °C for 5 mins. An equal volume of LB broth was then added and the cells incubated at 37 °C for 30 mins. The cell suspension was then spread onto an agar plate containing either amp or cam, dried at 37 °C, inverted and incubated at 37 °C o/n.

2.2.8 Electroporation of DH10B ElectroMax Cells

A 25 μl aliquot of DH10B ElectroMax cells was thawed on ice. Immediately prior to electroporation the cells were mixed with 1 μl of the ligation reaction and transferred to a pre-cooled electroporation cuvette (BioRad) and electroporated in the BioRad electroporation apparatus (set at 1.8 kV, 200 Ohm). Immediately after the electroporation 1 ml of LB broth was added to the cuvette and the cells transferred to a 10 ml round bottom tube. The cells were then incubated at 37 °C for 1 hr with agitation. At the end of the 1 hr incubation, aliquots of 500, 100 and 10 µl were spread on agar plates containing either amp or cam, dried at 37 °C, inverted and incubated o/n at 37 °C.

2.2.9 Transfer of DNA onto Nylon Membranes

Transformations or electroporations were performed as previously described (section 2.2.7 and 2.2.8, respectively). The cell suspension was spread onto LB plates containing either amp or cam and incubated o/n at 37 °C. Colonies were transferred (by sterile toothpicks) onto duplicate plates with gridded nylon filters and further incubated at 37 °C. Ten to sixteen hrs later the membrane was removed from one plate and the duplicate plate stored at 4 °C. The membrane was then placed onto Whatman 3MM blotting paper saturated with 5% SDS, 2 x SSC and dried in a microwave for 3 mins at the highest setting. The filter was then washed in 4 x SSC buffer at RT on a shaker for 30 mins and all debris carefully removed by gentle rubbing.

2.2.10 DNA Plasmid Preparation

(a) Minipreps

Single colonies were inoculated into 1 ml of LB broth containing either amp or cam and grown for 6 - 16 hrs on a shaker at 37 °C. Five hundred µl of each cell suspension was pelleted by micro-centrifugation, the supernatant aspirated and the pellet resuspended in 150 µl of LiCl lysis buffer and the DNA phenol/chloroform extracted. The aqueous phase (DNA) was then precipitated with 0.6 volumes of isopropanol, the DNA pellet washed with 70% EtOH and resuspended in 0.5 x TE buffer.

(b) Large-Scale DNA Plasmid Preparations

Prepared using the Qiagen 'Plasmid Midi-Kit' (Qiagen, Germany) according to the manufacturer's instructions.

2.2.11 Northern Blot

A 1.2% (w/v) agarose, 1 x MOPS gel with formaldehyde (15% v/v) was prepared. The RNA was denatured by adding 40 µl of the RNA loading buffer to 5 - 10 µg of RNA and incubating the reaction at 65 °C for 10 mins. The samples were then quenched on ice and loaded into duplicate sets of wells (one set was used for hybridisation and the other used for ethidium bromide staining) and electrophoresed at 1 Volt/cm o/n. At the end of the electrophoresis the gel was photographed alongside a ruler to provide a scale for later analysis of the autoradiograph, and the gel then cut in two. One set of samples was stained to allow assessment of sample integrity and the use of the rRNA (28s, 18s) as size markers, and the other duplicate set of samples was used for transfer onto a nylon membrane. Blotting and subsequent hybridisation were performed as described in *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1989; volume 1, unit 4.9).

2.2.12 Pre-Hybridisation and Hybridisation[1]

The filters were soaked in 4 x SSC buffer and incubated in pre-hybridisation buffer (50% (w/v) formamide, 4 x SSC, 10% (w/v) dextran sulphate, 2 x Denhardts solution, 0.2% (w/v) SDS and 100 μg/ml Herring Sperm DNA), for a minimum of 2 hrs at 42 °C. The denatured, random primed radioactive probe was added into the pre-hybridisation buffer and the membrane hybridised o/n at 42 °C. The membrane was then removed from the hybridisation solution and rinsed with 4 x SSC buffer at RT for 30 mins, and washed twice in 0.2 x SSC, 0.1% (w/v) SDS with agitation at 65 °C for 1 hr. The level of background signal was monitored with a Geiger-Muller counter and washing was repeated until excess signal was removed. The membrane was then blotted dry with Whatman 3MM paper, sealed in plastic and exposed to film at either RT or at minus 70 °C.

2.2.13 Random Primed Radioactive Labelling of DNA

Random primed radioactive labelling of DNA was performed using the High Prime reagent (Boehringer Mannheim, Germany) according to the manufacturer's instructions. Briefly, 100 ng of DNA was denatured at 95 °C and quenched on ice. Four μl of High Prime was then added to the reaction followed by 1 μl [$\alpha^{32}P$] dCTP. The reaction was incubated at 37 °C for 1 hr and terminated by the addition of 100 μl of 2.5 mM EDTA. The radiolabelled probe was then denatured at 95 °C for 3 mins, quenched on ice and added to the pre-hybridisation buffer.

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[1] For colony filters and northern blots

2.2.14 Isolation of Cytoplasmic RNA from Cells

Confluent 60-mm culture dishes of cells were harvested with PBS/0.01% (v/v) trypsin, 0.002% (v/v) versene solution. The trypsin was then neutralised by the addition of PBS/1% (v/v) FCS and the cells recovered by centrifugation at 500 x g for 5 min at RT. The supernatant was then aspirated and the cell pellet washed once with PBS and re-pelleted by centrifugation. The cell pellet was resuspended in 100 µl of 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 0.5% (v/v) NP40 and incubated on ice for 5 mins, and then centrifuged at maximum speed for 2 mins at 4 °C to pellet the nuclei.

Cytoplasmic RNA was isolated from the supernatant using the RNA isolation kit (Ambion®, USA) according to the manufacturer's instructions.

2.2.15 cDNA Synthesis

cDNA was prepared using the '1st Strand cDNA Synthesis Kit' (Boehringer Mannheim, Germany) for RT-PCR according to the manufacturer's instructions using total cytoplasmic RNA as the template.

2.2.16 Polymerase Chain Reaction (PCR) Analysis

All PCR reaction mixes were prepared in a dedicated pre-PCR room and the template added outside the pre-PCR room.

(a) For Cystic Fibrosis Mouse Confirmation

Confirmation of homozygote CF mice was based on two criteria, the presence of white dorsal incisors (a characteristic of CF mice; Wright *et al.*, 1996) 2 weeks after weaning, and PCR analysis. For PCR analysis a 2 mm piece of tail was removed 2 weeks after weaning. The piece of tail was transferred to a PCR tube and the DNA extracted using the 'DNeasy™ Tissue Kit' (Qiagen, Germany), according to manufacturer's recommended protocol. The DNA template (300 ng) was amplified in a 100 µl reaction in the presence of 1 x Taq buffer (Qiagen, Germany), 2.5 mM $MgCl_2$, 2 x Q buffer (Qiagen, Germany), 200 µM dNTPs, 125 ng of MCF-1 primer (section 2.1.15), 75 ng of MCF-2 primer (section 2.1.15), 200 ng of MCF-3 primer (section 2.1.15) and 1.5 U of *Taq* (Qiagen, Germany). The PCR reactions were performed in a Perkin Elmer Cetus DNA thermal cycler with tube control. The reaction was heated initially to 95 °C for 5 mins, followed by 35 cycles of: denaturation at 95 °C for 30 seconds (sec), annealing at 65 °C for 30 sec and extension at 72 °C for 30 sec. The last cycle was an additional extension at 72 °C for 3 mins.

The size of the PCR product for the normal CFTR allele is 277 bp and for the knockout allele ~220 bp.

(b) For Estimation of the LVCFTR Vector Titre

The primers Ext 4F and Ext 4R (section 2.1.15) were designed to amplify a 306 base portion (HIV-1 YU-2, Li *et al.*, 1992; bases 803-1109, GenBank accession number M93258) of the *gag* gene sequence in the LVCFTR vector.

DNA was prepared from NIH3T3 cells transduced with either 20 µl of LVCFTR vector (of unknown titre) or 20 µl of LVEYFP (containing 1 x 10^4 NIH3T3-TU) (as described in section 2.2.25) using the 'DNeasy™ Tissue Kit' (QIAGEN, Germany), according to the manufacturer's instructions.

Serial dilutions containing 1 µg, 100 and 10 ng of DNA prepared from cells transduced with either the LVCFTR vector or the LVEYFP vector were amplified in a 50 µl reaction with 5 U of *Taq* (Qiagen, Germany) in the presence of 1 x Taq buffer (Qiagen, Germany), 2.5 mM $MgCl_2$, 1 x Q buffer (Qiagen, Germany), 200 µM dNTPs and 1 µg of each of the primers Ext 4F and Ext 4R. The reaction was heated initially to 94 °C for 3 mins, followed by 35 cycles of: denaturation at 94 °C for 30 sec, annealing at 60 °C for 60 sec and extension at 72 °C for 30 sec. The last cycle was a further extension at 72 °C for 3 mins. PCR products were then analysed by agarose gel electrophoresis and the titre calculated by comparing the amount of product from the dilutions of the experimental sample (LVCFTR transduced cells) with that from the dilutions series of the sample of known titre (LVEYFP transduced cells).

(c) Reverse-Transcription PCR analysis of the LV Vector Constructs

The cDNA template (1 µg) was amplified in a 50 µl reaction in the presence of 1 x Taq buffer (Qiagen, Germany), 1 x Q buffer (Qiagen, Germany), 200 µM dNTPs and 300 ng of each primer (LacZa, LacZb, LacZc and MSD236; section 2.1.15) with 2.5 U of *Taq* (Qiagen, Germany). The reaction was heated initially to 94 °C for 3 mins, followed by 35 cycles of: denaturation at 94 °C for 45 sec, annealing at 55 °C for 60 sec and extension at 72 °C for 60 sec. The last cycle was a further extension at 72 °C for 4 mins.

LacZa, LacZb and LacZc represent antisense primers corresponding to the compliment of bases 1559 - 1580, 2098 - 2119 and 2638 - 2659 in the LacZ sequence

(GenBank accession number V00296), respectively. The MSD236 represents a sense primer to bases 684 - 704 in the HIV-1 YU-2 sequence (GenBank accession number M93258).

2.2.17 Purification of PCR Samples Prior to Sequencing

Prior to sequencing all PCR samples were purified using the 'Ultraclean PCR Clean-Up Kit' (MoBio Laboratories, USA) according to the manufacture's recommended protocol.

2.2.18 DNA Sequencing

DNA was sequenced using the 'BigDYE Terminator Cycle Sequencing Ready Reaction Kit' (PE applied Biosystems) according to the manufacturer's instructions. Approximately 300 ng of PCR product or 0.5 - 1 µg of plasmid was used as template with 4 µl of sequencing mix and 25 ng of primer in a total reaction volume of 20 µl. The sequencing reaction consisted of 25 cycles of: denaturation at 96 °C for 20 sec, annealing at 50 °C for 20 sec and extension at 60 °C for 3 mins. The products were then precipitated by the addition of 80 µl of 75% (v/v) isopropanol followed by incubation at RT for 15 mins in the dark. The sample was then centrifuged at max speed for 20 mins, the supernatant aspirated and the pellet washed with 250 µl of 75% isopropanol and briefly vortexed. After centrifuging at max speed for 5 mins, the supernatant was aspirated and the pellet air-dried. The PCR product was then sent for analysis to the Sequencing Centre of the Institute of Medical and Veterinary Science (Adelaide, AU).

2.2.19 Tissue Culture

Immortalised cells were sub-cultured when they reached confluence. Briefly, the growth media was aspirated and the cells washed twice with PBS. The cells were released from the substratum with either 0.1% (v/v) trypsin/0.02% (v/v) versene solution or PBS/0.01% (v/v) trypsin/0.002% (v/v) versene solution and dislodged by gently tapping the flask. The cells were harvested and recovered by centrifugation at 500 x g for 5 mins. The supernatant was then aspirated and the cell pellet re-suspended in growth media and sub-cultured, as desired, into new flasks. The cells were maintained at 37 °C in humidified air with 5% CO_2 unless otherwise stated. The number of passages was monitored and the cells were assayed for Mycoplasma contamination by Hoechst staining every 3 - 6 months and also immediately prior to freezing. No Mycoplasma was ever detected.

2.2.20 Cell Viability Assay

The Trypan Blue assay was used to determine the proportion of viable and non-viable cells in a whole cell population. Typically, viable cells appear clear when stained with the Trypan Blue solution, whereas non-viable cells will take up Trypan Blue and stain blue.

The cells were harvested as described in section 2.2.19 and a sample of cell suspension was mixed with an equal volume of Trypan Blue solution (diluted 1 in 10 in a 0.9% NaCl solution). The cells were then placed on a haemocytometer and the clear and blue cells counted in all squares as described in *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1989; volume 3, unit 11.5). The total number of cells was then calculated by multiplying the number of cells counted by 10^4 and then multiplied by 2 (the dilution factor for Trypan Blue). The percentage of viable cells was calculated using the following formula:

% Viable cells= number of viable cells/total number of cells x 100

2.2.21 Small-Scale Propagation of Adenovirus Vector Stocks

T-25 tissue cultures flasks (Costar) were seeded with 293 or 293T cells in DMEM/10% (v/v) FCS. The cells were allowed to reach 80% confluence, then one flask was harvested and the number of cells counted to estimate the amount of adenovirus (AdV) vector preparation to be added per flask to ensure a multiplicity of infection (MOI) of 10. The remaining flasks were then infected with an AdV vector preparation in 0.5 ml of DMEM/serum free (SF) using an MOI of 10. Typically, the number of cells per flask was ~3.5 x 10^6, so 3.5 x 10^7 LacZ forming units (lfu) (MOI= 10) was added in 0.5 ml of fresh DMEM/SF and the cells incubated for 1 hr at 37 °C. Five ml of DMEM/20% (v/v) FCS (37 °C) was then added to each flask and incubation continued at 37 °C for up to 48 hrs. The cells were harvested by gently tapping the flask, and then recovered by centrifugation for 10 mins at 800 x g at 4 °C. The supernatant was aspirated and the cell pellet re-suspended in 0.5 ml of 10% (v/v) glycerol in PBS. The cells were then lysed by three cycles of freeze-thaw (in liquid N_2-37 °C water bath, respectively). The cell lysate was then centrifuged for 25 mins at 6,000 x g and the supernatant (crude virus lysate) stored at minus 70 °C.

2.2.22 Determination of Adenovirus Vector Titre

293 cells were seeded in six-well plates at 6 x 10^5 cells per well in 2 ml of medium. Twenty-four hrs later the medium was aspirated and the cells exposed to serial dilutions of the AdV5CMVLacZ vector in SF medium (total volume 1 ml) for 2 hrs. FCS was then

added to a final concentration of 10% (v/v) and the cells incubated for an additional 18 hrs. The medium was then aspirated and the cells fixed with 1 ml of 0.5% glutaraldehyde in PBS for 10 mins at RT. The cell layer was then washed twice with 1 mM $MgCl_2$ in PBS for 15 mins at RT before being exposed to 1 ml of X-gal solution (1 mg/ml X-gal, 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$ and 5 mM $K_4Fe(CN)_6$ in PBS) for 3 - 5 hrs at 37 °C. The X-gal solution was aspirated when staining had reached sufficient intensity to allow easy numeration of LacZ positive cells. The number of LacZ positive cells in a selected surface area of 1.25 cm^2 (five 0.25 cm^2 squares) was counted in each 35 mm well (area= 9.4 cm^2). The titre of the AdV vector was calculated using the following formula:

Number of LacZ positive cells x dilution factor x total area (cm^2)/selected area (cm^2)= Ifu/ml

2.2.23 Conjugation of AdV5CMVLacZ to Cy3

Based on the protocol described by Leopold and colleagues (1998) an aliquot of the AdV5CMVLacZ vector preparation was thawed and the number of viral particles estimated by diluting 1 µl of the AdV5CMVLacZ vector in 700 µl of 1% SDS buffer and measuring its absorbance at 260 nm (OD_{260}= 1= 10^{12} particles/ml). For conjugation to the Cy3 dye ~1 - 5 x 10^{12} AdV vector particles in a total volume of 1 ml were used. One ml of AdV5CMVLacZ was transferred to a Slide-A-Lyzer® Cassette and dialysed against 4 changes of 10% (v/v) glycerol in PBS at 4 °C to remove the AdV vector storage buffer, as the presence of Tris inhibits the conjugation reaction (Dr R. J. Pickles, UNC at Chapel Hill, personal communication).

The dialysed AdV5CMVLacZ vector was then removed from the dialysis cassette and kept on ice. The Cy3 dye was reconstituted in 1 ml of conjugation buffer and 200 µl of Cy3 dye then incubated with the dialysed AdV5CMVLacZ for 2 hrs at RT in the dark. The AdV5CMVLacZ/Cy3 vector complexes were then dialysed against four changes of storage buffer (10% (v/v) glycerol, 10 mM Tris-HCl, 140 mM NaCl, 1 mM $MgCl_2$, pH 8.0) in the dark at 4 °C. The AdV5CMVLacZ/Cy3 (AdV/Cy3) vector preparation was then removed from the dialysis cassette and stored as 20 µl aliquots at minus 20 °C.

2.2.24 Introduction of Plasmid DNA into Mammalian Cells

(a) $CaPO_4$ Co-Precipitation (for Large-Scale Lentivirus Vector Stock Production)

293T cells were grown in T-75 flasks until confluent and were subcultured 1:3 2 days prior to transfection. Once the cells reached confluence they were harvested and seeded onto 100-mm culture dishes using 4.5 x 10^6 cells in 10 ml of DMEM/10% (v/v) FCS per dish for 16 hrs. The medium was then aspirated and the cells were fed with 10 ml of DMEM/10% (v/v) FCS. One hr later the cells were transfected with a mix of five different plasmids including 14 μg of the LV vector plasmid, 3 μg of pcDNA3gagpolml (Fuller and Anson, 2001), 0.2 μg of pcDNA3Tat101ml (Anson, unpublished), 14 μg of pHCMV-G (Yee *et al.*, 1994) and 4 μg of pCMV-*rev* (Lewis *et al.*, 1990). The DNA was made up to a volume of 540 μl with sterile H_2O before adding 60 μl of 2.5 M $CaCl_2$. The DNA-$CaCl_2$ solution was added drop-wise to an equal volume of 2 x HeBS buffer whilst vortexing. Once all the DNA-$CaCl_2$ solution was added, the tube was vortexed for 1 min and the solution immediately added drop-wise to the cells, mixed by swirling and maintained in culture o/n. Following the o/n incubation the transfection medium was removed and 14 ml of DMEM/10% (v/v) FCS was added to the cells. The LV vector supernatant was collected 24 hrs later, filtered through a 0.2 μm filter to remove cell debris and stored on ice prior to concentration.

(b) DOTAP Transfection

293T cells were grown in T-75 flasks until confluent and were subcultured 1:3 2 days prior to transfection. Cells were then harvested and 60-mm culture dishes seeded with 1.8 x 10^6 cells in 3 ml of DMEM/10% (v/v) FCS for 16 hrs. The medium was then aspirated and the cells fed with 7 ml of DMEM/10% (v/v) FCS and transfected as follows. Thirty μl of DOTAP was added to 90 μl of HBS buffer (solution A), and 6 μg of DNA was added to 114 μl of HBS buffer (solution B). Solutions A and B were then mixed and incubated at RT for 15 mins. The DOTAP/DNA complexes were then added drop-wise to the cells and the cells were then maintained in culture o/n. The transfection medium was then removed and replaced with 4 ml of DMEM/10% (v/v) FCS and incubation continued for further 24 hrs. The LV vector supernatant was then collected, filtered through a 0.2 μm filter to remove cell debris and stored on ice prior to concentration.

(c) Lipofectamine Transfection

293T cells were seeded in 60-mm culture dishes prior to transfection as described in section (b). Sixteen hrs later the medium was aspirated and the cells fed with 7 ml of DMEM/10% (v/v) FCS and transfected as follows. Briefly, 20 μl of Lipofectamine was added to 300 μl of DMEM/SF (solution A), and 6 μg DNA added to 300 μl of DMEM/SF (solution B). Solutions A and B were then mixed and incubated at RT for 45 mins. The

Lipofectamine/DNA complexes were then added drop-wise to the cells and maintained in culture o/n. The transfection medium was then removed and replaced with 4 ml of DMEM/10% (v/v) FCS and incubation continued for a further 24 hrs. The LV vector supernatant was then collected, filtered through a 0.2 μm filter to remove cell debris and stored on ice prior to concentration.

(d) Fugene-6 Transfection

293T cells were seeded in 60-mm culture dishes prior to transfection as described in section (b). Sixteen hrs later the medium was aspirated and the cells fed with 7 ml of DMEM/10% (v/v) FCS and transfected as follows. Briefly, 15 μl of Fugene-6 was added to 85 μl of DMEM/SF and incubated for 5 mins at RT. Six μg of DNA was then added drop-wise to Fugene-6 and incubated for a further 15 mins at RT. The Fugene-6/DNA complexes were then added drop-wise to the cells and incubated for 16 hrs. Following the 16 hr incubation the transfection medium was removed and replaced with 4 ml of DMEM/10% (v/v) FCS and incubation continued for a further 24 hrs. The LV vector supernatant was then collected, filtered through a 0.2 μm filter to remove cell debris and stored on ice prior to concentration.

2.2.25 Lentivirus-Mediated Cell Transduction

NIH3T3 or A549 cells were seeded in 6-well tissue culture plates at 1.2 x 10^6 cells per well in 2 ml of DMEM/10% (v/v) FCS. A549 cells were used because they give 10 - 20-fold higher titres than NIH3T3 cells, therefore allowing easier quantification of the viral titre. Six hrs later the medium was aspirated and the cells were transduced with the LV vector supernatant in a total volume of 2 ml per well in the presence of 4 μg/ml polybrene. Sixteen hours later, the cells were split 1:10 and maintained in culture. After 3 days the cells were assayed for marker gene expression. Briefly, the cells were fixed and either stained for LacZ gene expression (described in section 2.2.27) or harvested for FACS analysis (described in section 2.2.28).

2.2.26 Concentration of the Lentivirus Vector Supernatant

The LV vector supernatant was initially concentrated by ~10-fold by ultra-filtration in a 50 ml ultra-filtration Amicon stirred cell apparatus using a DIAFLO®Ultrafiltration ZM500 membrane (500K weight cut off) at 4 °C. The concentrated LV vector stock was then filtered through a 0.2 μm filter to sterilise, transferred into polyallomer centrifuge tubes and concentrated further by ~100-fold by ultra-centrifugation at 30,000 x rpm for 95 mins at 4 °C in a Beckman SW-60 rotor. The resulting pellet was typically re-suspended in 200 - 300 µl of PBS (1/1000 of the starting LV vector supernatant volume) and stored as 45 µl aliquots at minus 70 °C.

For determination of the viral titre of the LV vector stock, A549 or NIH3T3 cells were transduced with 1 µl of the concentrated LV vector in the presence of 4 µg/ml of polybrene, as described in section 2.2.25. Seven to ten days later the cells were assayed for gene expression using (i) the X-gal assay (if the cells were transduced with LVLacZ), (ii) FACS analysis (if the cells were transduced with LVEYFP) or (iii) PCR quantitative analysis (if the cells were transduced with LVCFTR).

2.2.27 X-gal Assay for LacZ Gene Expression

NIH3T3 cells grown on 6-well tissue culture plates were transduced with the LVLacZ vector as described previously (section 2.2.25). Seven days later the cells were washed twice with 1 mM $MgCl_2$ in PBS for 15 mins at RT before being exposed to 1 ml of X-gal solution for 16 hrs at 37 °C to detect LacZ gene expression. The X-gal solution was then aspirated and for each well the number of LacZ positive cells in a surface area of 0.75 cm^2 (three 0.25 cm^2 squares) was determined (as described in section 2.2.22).

2.2.28 Fluorescence Activated Cell Sorting (FACScan) Analysis

The LVEYFP transduced cells were harvested and pelleted as described in section 2.2.19. The cell pellet was then washed with PBS, the cells recovered by centrifugation and the supernatant discarded. The cells were resuspended in 190 µl of PBS and mixed with 10 µl of 10 µg/ml propidium iodide (PI) immediately prior to FACScan analysis. Data acquisition and subsequent analysis was performed by using the Cellquest version 3.0 software (Becton Dickinson). The non-viable cells were excluded by propidium iodide staining and by forward and side scatter properties. In all experiments a negative control of LVLacZ transduced cells was used to monitor the auto-fluorescence of cells. A fluorescence background of 1% was generally assumed for all samples (Fuller and Anson, 2001) and this value was deducted from all EYFP positive readings. In order to calculate the titre of the LVEYFP vector, the number of transduced cells (1.2 x 10^6 cells were typically seeded in a well of a six-well tissue culture plate) was multiplied by the percent of EYFP positive cells and adjusted according to the volume of the LV vector stock assayed.

2.2.29 Assay for Detection of Helper Virus

In order to monitor for replication competent retrovirus (RCR) the p24 levels present in the medium of LV-transduced A549 cells were monitored. Briefly, A549 cells were transduced with a sample of the concentrated LV vector stock (LVLacZa, LVLacZb, LVLacZc or LVCFTR), as described in section 2.2.25 and maintained in culture for 28 days. Twice a week a 1 ml sample from a confluent culture was collected and stored at minus 70 °C until all samples were collected. The medium from non-transduced A549 cells was also collected and used as a negative control. The samples were assayed for p24 using the 'HIV-1 p24 ELISA Kit' (NEN Life Sciences, USA) according to the manufacturer's instructions.

2.2.30 Detergent Treatment of Cells prior to Gene Transfer

All cell lines were seeded onto polycarbonate 24-well Transwell supports at 1.5 x 10^5 cells per support in 500 µl of growth media. The cells were allowed to reach confluence (~3 days) and the transepithelial resistance measured using the EVOM™ epithelial ohmmeter. One probe was inserted into the bottom reservoir and the other in the (upper) Transwell support taking care not to damage the membrane of the Transwell support. The medium was then aspirated and the cells were pre-treated with either polidocanol or LPC (both prepared in PBS) diluted in growth media. The control was PBS (as the detergent stocks were prepared in PBS) diluted in growth media. After the appropriate interval of treatment, the detergent solution was aspirated and the cells washed twice with 500 µl of DMEM/10% (v/v) FCS (37 °C).

One hr later the cells were infected with either the LV or the AdV vector preparations. One set of cells was transduced with the LVLacZ vector preparation in 0.5 ml of DMEM/10% (v/v) FCS in the presence of 4 µg/ml polybrene for 18 hrs. The other set of cells was infected with the AdV5CMVLacZ vector (MOI= 10) in 200 µl of DMEM/SF for 1 hr, and following the addition of 20 µl of FCS the incubation continued for a further 17 hrs. Both sets of cells were fixed and stained with X-gal as previously described (section 2.2.27 and 2.2.22, respectively), to reveal LacZ gene expression.

2.2.31 Air Liquid Interface Cultures of Rat Tracheal Epithelial Cells

(a) Harvesting and Dissociation of Tracheal Epithelial Cells

Female rats of two different strains (Sprague Dawley or Hooded Whistar) were used. Briefly, rats aged between 6 - 12 weeks were sacrificed by CO_2 asphyxiation and the heart bled transdiaphragmatically. The trachea was then immediately exposed and excised as described by Kaartinen and colleagues (1993). A cannula with a non-slip tip was made by slightly flaring the end of PE190 tubing on a flame heated scalpel blade and this cannula was then securely tied onto the uppermost tracheal ring. To provide distal access close to the carina the trachea was cut between the cartilage rings and a 2 cm piece of PE190 tubing with a flared, but sealed, end inserted loosely into the trachea. The trachea was first flushed through with 1% pronase solution (PBS/1% (w/v) pronase) and then filled with the 1% pronase solution, all *via* the proximal cannula. The distal tube was then tied in place using a 3-0 silk suture. The proximal tubing was then doubled over and clamped with a bulldog clip, which helped to seal and slightly inflate the trachea. The trachea was then placed in a 50 ml tube filled with F12 media (supplemented with penicillin and streptomycin) and incubated at 4 °C o/n.

(b) Isolation and Culture of Tracheal Epithelial Cells

Following the 16 hr incubation the bulldog clip was removed from the proximal tubing and the sealed cannula cut open. The trachea was flushed with 2 ml of F12/5% (v/v) FCS to recover the cells and to also terminate the digestion reaction. The cell suspension was filtered through a 50 μm filter and the cells recovered by centrifugation at 600 x g for 10 mins at 10 °C. The supernatant was then removed and the pellet resuspended in 3 ml of F12 media, which contained 30 mg BSA and 1.5 mg DNaseI, re-filtered through a 50 μm filter and incubated on ice for 5 mins. The cells were recovered by centrifugation at 600 x g for 10 mins at 10 °C and the cells re-suspended in 2 ml of high-protein media (section 2.1.7). The cell number and viability was determined by the Trypan Blue exclusion assay (section 2.2.20). Typically 7×10^5 - 1×10^6 cells per trachea were recovered. Each collagen-coated Transwell support of a 12-well plate was seeded with 6×10^4 cells in 500 μl of high-protein media. In the bottom reservoir 1 ml of high-protein media/5% (v/v) FCS was added. The presence of FCS in the bottom reservoir establishes a physical gradient that greatly favours attachment, survival and growth of the airway epithelial cells (Dr S. H. Randell, UNC at Chapel Hill, personal communication). The cells were maintained in humidified air at 35 °C with 3% CO_2 for 24 hrs. The medium was then aspirated from the Transwell support and the bottom reservoir, and low-protein media was added to both compartments. The medium in both the Transwell support and bottom reservoir was replenished every 2 days for the duration of the culture.

2.2.32 Air Liquid Interface Cultures of Explanted Rat Nasal Septa

Rats were killed by CO_2 asphyxiation and the heart bled transdiaphragmatically. The heads were then removed from the carcass and the septum excised based on the method described by Fannuchi.and colleagues (1999). Briefly, after removal of the skin, the thin bone structure of the dorsal region of the nose was cut free and peeled back using fine scissors. The anterior nasal septum, located approximately between the anterior surface of the incisor teeth and the more posterior incisive papilla, was excised and washed with 0.9% NaCl solution to remove any blood. The excess cartilage present around the rat septum was removed and cilial beat confirmed by viewing under a dissecting microscope using reflected light from a fiber light unit. The fiber light was adjusted to shine at a low-medium angle to illuminate the cilia 'rippling' the fluid on the exposed surface of the septum. Each septum was placed onto a polycarbonate 12-well Transwell support and a drop of septum-medium (enough to keep the septum moist) was added onto the septum surface and 350 µl of septum-medium added to the bottom reservoir (enough to keep the membrane support moist). The septum was maintained in humidified air at 35 °C with 3% CO_2 for up to 14 days. The growth media was replaced with fresh septum-medium every 24 hrs and cilial beat monitored every 3 days.

2.2.33 Instillation of Solutions into the Mouse Nasal Cavity

C57Bl/6 and *cftr*^{tm1Unc} mice were used under the approval of the Animal Ethics Committees of both the WCH and the University of Adelaide.

C57Bl/6 mice (6 - 7 weeks of age) or homozygote *cftr*^{tm1Unc} mice (8 - 20 weeks of age) were weighed and their tails colour-coded for blinding purposes. The mice were then anaesthetised intramuscularly (i. m.) with 1 µl/g body weight for the C57Bl/6 mice, and 0.7 µl/g body weight for the *cftr*^{tm1Unc} mice, of a 3:2 mixture of xylazil (20 mg/ml): ketamine (100 mg/ml). Body temperature was maintained during anaesthesia with a heat pad or lamp, and during the recovery period the mice were placed in a 35 °C air chamber. For dosing, mice were suspended from their dorsal incisors (hind quarters supported) and pre-treatment solutions delivered as a bolus using a gel-loading tip (Finnpippette) into the right nostril. Typically 30 mins after the initial anaesthetic dose, mice were re-anaesthetised with half the starting anaesthetic dose. One hr after the detergent pre-treatment, the LV vector (or the appropriate control solution) was instilled as two 10 µl aliquots over 2 - 3 mins. The mice were monitored for respiratory distress and any loss of treatment solution was noted. Mice were weighed daily for 7 days and observed for signs of distress over the duration of the experiment.

2.2.34 Processing of the Mouse Head for LacZ Gene Expression

At the designated time points mice were sacrificed by CO_2 asphyxiation. The heart was bled transdiaphragmatically to minimise the amount of blood to the head vasculature as well as blood spillage into the airway lumen, as both interfere with X-gal staining (Johnson *et al.*, 2000). The head was then separated from the carcass and the skin and eyes subsequently removed. The soft portion of the nose tip was then snipped off and the head flushed immediately with 2% PFA/0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) *via* the tracheal remnant and kept on ice for 2 hrs. The head was then washed twice with 1 mM $MgCl_2$ in PBS for 15 mins and then exposed to the X-gal solution for 6 - 8 hrs in the dark with intermittent flushing of the nasal cavity though the tracheal remnant. At the end of the incubation the head was rinsed with H_2O for 15 mins and then fixed with 10% (v/v) NBF for 24 hrs. The head was then decalcified in DeCal solution for 22 hrs, washed under running H_2O for 30 mins and stored at RT in 70% EtOH.

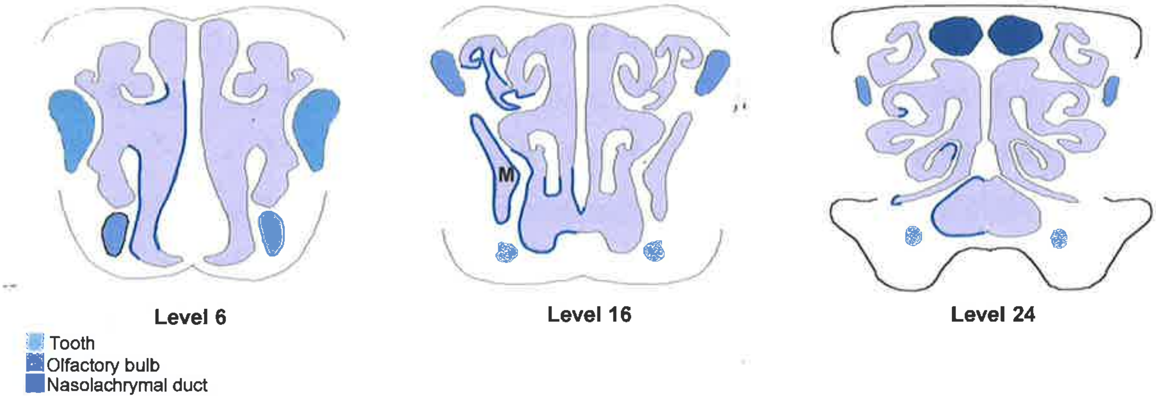

Figure 2.1: Diagrams of the Cross-Sections of the Murine Nasal Airways.

Three cross-sections were taken at Level 6: immediately posterior to the dorsal incisor, level 16: where the two nasal airways coalesce into the nasopharyngeal duct and Level 24: at the rear of the head. The bolded areas indicate the approximate presence of respiratory epithelium (only left side is noted). M: maxillary sinus. *Adapted from Mery et al., 1994.*

The jaw and tongue were removed and the right side marked with a strip of black ink, which was made permanent by dabbing the marked head region in Carnoy's fixative solution. The softened heads were sectioned with disposable microtome blades in three standard sections as previously described (Parsons *et al.*, 1998). These sections were taken at; Level 6: immediately posterior to the dorsal incisor, Level 16: where the two nasal airways coalesce into the nasopharyngeal duct, and Level 24: at the rear of the head (Mery *et al.*, 1994) (Figure 2.1).

The 3 cross-sections were placed in sectioning cassettes, anterior face down, and embedded in wax. The number of LacZ positive cells was counted in all 3 standard cross-sections counterstained with Safranin (Saf-O), whilst the types of LacZ positive cells in respiratory and transitional epithelium were determined in the standard cross-sections counterstained with H/E (Parsons *et al.*, 1998).

2.2.35 Safranin-O and Haematoxylin/Eosin Histological Staining

Saf-O counterstains the whole tissue non-specifically, with the contrast produced allowing for the number of LacZ positive cells to be easily counted. Haematoxylin stains nucleic acids while eosin stains both the cytoplasm and the extracellular organelles. The combination of both these stains allows for the identification of the type of LacZ positive cells.

Typically, the wax tissue sections were hydrated in water and counterstained lightly with either Saf-O for 15 seconds or haematoxylin (H) for 2 mins and then washed with water. The H-stained sections were then exposed to Li_2CO_3, washed with water, then stained with eosin (E) for 2 mins and again washed with water. Both the Saf-O and H/E stained sections were dehydrated through alcohols and cover-slipped using DPX.

2.2.36 The *cftr*^{tm1Unc} Mouse Colony

The *cftr*^{tm1Unc} transgenic mouse was generated by Snouwaert and colleagues in 1992 at UNC, Chapel Hill. The CF colony that began in 1993 in the WCH was established with 3 breeder pairs purchased from the Jackson Labs (USA) and is currently housed in the PC-2 facility of the Animal House in the WCH. Breeder cages are comprised of a fertile homozygote CF male and a heterozygote CF female, resulting in a nominal 75% probability of a homozygote CF mouse birth. New breeder pairs were set up when the numbers of mice born were low (1 - 2 per litter) or unexpectedly high numbers of newborn mortalities (> 60%) were observed. The CF mouse colony was fed with standard mouse breeder cubes and given 0.045% (w/v) ColonLytely in their drinking H_2O to prevent the fatal obstructive gut disease associated with the homozygote CF phenotype. Homozygote CF mice were positively identified by tail PCR (described in section 2.2.16

(a) and/or by the distinctive white colour of their dorsal incisors (Wright *et al.*, 1996) appearing 2 - 4 weeks after weaning (heterozygote CF mice have yellow incisors). In our hands, as well as in other laboratories, this characteristic has proven to be as accurate as PCR analysis for the determination of CF genotype and this has also been shown in other labs.

2.2.37 Measurement of Mouse Nasal Transepithelial Potential Difference

Mice were anaesthetized as previously described (section 2.2.33), suspended from their dorsal incisors (hindquarters supported) and a subcutaneous needle-agar bridge (as a reference electrode) was placed in the abdomen of the mouse. A heat-drawn PE10 polyethylene cannula (marked with a fine tip permanent marker at 2.5 mm and 5.0 mm to allow accurate placement of the cannula tip) was inserted to the designated depth in the treated nostril and connected to a perfusion-recording apparatus (consisting of a modified dual syringe pump (IVAC 770), a WPI Isomillivoltmeter and a chart recorder). The syringe pump was loaded with two 1 ml Hamilton syringes containing either the basal or the low-Cl^- perfusion solutions and was connected to the tubing system. The cannula was inserted into the treated right nostril of the mouse at ~3 mm (this depth was 2 mm shallower than that used in previous studies (5 mm), Parsons *et al.*, 1998) in an effort to improve the probability of recording electrical potentials from only respiratory epithelium in the nose (Parsons *et al.*, 2000b). Infusion of the basal solution (~2.3 µl/min) was initiated and a Rikadenki chart recorder was used to record the transepithelial potential difference (TPD) readings until a stable TPD value was recorded (a plateau of at least 1 min was required). The infusion solution was then switched to the low-Cl^- solution (NaCl replaced with Na gluconate) and a new TPD value recorded. The infusion solution was switched back to basal and an electrical potential reading was taken with the cannula and reference electrode immersed in the 0.9% NaCl solution; typically the drift value obtained was in the range of 0.1 - 0.9 mV.

The mice were monitored for respiratory distress and excess fluid present in the nostril was removed either by suction or by wicking with fine twists of tissue. Once readings were completed the mice were placed in an air chamber set at 35 °C and allowed to recover. In addition, two untreated homozygote *cftr*$^{\mathrm{tm1Unc}}$ mice were used for blinding purposes and as negative controls at each TPD assessment point.

The treatment category of the *cftr*$^{\mathrm{tm1Unc}}$ mice was blinded by tail colour re-coding prior to the TPD recordings. The TPD values were measured from chart paper recordings and the ΔPD value was calculated by subtracting the TPD value recorded under basal conditions from the TPD value recorded under low-Cl^- conditions.

2.2.38 Statistical Analysis

Statistical analysis of the data presented in this thesis project was performed using the SigmaStat 2.03 program (SPSS, Chicago, Illinois). Statistical significance was set at $P = 0.05$ and a statistical power greater than 0.80 was required (if power did not reach 0.80 it is noted). When data failed normality tests, where possible, these were transformed to normality using standard statistical transforms supplied by the software or non-parametric measures were used. Results in this thesis project are presented either as a mean ± standard deviation or as a mean ± standard error (SEM). Students t-test was used for two-group comparisons and multiple treatment groups were analysed by one-way analysis of variance (ANOVA) using post-test multiple comparisons to identify specific group differences. Changes in the proportions of LacZ positive cell types (section 6.4) were analysed by logistic regression analysis using GenStat, Release 4.2, 5th Edition (VSN International Oxford, UK).

CHAPTER 3

MODEL SYSTEMS OF HUMAN AIRWAY EPITHELIUM

Preface

This chapter describes the various cell culture models of airway epithelium that were utilised to evaluate the efficiency of adenovirus- and lentivirus- mediated gene transfer. The use of these cell culture systems allowed for the preliminary assessment of the effects, and the dose-dependence of detergent pre-treatment of cell cultures on the level of viral-mediated gene transfer. In the first instance submerged cell cultures were used. These cultures allowed the initial evaluation of the efficiency of AdV- and LV-mediated gene transfer. It became apparent, however, that cell culture systems that better modelled the intact airway epithelium were also required. Consequently, cell culture systems grown at air liquid interface conditions were trialed. In these cell culture systems the efficiency of LV-mediated gene transfer was examined and methods to improve gene transfer were explored (Chapter 5). Based on the results of these experiments gene transfer protocols were developed for *in vivo* studies (Chapter 6).

3.1 Immortalised Cell Lines

3.1.1 Introduction

Initially the cell culture systems used to assess the efficiency of the AdV- and LV-mediated gene transfer protocols, utilised immortalised epithelial cell lines, which grow on a substratum submerged in medium. These cell culture systems allowed for a variety of parameters that influence gene transfer to be tested. In particular, the optimal duration of exposure of the cells to agents that improve the efficiency of gene transfer was evaluated. Although several different immortalised epithelial cell lines were available, for the gene transfer experiments described here attention was focused on using epithelial cell lines able to form tight-junctions (TJ's), thus providing the polarisation characteristic of the *in vivo* airway epithelium (i.e. physical and functional separation of the apical/basolateral surfaces). Cell lines chosen for their reported ability to form TJ's included MDCK, 16HBE and CFBE41o$^-$ (Table 3.1). For control and comparison purposes, a number of cell lines that do not form TJ's were chosen, these were 1HAE, CFPAC-1 and A549 (Table 3.1).

Table 3.1: Properties of the Epithelial Cell Lines Used for the Evaluation of Viral-Mediated Gene Transfer.

Cell line	Type	Tight junctions[1]	Reference
MDCK	Canine kidney epithelium	✓	Gaush *et al.*, 1966
16HBE	Human bronchial epithelium	✓	Gruenert *et al.*, 1988
1HAE	Human airway epithelium	-	Cozens *et al.*, 1992
CFPAC-1	CF (ΔF508) pancreatic epithelium	-	Schoumacher *et al.*, 1990
A549	Human lung carcinoma	-	Lieber *et al.*, 1976
CFBE41o$^-$	CF (ΔF508) bronchial epithelium	✓	Prof. Gruenert, UCSF[2]

[1]Reported; [2]personal communication. (✓) presence; (-) absence.

3.1.2 Methods and Results

The cell lines listed in Table 3.1 were grown and harvested based on the standard cell culture methods described in section 2.2.19. To ensure optimal growth the growth media of all cell lines was supplemented with 10% (v/v) FCS, except for the growth media of A549 cells which was supplemented with 5% (v/v) FCS (Table 3.2). Cells were grown to confluence and sub-cultured 1:3 two days prior to the experiments to ensure a single cell suspension and consequently a uniform cell monolayer. In order to verify whether the selected cell lines did in fact form tight or leaky intercellular junctions they were grown on collagen-coated Transwell support membranes to confluence and the transepithelial resistance (TR) of each cell line was measured using the EVOM™ apparatus as described in section 2.2.30. The TR of each cell line was calculated using the following formula supplied by WPI:

$$(R_{sample} - R_{control}) \times \Omega \times \pi r^2 = \text{resistance per area } (\Omega cm^2)$$

R= Resistance, Ω= Ohm

A TR of > 600 Ωcm^2 was considered to verify TJ formation (Table 3.2). As a control value the resistance of the membrane itself in culture media was measured; this was typically ~100 Ωcm^2.

Table 3.2: Cell Culture Conditions for the Growth of Immortalised Epithelial Cell Lines.

Cell line	Media	FCS % (v/v)	BSA (mg/ml)	TR (Ωcm^2)
MDCK	DMEM	10	-	> 1000
16HBE	MEM	10	-	~600
1 HAE	DMEM	10	-	100
CFPAC-1	DMEM	10	-	100
A549	DMEM	5	-	100
CFBE41o⁻	MEM	10	1	100

(-) absence.

Another simple indicator of the formation of TJ's is the rate of flow of media from the basolateral side to the apical side of the epithelial cell monolayer, which was monitored once confluence had been reached. Formation of TJ's does not allow the flow (leakage) of media from the basolateral to the apical reservoir.

Both the MDCK and 16HBE cells formed TJ's. MDCK cells exhibited a TR of > 1000 Ωcm^2, compared to ~600 Ωcm^2 for the 16HBE cells. Contrary to expectation, the CFBE41o$^-$ cells never fully differentiated to ciliated cells or formed TJ's. Subsequently, it was confirmed that this cell line had lost its ability to differentiate into a polarised airway epithelium layer (Prof. D. C. Gruenert, UCSF, San Francisco, personal communication) and it was therefore excluded from further experiments. The 1HAE cell line that has been previously reported to develop TJ's and differentiate (Cozens *et al.*, 1992) also did not form TJ's and was therefore used as a control, non-polarised, cell line in the gene transfer experiments.

3.1.3 Discussion

The advantages of the immortalised epithelial cell lines described here are that they are easy to culture and they provide an infinite resource of either polarised or non-polarised cells. Furthermore, some of these epithelial cell lines, such as CFBE41o$^-$, can also be grown at air liquid interface. The use of submerged cultures also allows for easy manipulation of the cells. Gene transfer experiments using these culture systems can provide valuable information on the various parameters that influence the efficiency of gene transfer and also allows for partial optimisation of protocols prior to *in vivo* experimentation.

The main disadvantage associated with the use of immortalised epithelial cell lines is that they do not provide an accurate model of the intact airway epithelium with respect to the formation of cilia, mucus production and associated MCC mechanisms. They are therefore limited as a model system of the intact airway epithelium when compared to both cultures of primary airway epithelial cells and animal models. In addition, the cell lines, such as the CFBE41o$^-$, that had been expected to provide the best immortalised cell culture model in fact did not, reflecting the difficulty of establishing and maintaining previously developed immortalised airway epithelial cell lines with the desired characteristics of the intact airway epithelium.

Although primary airway epithelial cell cultures are good models of the intact airway epithelium with respect to morphology and function, these are more difficult to establish and more labour intensive, than the immortalised epithelial cell cultures. Nonetheless, the perceived usefulness of *in vitro* cell culture systems that mimic the intact airway epithelium lead to the use of the air liquid interface cell cultures discussed in the next section.

3.2 Air Liquid Interface Cultures of Tracheal Epithelial Cells

3.2.1 Introduction

Human airway epithelial cells particularly from CF patients should provide an excellent cell culture system to evaluate the efficiency of gene transfer *in vitro* prior to *in vivo* experimentation. However, the limited availability of human airway tissue, as well as the large numbers of cells required to establish these cultures, continues to limit the usefulness of this approach. As a result, airway epithelial cells derived from animal species have been used to provide cell culture systems able to model many aspects of the intact human airway epithelium. For example, tracheal airway epithelial cells derived from mouse (Kumar *et al.*, 1997), rat (Kaartinen *et al.*, 1993), dog (Kondo *et al.*, 1991), pig (Whitcutt *et al.*, 1988), cow (Kondo *et al.*, 1993), rabbit (Jetten *et al.*, 1987; Tournier *et al.*, 1998) and hamster (Niles *et al.*, 1990) have all been used to model the human airway epithelium.

In an attempt to provide a cell culture system more representative of the intact airway epithelium, the technique of growing cells under air liquid interface (ALI) conditions was established. These specialised *in vitro* cultures of nasal, tracheal or bronchial airway epithelial cells have provided researchers with unique insights into airway epithelial cell function and morphology (Gray *et al.*, 1996). It has been reported that when airway epithelial cells are grown under ALI conditions the majority (> 50%) of the membrane supports seeded are able to form TJ's, differentiate into secretory and ciliated cells, and an overlaying mucus layer and associated MCC mechanisms then become apparent.

The cell culture method described by Kaartinen and colleagues (1993) was used in our lab to establish ALI cultures utilising epithelial cells isolated from the rat trachea. Following enzymatic dissociation of the trachea, the epithelial cells were seeded onto collagen-coated Transwell support membranes as described in section 2.2.31. It has been reported that the rat tracheal epithelial (RTE) cells initially behave like basal cells. That is they differentiate into ciliated cells with immature cilia before becoming well-differentiated ciliated cells within 10 - 14 days in culture (Kaartinen *et al.*, 1993). When confluent cultures of RTE cells are exposed to air in a fully humidified environment they typically form a polarised epithelium, which consists mainly of columnar cells and possesses physiological properties similar to those of the intact airway epithelium with respect to (i) the formation of TJ's, (ii) the presence of an overlaying mucus layer and

(iii) a synchronous cilial beat. Each of these characteristics is a key component of the intact ciliated airway epithelium.

3.2.2 Methods and Results

(a) Isolation of Epithelial Cells from the Rat Trachea

Airway epithelial cells were isolated from the trachea of either Sprague Dawley or Hooded Wistar rats as described in section 2.2.31 (a). The number of cells isolated from each trachea in these experiments was less than anticipated; only 3 - 4.5 x 10^5 cells were recovered per trachea, rather than the ~1 x 10^6 cells expected (Dr S. H. Randell, UNC at Chapel Hill, personal communication). The low number of cells dissociated from the tracheal epithelium may have been due to the inadequate inflation of the trachea during the enzymatic digestion process (described in section 2.2.31 (a)). When the trachea is gently inflated it becomes more exposed to the digesting action of the pronase solution, thus resulting in the isolation of a higher number of epithelial cells. Despite the recommended procedures (Dr. S. H. Randell, personal communication) for the inflation of the trachea being followed, in our hands the trachea did not remain fully inflated throughout the digestion period.

Although a low number of epithelial cells was isolated from the rat trachea, the cells were re-suspended in the high-protein medium (section 2.1.7), and seeded on Transwell support membranes (SA= 1cm^2) at a density of 3 x 10^4 cells. The cells were fed with high-protein medium/5% (v/v) FCS from the bottom reservoir based on the recommended protocols (Dr S. H. Randell, personal communication). Twenty-four hrs later the growth media in both compartments was replaced with low-protein media (as described in section 2.2.31 (b)). As the cells did not reach confluence within the expected 7 days, various cell seeding densities ranging from 0.2 - 1 x 10^5 cells per Transwell were then assessed. In these experiments it was found that the density that promoted maximal cell expansion was 6 x 10^4 cells per Transwell. Interestingly, seeding densities higher than 6 x 10^4 cells per Transwell resulted in the formation of small clusters of ciliated cells (located principally in the centre of the Transwell), which failed to adhere to the membrane.

(b) Growth of the Rat Tracheal Epithelial Cells

Collagen, a component of the lamina propia, improves attachment, growth and differentiation of the RTE cells (Kaartinen *et al.*, 1993; Davenport and Nettesheim, 1996a/b). Consequently, polycarbonate Transwell support membranes, which were coated with rat-tail collagen, were trialed in an attempt to improve the growth rate of the RTE cells. The rate of both cell attachment and growth was improved and islets of ciliated cells were formed. However, these cells began to detach from the membrane of the Transwell within ~21 days of seeding. Since collagen dissociates at 37 °C (Kaartinen *et al.*, 1993), the culture incubation temperature was lowered by 2 degrees to 35 °C. The lower temperature did improve the rate of epithelial cell growth such that more than half of the rat-tail collagen-coated Transwells seeded with RTE cells reached 80% confluence within 8 days. However, none of these Transwells ever reached confluence.

Comparison of the growth rate of the airway epithelial cells seeded on either the commercially available Transwell supports or on the Transwell supports coated in house with rat-tail type I collagen indicated that the rate of cell attachment and growth was the same. For convenience, the commercially available collagen-coated Transwell supports were used for all subsequent RTE cell cultures.

(c) Bovine Pituitary Extract: an Important Constituent of the Rat Tracheal Epithelial Cell Growth Medium

One of the most important constituents of the RTE cell growth medium is considered to be the bovine pituitary extract (BPE), which stimulates cell growth (Dr. S. H. Randell, personal communication). The BPE that was added into the growth medium of the RTE cells was isolated in house from whole bovine pituitaries. As the activity of the BPE could not be assayed directly, the effect of various dilutions of BPE ranging from 1 - 10% (v/v) in the RTE growth medium was directly tested in culture. The 2% (v/v) BPE in the growth medium promoted the highest rate of cell growth.

During the course of the culture period precipitates, thought to have originated from the BPE, were formed when the growth media was warmed to 35 °C, but the reason for this was not clear. In an attempt to minimise the formation of these precipitates, BPE was also obtained from a commercial source (Sigma® Chemicals Co.). This BPE formulation did not form precipitates when the RTE growth medium was warmed to 35 °C and no significant difference in the rate of growth of cells was apparent when the commercially sourced BPE was substituted for the in house BPE. For convenience, the commercial BPE was used for subsequent growth medium formulation.

No attempt was made to alter the concentrations of the other additives and hormones present in the RTE cell growth medium formulation as recommended by Kaartinen *et al.* (1993). The mitogenic components present in the RTE cell growth medium were the (i) cholera toxin, (ii) the epidermal growth factor (EGF) and (iii) the

BPE (Clark *et al.*, 1995). EGF has been reported to activate the BPE (Lechner and LaVeck, 1985), consequently stimulating airway epithelial cell proliferation. Insulin was also added in the growth media as it too acts as a mitogenic agent essential for airway epithelial cell proliferation (Kaartinen *et al.*, 1993). Hydrocortisone and retinoic acid were added as they promote differentiation of the dissociated airway epithelial cells into columnar and ciliated cells, respectively (Kaartinen *et al.*, 1993). The broad-spectrum antibiotics, penicillin and streptomycin were added to prevent culture infection. Although on rare occasions there were incidents of fibroblast contamination their growth was suppressed by (i) a lower culture temperature (35 °C) and (ii) the removal of FCS from the growth media as recommended by Werner and Kissel (1995) and Lechner *et al.* (1982), respectively. The removal of FCS had no inhibitory effect on the proliferation rate of the epithelial cells.

3.2.3 Discussion

The RTE growth medium consisted of various hormones and mitogenic factors to ensure optimal growth and differentiation of the RTE cells when these were grown under ALI conditions. In an attempt to improve the sub-optimal growth of the RTE cells in our lab, various cell seeding densities were examined for their ability to enhance proliferation of the RTE cells to confluence. Additionally, the effect of collagen-coated membranes on the growth of cells was also explored. It was found that the density that promoted optimal cell growth was 6 x 10^4 cells per cm^2; the RTE cells, however, never reached confluence. The two different types of collagen coated Transwells did not appear to have an impact on the growth rate of the RTE cells. In addition, no difference in the proliferation of the RTE cells was apparent when the in house BPE preparation, which formed precipitates when warmed to 35 °C, was substituted for a commercial sourced BPE formulation.

It is clear that RTE ALI cultures can provide an excellent *in vitro* model of the intact airway epithelium. Although various attempts were made to further optimise the protocol for isolating and growing RTE cells under ALI conditions, in these experiments the RTE cell cultures never reached confluence. Nonetheless, the phenotype of the dissociated airway epithelial cells was seen to differentiate such that islets of ciliated cells were formed. The difficulties associated with establishing this cell culture system prompted the development of an alternative *in vitro* approach to modelling the intact airway epithelium. This approach was based on the use of explanted rat nasal septum cultures also grown under ALI conditions.

3.3 Air Liquid Interface Cultures of Explanted Nasal Septa

3.3.1 Introduction

As the success rate of the RTE cell cultures was low (section 3.2), another model of the intact airway epithelium that could also be grown under ALI conditions was investigated. Specifically, an ALI cell culture system utilising explanted rat nasal septa was established based on a method described by Fanucchi and colleagues (1999). The advantage of this ALI culture system over the RTE ALI culture system, described previously, is that it does not rely on the dissociation of the airway tissue (trachea) that was necessary for the isolation of the RTE cells. This means that the nasal septum can be used for experimentation immediately after removal. Furthermore, the technique used to excise the nasal septum was simple and gentle, thus ensuring minimal damage to the epithelium surface.

3.3.2 Methods and Results

The nasal septum was excised from rats as described in section 2.2.32 and the thin respiratory region of the septum, which consisted mainly of ciliated epithelium was then placed on a polycarbonate Transwell support membrane. The success of the septum cultures was measured by the longevity of cilial beat and by the lack of bacterial/fungal infections.

Cilial beat across the nasal septum epithelium was assessed immediately after removal and used as an index to monitor septum viability every three days, as described in section 2.2.32 (see CD, Clip 1). The surface of the septum was uneven similar to a wave-like crest and trough pattern. The clearance function provided by the cilial beating was assessed by introducing a 4 μl preparation of titanium-dioxide particles (median diameter ~0.19 μm) suspended in 10% (v/v) glycerol in PBS at 35 °C, n= 2 and viewing under a dissecting microscope as described in section 2.2.32. It was apparent that the cilial beat frequency, as well as the cilial co-ordination, was not the same throughout the surface of the septum. In one sample a spiral-like circulation effect (diameter ~110 μm) became visible within 3 mins of titanium-dioxide particle deposition on the apical surface of the septum (see CD, Clip 2; a more detailed description is provided in “Clip 2-legend.txt”). In other regions, titanium-dioxide clumps appeared immobilised on the septum surface suggesting absence of MCC. The reasons for this are not clear but this may be due to damage to the septum surface during excision. Using the titanium-dioxide solution it was found that lowered temperatures (~24 °C) resulted in a slower and less co-ordinated cilial beating (data not shown). The 10% (v/v) glycerol in PBS solution alone (the standard suspension buffer for AdV vector stocks) did not have an inhibitory effect on the rate of MCC when applied onto the nasal septum (n= 2) (data not shown).

Based on the method of Fanucchi *et al.* (1999) the septa were maintained in culture for up to 14 days at which time they appeared to be well preserved (Figure 3.1).

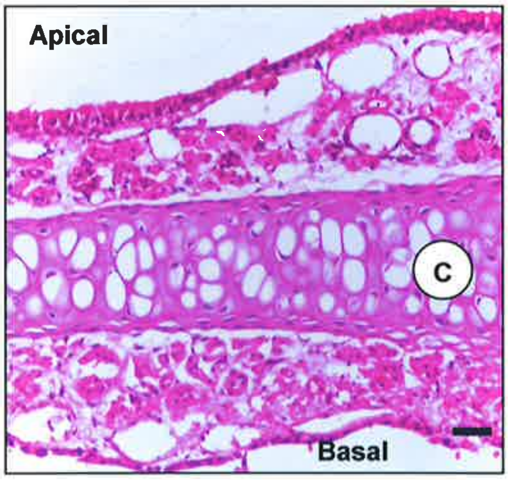

Figure 3.1: Morphology of the Rat Nasal Septum Fourteen Days After Excision.
The apical side of the columnar-like epithelial layer is reasonably well preserved with maintenance of cilial structure. In contrast the basal side (i.e. septum face that was in contact with the Transwell membrane) of the epithelium layer is not well preserved, appearing more squamous-like. Blood vessels are present in both surfaces and appear expanded as a result of maintenance in culture. The septum cartilage (C) is well preserved and appears normal. The septum sections were stained with H/E as described in section 2.2.35. This figure is representative of septa from n= 12 animals. Scale bar= 100 μm.

3.3.3 Discussion

The most important feature of the rat nasal septum cultures is that, unlike the RTE cell cultures, they do not rely on the complex process of dissociation and then re-constitution of intact, fully functional airway epithelium. As such they provide a cell culture model that closely resembles the *in vivo* airway epithelium. The longevity of this model system was described by Fannuchi and colleagues (1999) as approximately 14 days *in vitro*, and similar integrity was found here. An advantage of these cultures is that they can be utilised almost immediately after removal compared to the much longer generation and culture period (~4 weeks) necessary to obtain fully differentiated cultures of RTE cells.

The culture system using explanted rat nasal septa had some disadvantages. These were (i) the technique was limited to one sample per animal (although micro-dissection techniques could be applied to divide the excised tissue into several smaller sections), (ii) there was difficulty in controlling and monitoring accurate application of solutions onto the apical surface of the septum and (iii) the septum had a relatively short life (14 days), which did not permit long-term gene transfer studies to be completed.

Other explant culture systems suitable for the evaluation of parameters that influence gene transfer have also been reported. These cell culture systems have made use of explanted tracheas (from *cftr*$^{\mathrm{tm1Unc}}$ mice or sheep) to provide a model of the intact upper airway epithelium (Scott *et al.*, 2000; Kitson *et al.*, 1999). The mouse tracheal epithelium however, is considered to be an inadequate model of the CFTR malfunction (Grubb and Boucher, 1999). Additionally, the culture systems described by Scott *et al.* (2000) and Kitson *et al.* (1999) have a very short life; the trachea was only viable for 24 hrs. Therefore, even short-term effects of successful gene transfer would be difficult to assess. Recently, another cell culture system was reported by Sersale and colleagues (2001). This culture system utilised airway cell outgrowths from explanted human nasal polyps. However, as nasal polyps are generally regarded an abnormal airway epithelial tissue, the general applicability of findings using this culture system is questionable.

Although no cell culture system can provide an accurate model of the intact airway epithelium, the cell culture systems (the immortalised epithelial cells, the ALI RTE cells and the explanted rat nasal septa) used in this project did provide valuable information on the factors affecting LV-mediated gene transfer prior to animal testing.

3.4 Animal Models of the Human Airway Epithelium

3.4.1 The C57Bl/6 Mouse

The use of marker genes (i.e. β-galactosidase, LacZ, and EYFP) allows for gene transfer protocols to be developed and optimised in a non-CF animal model, such as the C57Bl/6 mouse, prior to the application of the developed protocols for CFTR gene transfer in transgenic CF mice. The majority of gene transfer studies in the mouse have been performed in the nasal airways. This is because the cell types that line some regions of the mouse nasal airway epithelium resemble the epithelial cells that line the human lung airway (Grubb and Boucher, 1999). Moreover, the nasal cavity provides an easily accessible area in which the instillation of various solutions can be readily controlled. Finally, the non-treated nostril can generally be used as a within-animal control.

In the gene transfer studies described in Chapter 6, the nasal airway of C57Bl/6 mice was used to optimise the protocol for LV-mediated gene transfer *in vivo* using the LacZ marker gene prior to the evaluation of these protocols for CFTR gene transfer in the nasal airway of *cftr*$^{\mathrm{tm1Unc}}$ mice.

The C57Bl/6 mice used in this thesis project were typically 6 - 12 weeks old and there were no cases of anaesthesia mortality throughout the duration of the project. The virus preparation was delivered to the airway as two 10 μl bolus instillations, as described in section 2.2.33 and only very rarely was respiratory difficulty or distress (which was transient) associated with solution instillation. During the course of this study only one unexpected death during virus instillation was recorded. Upon dissection of the nasal cavity of this mouse it became apparent that the left (non-treated) nostril was blocked by an anatomical malformation, which prevented adequate airflow during virus dosing.

In order to quantify LacZ gene transfer, 3 standard cross-sections (Parsons *et al.*, 1998) were taken at Levels 6, 16 and 24 of the decalcified mouse head (Mery *et al.*, 1994) as described in section 2.2.34. Initial assessment of LacZ gene transfer was by microscopic *en face* examination of grossly sectioned blocks of nasal tissue prior to paraffin embedding and histological processing. The use of the LacZ marker gene allowed the pattern of cell transduction and the persistence of gene expression within the various time frames studied (Chapter 6, Part A) to be assessed, and helped establish the dose settings to be used for gene transfer protocols in CF mouse nasal airways.

3.4.2 The Transgenic Cystic Fibrosis Mouse Model

The cloning of the murine CFTR gene (Tata *et al.*, 1991) allowed the generation of mouse models of CF by gene targeting. A number of CF knockout mouse models have been generated using this technique (Snouwaert *et al.*, 1992; Ratcliff *et al.*, 1993; O'Neal *et al.*, 1993; Hasty *et al.*, 1995), as well as mice homozygous for either the ΔF508 CF gene mutation (Colledge *et al.*, 1995; Zeiher *et al.*, 1995; VanDoorninck *et al.*, 1995) or the G551D CF gene mutation (Delaney *et al.*, 1996). The null $cftr^{tm1Unc}$ mouse (Snouwaert *et al.*, 1992) was chosen for the gene transfer studies performed in this thesis project to evaluate the efficiency of LV-mediated CFTR gene transfer *in vivo*.

The murine CFTR gene in the $cftr^{tm1Unc}$ mouse model contains an in-frame stop codon in the coding region of exon 10 (Snouwaert *et al.*, 1992). The resulting phenotype of the $cftr^{tm1Unc}$ mouse is severe (Snouwaert *et al.*, 1992) and is similar to the phenotype conferred by the ΔF508 CF gene mutation observed in humans, as described in section 1.1.2. The complete absence of CFTR in the $cftr^{tm1Unc}$ mouse allows clear assessment of the success of the experimental manipulations designed to introduce the CFTR gene. A $cftr^{tm1Unc}$ mouse colony has been available on site (WCH Animal House) since 1993.

(a) Management of the $cftr^{tm1Unc}$ Mouse Colony

The $cftr^{tm1Unc}$ mice of the WCH colony (described in section 2.2.36) were identified 2 weeks after weaning by observation of the colour of their dorsal incisors (Wright *et al.*, 1996) (CF mice exhibit chalky white dorsal incisors) coupled with PCR analysis, as described in section 2.2.16 (a) (Figure 3.2).

The pancreatic disease and subsequent intestinal obstruction is associated with mortality in $cftr^{tm1Unc}$ mice if left untreated. This can be largely prevented by the addition of 0.045% (w/v) ColonLYTELY (a polyethylene glycol-based solution) to their drinking water. Despite this intervention, however, approximately 15% of the $cftr^{tm1Unc}$ pups died before weaning (data not shown). The surviving $cftr^{tm1Unc}$ mice were approximately 20% smaller in weight than their heterozygote littermates, but did not display additional health problems. Following weaning (typically 3 weeks after birth) unexpected deaths of $cftr^{tm1Unc}$ mice occurred very rarely during the course of this thesis project.

The $cftr^{tm1Unc}$ mice used in the LV-mediated CFTR gene transfer studies (Chapter 6, Part B) were on average between 2 - 6 months old. It was initially found that the $cftr^{tm1Unc}$ mice were more likely to die during recovery from anaesthesia, compared with the C57Bl/6 mice. Anecdotally, such deaths are not uncommon during TPD studies

(Dr. D. W. Parsons, personal communication). This was vastly overcome by lowering the first anaesthetic dose by 30% (from 1 µl/g to 0.7 µl/g body weight of a 3:2 mixture of xylazil (20 mg/ml): ketamine (100 mg/ml)) and limiting the anaesthesia time to less than 1 hr. In addition, placing the treated *cftr*$^{\mathrm{tm1Unc}}$ mice in an air chamber pre-warmed to 35 °C assisted recovery from anaesthesia.

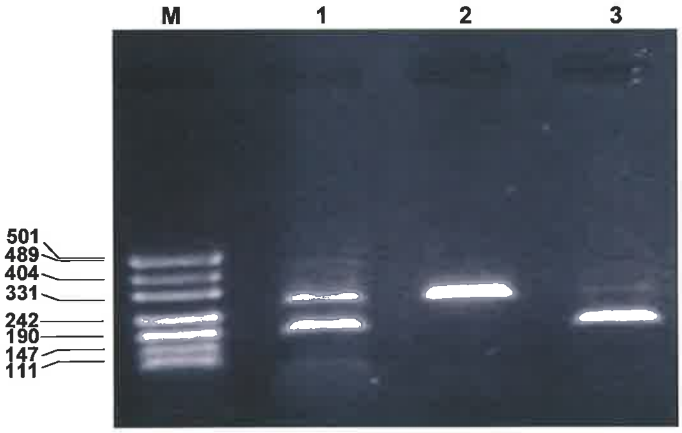

Figure 3.2: Example of Genotype Analysis of Mice from the CF Mouse Colony.
A 2 mm piece of tail was removed from mice 2 weeks after weaning and the DNA extracted using the DNeasy™ kit. The DNA template was then amplified as described in section 2.2.16 (a). The size of the PCR product for the normal CFTR allele is 277 bp and for the knockout allele ~220 bp. The PCR products were analysed on a 2% agarose, 1 x TBE gel. M: pUC19/*Hpa*II markers, the sizes of the relevant bands are shown to the left of the figure. Lane 1, the presence of two bands of sizes 277 bp and ~220 bp is indicative of a heterozygote CF colony mouse; lane 2, the presence of one band of 277 bp is indicative of a non-CF mouse (control: C57Bl/6 mouse); lane 3, the presence of one band of ~220 bp is indicative of a *cftr*$^{\mathrm{tm1Unc}}$ mouse.

(b) Transepithelial Potential Difference Measurements Across the Mouse Nasal Airway Epithelium

The nasal airway of the CF mouse is an excellent site to evaluate CFTR gene transfer as it displays the electrophysiological signature of CF, which conversely is not present in the CF mouse lung (Grubb and Boucher, 1999). As described in Chapter 1 (section 1.2.5 (b)), the nasal airway epithelium exhibits hyperabsorption of Na^+ and decreased Cl^- secretion, which is typical of the human CF lung epithelium.

Electrophysiological differences between the *cftr*$^{\mathrm{tm1Unc}}$ and heterozygote CF colony mice were assessed as described in section 2.2.37 and were similar to those reported by Grubb and colleagues (1994a). The basal transepithelial potential difference

(TPD) measurement (a general index of Na^+ transport) was raised in *cftr*^{tm1Unc} mice (-25.9 ± 1.8 mV, n= 6), when compared to both normal C57Bl/6 mice (-6.5 ± 0.3 mV, n= 3) and heterozygote CF colony mice (-4.5 ± 0.5 mV, n= 8). Perfusion with low Cl^- solution through the TPD measurement cannula was then used to measure the relative permeability of the nasal epithelium for Cl^-. The *cftr*^{tm1Unc} nasal epithelium depolarised (-19.8 ± 2.0 mV, n= 6) in response to perfusion with the low Cl^- solution. In contrast, perfusion with the low Cl^- solution resulted in a large hyper-polarisation in both normal (-23 ± 3.3 mV, n= 3) and heterozygote CF colony mice (-21 ± 1 mV, n= 8).

As there was a clear difference in the TPD responses in CF mouse nasal airway compared to heterozygote or normal mice, this methodology was used to monitor the success of LV-mediated CFTR gene transfer *in vivo*. The level of CFTR gene transfer was assessed by calculating the difference, designated as ΔPD, between the TPD value recorded under perfusion with the basal solution and the TPD value recorded under perfusion with the low-Cl^- solution in the LV-treated mouse nostril (Figure 3.3) as described in section 2.2.37. Successful CFTR gene transfer was measured as decreased (i.e. more negative) ΔPD values, which reflect functional correction of the CFTR-mediated Cl^- secretion defect (Parsons *et al.*, 1998).

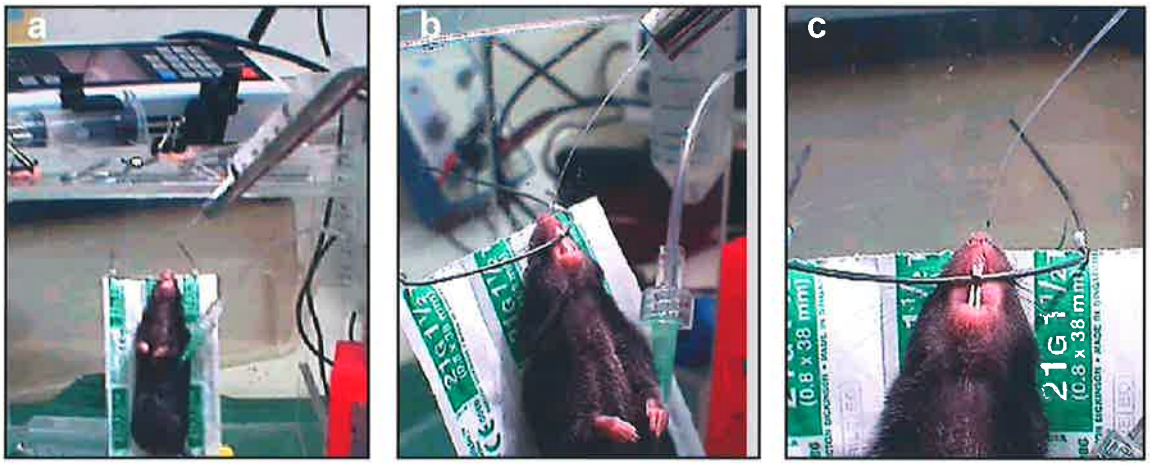

Figure 3.3: TPD Measurements Across the Mouse Nasal Airway Epithelium.
(a) An anaesthetised mouse (suspended from its dorsal incisors, during the TPD measurements is shown. The IVAC-770 infusion pump is shown in the background, **(b)** The reference cathode placed subcutaneously in the mouse abdomen and the heat-drawn fine PE10 polyethylene tubing cannula inserted into the treated nostril are shown, **(c)** The cannula was inserted at a depth of ~3 mm into the treated nostril in an effort to improve the probability of recording electrical potentials from only the respiratory epithelium (Parsons *et al.*, 2000b).

The main goal of the CFTR gene transfer studies (Chapter 6, Part B) was to assess the long-term persistence of CFTR gene expression across the LV-treated *cftr*$^{\mathrm{tm1Unc}}$ mouse nasal airway epithelium. In this vein the ΔPD assay, which involved repeated TPD measurements over long time-frames provided a simple measure of the level and persistence of recovery of the Cl^- secretion function. cAMP agonists were not included in the basal/low Cl^- perfusion solutions, since previous studies (Grubb *et al.*, 1994a) demonstrated that these are not necessary to accurately measure CFTR gene-mediated correction of Cl^- transport. To assess the effect of LV-mediated CFTR gene transfer on Na^+ hyperabsorption associated with CF, Na^+ transport correction was initially attempted using standard methods, i.e. perfusion with basal/low Cl^- solutions that were supplemented with the Na^+ channel blocker amiloride. However, the prolonged anaesthesia time experienced by the LV-treated *cftr*$^{\mathrm{tm1Unc}}$ mice during these extended TPD measurements caused an unacceptably high rate of mortality. For this reason only the effect of LV-mediated CFTR gene transfer on the Cl^- secretion defect was evaluated in this thesis project.

In future it will be important to improve the recovery of CF mice post-ΔPD assessment of the Na^+ transport defect, which currently can require a long anaesthesia time (up to 1 hr). This will not only minimise animal mortality but also allow for the evaluation of the effect of LV-mediated CFTR gene transfer on the Na^+ transport impairment present in CF airways.

CHAPTER 4

ADENOVIRUS-MEDIATED GENE TRANSFER *IN VITRO*

Preface

In order to investigate the various factors influencing efficient viral gene delivery, an AdV vector carrying the LacZ marker gene sequence was utilised in gene transfer studies *in vitro*. The information gained from these AdV gene transfer studies was then used to develop and optimise protocols for LV-mediated gene transfer to the airway epithelial cells initially *in vitro* and then *in vivo*. This approach was based on the expectation that the airway surface barriers that influence AdV-mediated gene transfer would similarly influence LV-mediated gene transfer.

This chapter is divided into two parts. Part A addresses the generation of the AdV vector stocks, whilst Part B presents and discusses the results from gene transfer studies performed in immortalised epithelial cell lines.

Part A

Technical Issues

4.1 Propagation of Adenovirus Vector Stocks

4.1.1 Introduction

The most widely used protocols for the propagation of E1-deleted adenovirus (AdV) vectors make use of the cell line 293 (Graham *et al.*, 1977). This cell line was produced by immortalisation of human embryo kidney cells using the AdV early region viral genes (E1a and E1b) (Graham *et al.*, 1977). As a result, 293 cells provide the essential E1 functions *in trans*.

The AdV vector used in the gene transfer studies described here was a replication-deficient AdV vector serotype 5 in which the E1 region has been replaced with the LacZ marker gene sequence and is under the transcriptional control of the cytomegalovirus (CMV) promoter. This vector was designated AdV5CMVLacZ.

An unexpected but interesting finding during the production of the AdV vector stocks was that propagation of the AdV vector in 293T cells, a derivative of the 293 cell line into which the temperature-sensitive simian virus 40 (SV40) T-antigen has been inserted (DuBridge *et al.*, 1987), could result in crude AdV vector lysates with higher viral titres compared to those of AdV vector lysates propagated in 293 cells.

4.1.2 Methods

Twenty-seven T-25 tissue culture flasks were seeded with either 293 cells (at 2.2 x 10^6 cells per flask) or 293TMJ cells (at 1.8 x 10^6 cells per flask) in 5 ml of DMEM/10% (v/v) FCS. Twenty hrs later the medium was aspirated and 500 µl of DMEM/SF containing 3.5 x 10^7 lfu (MOI= 10) of AdV5CMVLacZ was added. One hr later 5 ml of DMEM/10% (v/v) FCS was added to each flask and the incubation continued for up to 48 hrs. At the time intervals indicated in Figure 4.1 three flasks from each cell line were harvested, the cells recovered by centrifugation, and the resulting cell pellet immediately cooled on ice and then stored at minus 70 °C. Once the cells from all time points had been collected they were thawed and lysed, as described in section 2.2.21, and the titre of the AdV vector lysates then determined, as described in section 2.2.22.

4.1.3. Results

Due to the different growth rate between the 293 and the 293T (and 293TMJ) cells, the number of 293 cells seeded needed to be approximately 25% more than the number of 293T (and 293TMJ) cells seeded, so that both cell lines were at ~ 80% confluence 20 hrs after seeding, which was the recommended density for the propagation of AdV vector stocks (Dr. R. Krishnan, Queen Elizabeth Hospital, Adelaide, personal communication). Although the kinetics of the AdV vector propagation were similar in both cell lines at the earlier time points (Figure 4.1), AdV vector production rates diverged 16 hrs after infection.

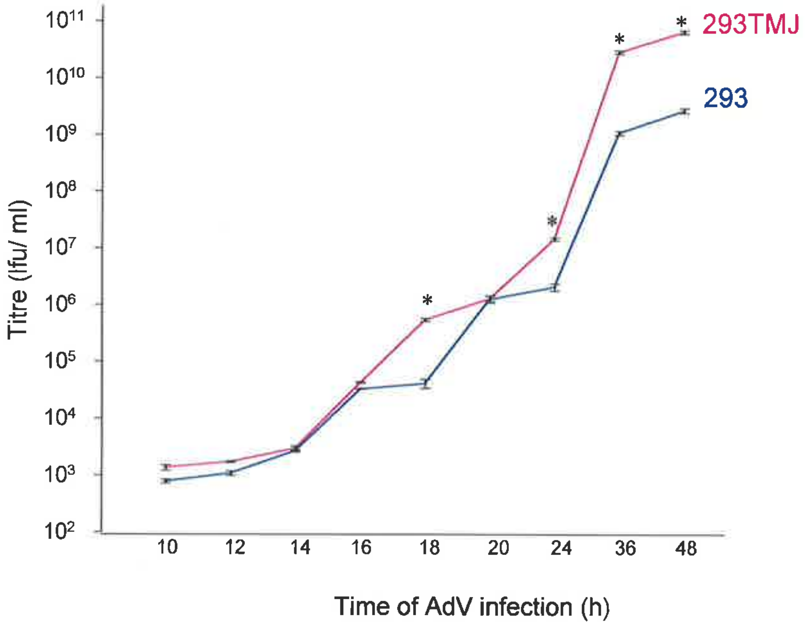

Figure 4.1: The Kinetics of the AdV5CMVLacZ Vector Propagation in 293 and 293TMJ Cells. Confluent cultures of 293 and 293TMJ cells were infected with 3.5 x 10^7 Ifu of AdV5CMVLacZ (MOI= 10) for 1 hr. Fresh DMEM/10% (v/v) FCS was added to each flask and the cells maintained in culture for up to 48 hrs. Cells were harvested at 10 - 48 hrs following AdV vector infection and the titre of the AdV vector determined as described in section 2.2.22. Values are presented as a mean of 3 samples ± standard deviation. The standard deviation is represented in the graph as an error bar. * *P*< 0.05, Students t-test.

Twenty-four hrs post-infection the titre of the AdV vector propagated in the 293TMJ cells was higher (8-fold) than that of the AdV vector propagated in the 293 cells (*P*< 0.05, Students t-test). By 48 hrs post-infection this difference had increased significantly (> 20-fold) (*P*< 0.05, Students t-test).

This experiment was then repeated using 293T cells obtained directly from the ATCC using the methods described in section 4.1.2. No significant difference in the level of viral titre of the AdV vector propagated in either the 293 or the 293T cells was apparent in this experiment (Figure 4.2). The viral titres of the AdV vector propagated in 293 cells were consistent with the titres of the AdV vector propagated in 293 cells obtained in the previous experiment (Figure 4.1).

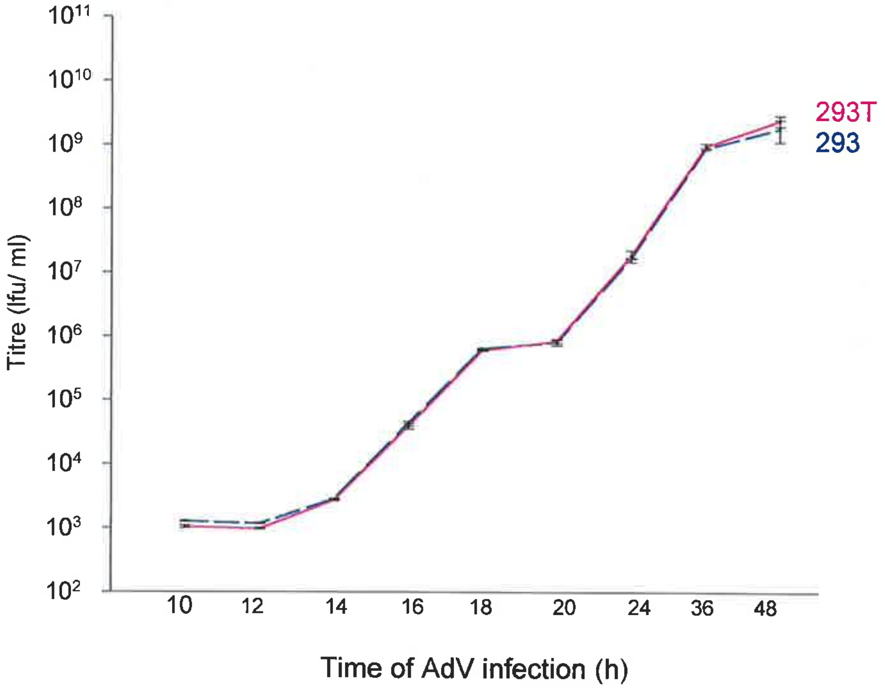

Figure 4.2: The Kinetics of the AdV5CMVLacZ Vector Propagation in 293 and 293T Cells. Confluent cultures of 293 and 293T (ATCC) cells were infected with 3.5 x 10^7 Ifu of AdV5CMVLacZ (MOI= 10) for 1 hr. Fresh DMEM/10% (v/v) FCS was added to each flask and the cells maintained in culture for up to 48 hrs. Cells were harvested at 10 - 48 hrs following AdV vector infection and the titre of the AdV vector determined as described in section 2.2.22. Values are presented as a mean of 3 samples ± standard deviation. The standard deviation is represented in the graph as an error bar.

4.1.4 Discussion

The 293T cell line is a derivative of the 293 cell line into which the gene for a temperature sensitive SV40 T-antigen mutant has been inserted (DuBridge *et al.*, 1987). As a consequence, 293T cells produce large amounts of T-antigen when maintained at 37 °C (DuBridge *et al.*, 1987). It is well known that a block in the expression of the late viral proteins prevents propagation of wild type human AdV in monkey cells (Lewis and Rowe, 1970). This block can be overcome by the helper functions provided by the large T-antigen of SV40 (Lewis and Rowe, 1970). The SV40 helper activity is an early event and has been shown to be a property of the T-antigen. It has been reported that the carboxyl-terminal domain of the SV40 T-antigen is the *trans*-activating enhancing factor (Horwitz, 1990). It appears, therefore, that the activity of this *trans*-activating enhancing factor could also be responsible for the increased replication of AdV in 293T cells. The fact that these results could not be duplicated in the 293T cells obtained directly from the ATCC suggests that the expression of the SV40 T-antigen *per se* does not enhance AdV vector replication in human cells. Therefore, it appears as though another variable that had evolved in the 293TMJ cell clone permitted the propagation of higher titre AdV vector lysates. Although this finding suggested that AdV vector titres could be improved through the use of the 293TMJ cell line, this was not explored any further mainly due to the lack of dedicated resources.

In summary, the results of the experiments described here suggest that propagation of the AdV vector in certain variants of the 293T cell line can provide substantially higher viral titre vector lysates. With further investigation this approach could substantially reduce the cost and/or time involved in AdV vector stock propagation.

Part B

Optimisation of Adenovirus-Mediated Gene Transfer

4.2 Modulation of the Airway Epithelium Surface Barriers to Improve Adenovirus-Mediated Gene Transfer

4.2.1 Introduction

As reviewed in Chapter 1 (section 1.3.6, c) it is apparent that modulation of the barriers of the airway epithelium can significantly improve gene transfer *in vitro*. Utilising a method described by Parsons and colleagues (1998) the synthetic detergent polidocanol (section 1.3.7, a) was investigated here for its ability to improve viral-mediated gene transfer. The adenovirus vector was used in these gene transfer studies as a model viral vector to assess both the effects of polidocanol treatment on the efficiency of viral-mediated gene transfer *in vitro*, and also to begin to understand the mechanisms that may underlie detergent action on the apical surface of the epithelium. Various immortalised epithelial cell lines that model aspects of the intact airway epithelium were chosen to ensure that the results obtained *in vitro* were pertinent to achieving efficient viral-mediated gene transfer *in vivo*.

Although the mechanisms by which polidocanol acts to enhance AdV-mediated gene transfer are not entirely known, it has been reported that polidocanol can transiently open the TJ's of the airway epithelium *in vivo* (Parsons *et al.*, 1998). Polidocanol has also been shown to reversibly inhibit MCC transport (Gizurarson *et al.*, 1990). The combination of these two effects may act to both increase the residence time of the AdV vector particles on the apical surface of the airway epithelium, and to also improve access of the AdV vector particles to their relevant, CAR, receptors located on the basolateral surface (Bergelson *et al.*, 1997).

4.2.2 Methods and Results

To determine whether treatment of the apical cell surface could enhance AdV-mediated gene transfer, the effect of various concentrations of polidocanol on different cell lines was tested. The immortalised epithelial cell lines (MDCK, 16HBE, 1HAE, CFPAC-1 and A549) (section 3.1) were seeded on 12-well polycarbonate Transwell support membranes in growth media. The formation or otherwise of TJ's was monitored and confirmed by measurement of the transepithelial resistance (TR), as described in section 3.1.

(a) Effect of Polidocanol Treatment on Cell Viability

Initially, various doses of polidocanol were evaluated on each cell line to establish the maximum concentration of polidocanol that could be used without causing cell mortality, as assessed by the Trypan Blue exclusion assay (described in section 2.2.20). Briefly, the cells were grown to confluence and were then treated with various doses of polidocanol for different contact times as described in section 2.2.30. In each case the polidocanol preparation was applied to the apical surface of the cells, then aspirated and the cells washed twice with growth media to remove any residual polidocanol solution. With the exception of the MDCK cells, the 0.1% dose of polidocanol at any contact time resulted in a high degree of cell mortality (> 60%). As a result, the dose of polidocanol that resulted in low cell mortality (< 5%) was chosen as the treatment dose for the particular cell line (Table 4.1).

Table 4.1: Polidocanol Concentrations and Contact Times Used in the Adenovirus-Mediated Gene Transfer Studies.

Cell lines	Contact time (mins) of cells with various polidocanol concentrations		
	0.01%	0.05%	0.10%
MDCK	-	3, 5	2
16HBE	5, 10, 15	3	-
1HAE	5, 10, 15	-	-
CFPAC-1	5, 10, 15	-	-
A549	5, 10, 15	-	-

The cells were seeded on polycarbonate Transwell support membranes until confluent. The medium was then aspirated from the Transwell and the cells exposed to polidocanol (0.01 - 0.1%) for 2 - 15 mins as described in section 2.2.30. (-) not tested.

For the MDCK cells only, their TR was monitored during growth (section 3.1) and as shown in Table 4.2. The results shown in Table 4.2 demonstrate that the effect of 0.05% polidocanol on the TR is rapid, resulting in a significant (50%) reduction in TR within 3 mins of application; the decrease in TR continued for at least 1 hr, as shown in Table 4.2

(*P*< 0.001, ANOVA, SNK). The effect of polidocanol on TR was reversible; TR returned to pre-treatment values within 24 hrs of polidocanol treatment. PBS treatment did not significantly alter the TR (*P*= 0.481, ANOVA, SNK, n= 3), although statistical power (0.05) was inadequate for analysis.

Table 4.2: Effect of Polidocanol Treatment on the T_R of Polarised MDCK Cells.

	Prior to treatment	3 mins after treatment	1 hr after treatment	24 hrs after treatment
0.05% polidocanol for 5 mins	1059 ± 55	615 ± 95	309 ± 51	1051 ± 55
PBS	1017 ± 58	981 ± 35	962 ± 37	1056 ± 129

The cells were seeded on polycarbonate Transwell support membranes until a TR > 1000 Ωcm^2. The medium was then aspirated from the Transwell and the cells treated with 0.05% polidocanol for 5 mins. The polidocanol solution was then aspirated and the cells washed twice with PBS (37 °C). The TR of the MDCK cells was then monitored as shown. The values presented are the mean of the TR (Ωcm^2) of 3 samples ± standard deviation. Rx: treatment.

(b) Effect of Polidocanol Treatment on Adenovirus-Mediated Gene Transfer

One hr after polidocanol treatment the epithelial cells were infected with the AdV5CMVLacZ vector (MOI= 10) as described in section 2.2.30. Eighteen hrs later the cells were fixed and stained with X-gal solution to reveal LacZ gene expression. The number of LacZ positive cells was then counted as described in section 2.2.22; these results are summarised in Figure 4.3 (a, b).

For the 1HAE, CFPAC-1 and A549 cell lines, all of which are non-polarised, there was no significant difference on the level of AdV-mediated gene transfer between any of the polidocanol treatment groups and the control group (PBS treatment) [CFPAC-1 (*P*= 0.722); 1HAE (*P*> 0.05); A549 (*P*= 0.055); ANOVA, SNK, n= 3] (Figure 4.3; a).

For the 16HBE cell line there was 1-½ fold increase in the number of LacZ positive cells after treatment with the 0.05% dose of polidocanol for 3 mins (Figure 4.3; b), compared to treatment with either the 0.01% dose of polidocanol (at all contact times) or PBS (control) (Figure 4.3; a) (*P*< 0.05, ANOVA, SNK, n= 3). Furthermore, there was also an increase (1.4-fold) in the number of LacZ positive cells after treatment with 0.01% polidocanol for either 10 mins or 15 mins compared to the control cells (*P*< 0.05, ANOVA, SNK, n= 3).

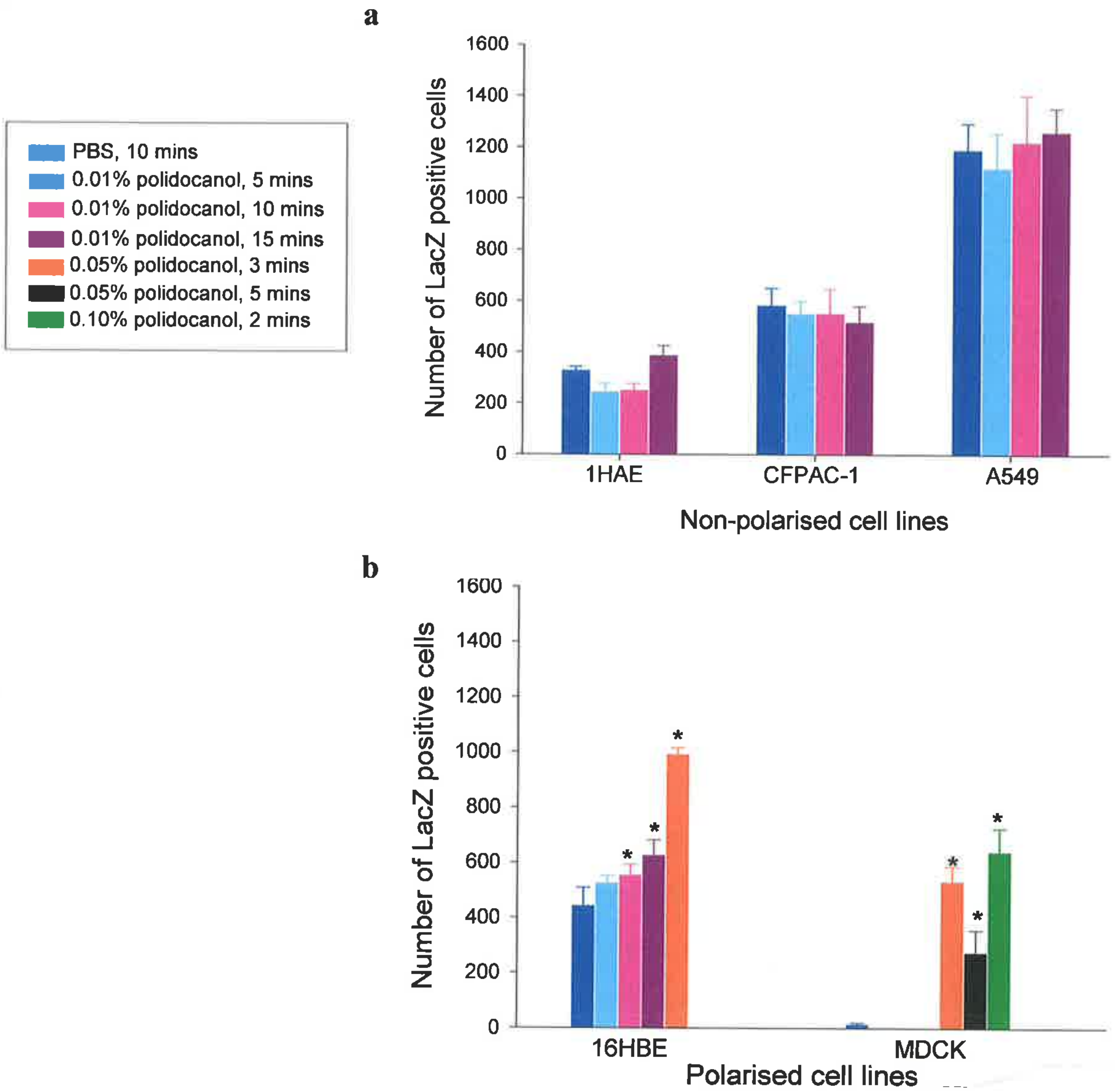

Figure 4.3: AdV-Mediated LacZ Gene Transfer Following Polidocanol Treatment of Cells. The cells were seeded on polycarbonate Transwell support membranes until confluent. The medium was then aspirated from the Transwell and the cells treated with polidocanol for the specified contact time. One hr later the cells were infected with the AdV5CMVLacZ vector (MOI= 10) as described in section 2.2.30. Eighteen hrs later the cells were fixed and stained with X-gal solution to reveal LacZ marker gene expression. The values presented are the mean of the total number of LacZ positive cells in 3 samples ± standard deviation. Control was treatment with PBS for 10 mins and exposure to the AdV vector 1 hr later. * *P*< 0.05 (ANOVA, SNK, n= 3).

For the MDCK cells, the 0.1 and 0.05% doses of polidocanol resulted in significantly improved levels of gene transfer compared to the control-treated MDCK cells ($P< 0.05$, ANOVA, SNK, n= 3). Interestingly, the increased contact time (5 min) of the 0.05% dose of polidocanol on the MDCK cells resulted in significantly reduced gene transfer compared to the shorter contact time of 3 mins ($P< 0.05$, Students t-test). The reason for this was most likely the detachment of the cells from the surface of the Transwell support, which occurred a few hours after this level of polidocanol treatment.

The results of these experiments suggest that polidocanol treatment of polarised cells (such as MDCK and 16HBE) prior to AdV vector exposure can result in improved levels of gene transfer compared to PBS-treated (control) cells. In non-polarised cells (such as 1HAE, CFPAC-1 and A549) treatment with polidocanol had no significant effect on the level of AdV-mediated gene transfer.

4.2.3 Discussion

The results of these experiments demonstrated that exposure of non-polarised cells to polidocanol prior to AdV vector infection did not improve the level of gene transfer. In contrast, polidocanol treatment of polarised cells (MDCK and 16 HBE) prior to AdV vector exposure improved the degree of gene transfer compared to control-treated cells (PBS treated and polidocanol treated non-polarised cells).

Although the transient opening of the epithelial TJ's was not directly shown here (e.g. by electron microscopy), polidocanol treatment of the polarised MDCK cells resulted in a significant reduction of the TR, an effect that is consistent with disruption of the epithelial TJ integrity (Parsons *et al.*, 1998).

In conclusion, the results of this study suggested that polidocanol treatment transiently permeabilised the TJ integrity of polarised cells. This approach increased access of the AdV vectors to the basolateral surfaces where their relevant receptors are located, consequently improving AdV-mediated gene transfer.

4.3 Transfer of Fluorescence-Labelled Adenovirus Vectors into Polarised Epithelial Cells Following Polidocanol Treatment

4.3.1 Introduction

The results of the experiments described in section 4.2 are consistent with published *in vivo* findings, which showed that polidocanol treatment of the apical surface of the airway epithelium can significantly enhance the efficiency of AdV-mediated gene transfer (Parsons *et al.*, 1998). Although none of the immortalised epithelial cell lines used in the studies described in section 4.2 were characterised by the presence of the overlaying mucus layer, which is typically present on the airway epithelium, the MDCK cell line possesses another barrier to viral gene transfer, the glycocalyx.

The glycocalyx is located on the apical surface of the airway epithelium. The potential role of this negatively-charged structure on AdV-mediated gene transfer has only recently received attention (Pickles *et al.*, 2000) and has been shown to limit viral-mediated gene transfer. In particular, AdV vector particles can become trapped in this "sticky" mesh-like structure (Pickles *et al.*, 2000) rendering unable to bind to their respective receptors located on the basolateral surface.

The experiments presented in this section were designed to examine the degree of TJ opening by assessing the proportion of AdV vector particles present in the basolateral regions of polarised MDCK and 16HBE cells following polidocanol treatment. The time course of AdV vector entry was monitored using AdV vector particles conjugated to a fluorescent dye immediately after polidocanol treatment.

4.3.2 Methods

(a) Preparation of Cells for Analysis by Fluorescence Microscopy

Fluorescence microscopy was used to examine the localisation of fluorescent AdV vector particles within the polidocanol-treated cells. A549, 16HBE and MDCK cells were each plated onto 24-well polycarbonate Transwell supports at a seeding density of 1.5 x 10^5 cells per Transwell. The non-polarised cell line (A549) was used as a control. When the A549, 16HBE and MDCK cells had reached confluence, and the 16HBE and MDCK cells became polarised (TR 16HBE ~ 600 Ωcm^2, TR MDCK > 1000 Ω cm^2) they were treated with the optimal dose of polidocanol, determined previously, for maximal AdV-mediated gene transfer (16HBE (n= 3) and MDCK cells (n= 3): 0.05% for 3 mins; A549 cells (n= 3): 0.01% for 15 mins). Immediately after polidocanol treatment the cells were washed with PBS (37 °C) to remove any residual polidocanol solution and exposed to 2.8×10^9 particles of the AdV5CMVLacZ vector labelled with the carboxymethyl-indocyanine dye Cy3 (AdV/Cy3) (prepared as described in section 2.2.23). Control treatment for all cell lines was performed with PBS for 5 mins (n= 3).

At 20 and 60 mins after exposure of the polidocanol-treated cells to the AdV/Cy3 vector particles, the apical surface was washed three times with PBS (37 °C) to remove any unbound virus particles and the cells were fixed immediately with 4% (w/v) paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (pH 7.3) for 15 mins at RT. The control (PBS) treated cells were fixed 20 mins after exposure to the AdVCy3 vector particles. The fluorescence of any surface-bound AdV/Cy3 vector particles was quenched using the Trypan Blue solution, as described by Chu and colleagues (1999). Briefly, the samples were treated with 0.1% (w/v) Trypan Blue solution in 0.9% NaCl solution for 5 mins on ice, the solution then aspirated, the cells washed once with PBS and the cell layer air-dried.

The membrane with the attached cells was then removed from the Transwell support and placed on a glass slide. A drop of anti-fade solution containing 1 μg/ml of 4',6'-diamidino-2-phenylindole (DAPI) (used to identify the cell nuclei) was added onto the membrane, which was then covered with a coverslip. The cells were analysed by fluorescence microscopy (Figure 4.4).

(b) Preparation of Cells for Analysis by Confocal Fluorescence Microscopy

Confocal fluorescence microscopy and X-Z sectioning was used to examine the diffusion of the AdV/Cy3 vector particles along the lateral walls of the polidocanol-treated MDCK cells. MDCK cells were plated onto 24-well polycarbonate Transwell supports at a seeding density of 1.5×10^5 cells per Transwell (n= 3). When the cells became polarised they were incubated with 10 mM carboxyfluorescein diacetate-succinimidyl ester (CFDA-SE) in DMEM/SF for 15 mins at 37 °C to provide a marker of cell viability. CFDA-SE passively diffuses into cells where cleavage of its acetate groups by intracellular esterases generates a highly fluorescent signal that is an indicator of live cells. The cells were then treated with 0.05% polidocanol for 3 mins, washed with PBS twice and then exposed to 2.8×10^9 AdV/Cy3 vector particles for 20 mins. The apical surface of the cells was washed twice with PBS (37 °C) to remove any unbound virus particles and the cells fixed immediately as described in section (a). The membrane with the attached cells was then removed from the Transwell support, placed on a coverslip and the cells analysed by confocal fluorescence microscopy.

4.3.3 Results

(a) Fluorescence Microscopy of Cells Exposed to the AdV/Cy3 Vector

Small numbers of AdV/Cy3 fluorescent signals were detected by fluorescence microscopy in the PBS-treated 16HBE cells (Figure 4.4; d), but these were not detected in the PBS-treated MDCK cells (Figure 4.4; g). Following polidocanol treatment of the 16HBE and MDCK cells there was an increase in the number of AdV/Cy3 (Red) signals with the majority of these located over and adjacent to the cell nuclei (Blue). Furthermore, the number of observed Cy3 signals was higher 60 mins after AdV/Cy3 vector infection of either 16HBE or MDCK cells (Figure 4.4; f, i, respectively), compared to the numbers seen 20 mins post-AdV/Cy3 infection (Figure 4.4; e, h, respectively).

These results suggest that although opening of the TJ's was initiated soon after treatment with polidocanol, the degree of TJ permeabilisation appears to be time-dependent. TJ permeabilisation had begun within 20 mins and continued to at least 60 mins post-polidocanol treatment, as indicated by the presence of the AdV/Cy3 vector particles, (Figure 4.4; e, h and f, i, respectively).

In contrast polidocanol treatment of the non-polarised A549 cells did not alter the number of AdV/Cy3 vector particles present in the cells when compared to the PBS-treated cells (Figure 4.4; a, b, c). These results are in agreement with previous findings (Figure 4.3; a), which demonstrated that polidocanol treatment of the A549 cells prior to exposure to the AdV vector did not improve gene transfer.

(b) Confocal Fluorescence Microscopy of Cells Exposed to the AdV/Cy3 Vector

It was obvious, under fluorescence microscopy, that AdV/Cy3 vector particles had outlined the edges of the polidocanol pre-treated MDCK cells in a pericellular-like fashion (Figure 4.5). Using X-Z confocal imaging analysis, a series of sixteen consecutive confocal Z-plane images (1 µm apart) were captured moving from the apical to the basolateral surface. These fluorescence images showed that some AdV/Cy3 vector particles had penetrated from the apical surface along the lateral cell walls towards the basal regions of the cell. This is apparent when the entry of an AdV/Cy3 vector particle cluster (arrows) is followed from image 4, Figure 4.5, at a depth of 1 µm, through to image 11, at a depth of 8 µm.

The average height of a MDCK cell is ~12 µm and in this culture system the nucleus is located close to the basolateral surface. It is therefore, more than likely that the AdV/Cy3 vector particles entered the cells *via* the permeabilised TJ's, as previously shown *in vivo* (Parsons *et al.*, 1998) and settled close to the cell nucleus, as demonstrated in Figure 4.4 (h, i). AdV/Cy3 signals were absent from the MDCK cell cultures not pre-treated with polidocanol (data not shown).

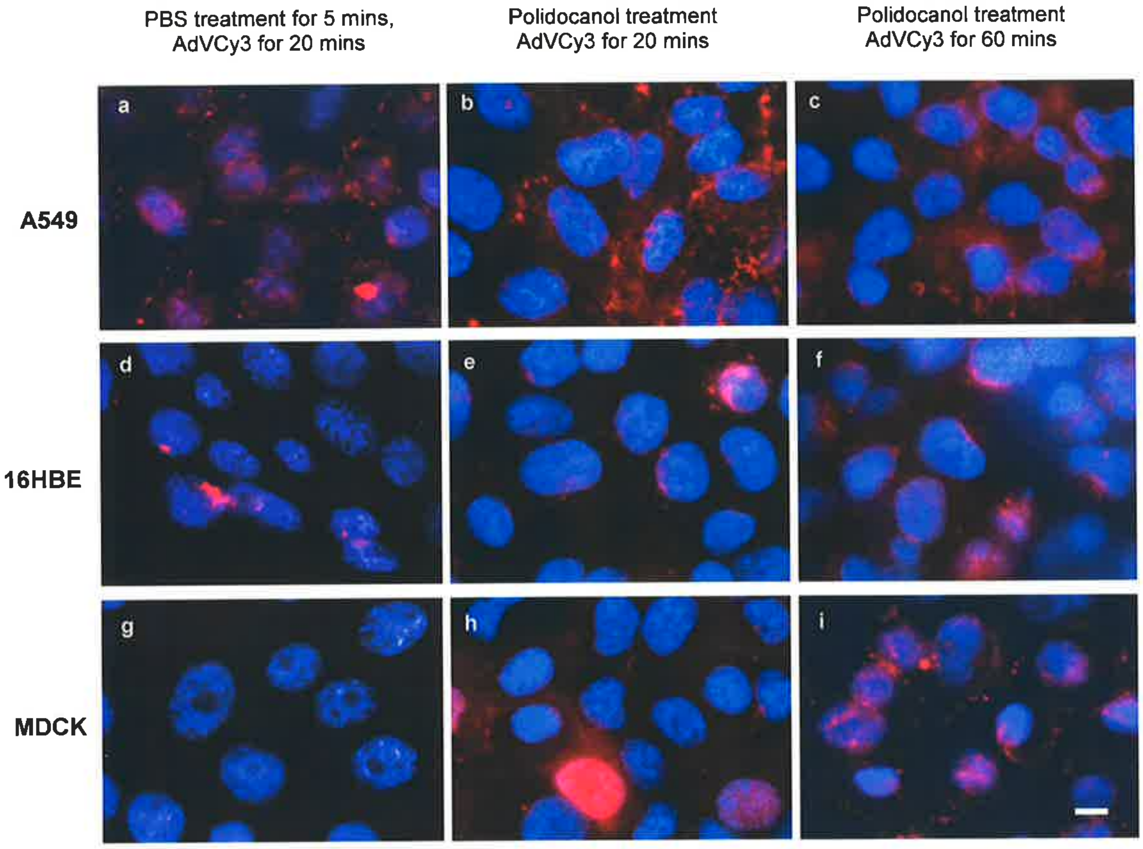
PBS treatment for 5 mins, AdVCy3 for 20 mins
Polidocanol treatment AdVCy3 for 20 mins
Polidocanol treatment AdVCy3 for 60 mins
A549
16HBE
MDCK
a
b
c
d
e
f
g
h
i

Figure 4.4: Fluorescence Analysis of the AdV/Cy3 Vector Particle Localisation in the DAPI-Stained Cell Nuclei Following Polidocanol Treatment.

The A549, 16HBE and MDCK cells were treated with polidocanol, washed twice with PBS and exposed to AdVCy3 vector particles. The cells were then fixed at 20 and 60 mins post- AdV vector infection. Cells were stained with DAPI to identify the nuclei, which appeared Blue, and the AdV vector particles, which were conjugated to Cy3, appeared Red. Scale bar= 10 μm.

4.3.4 Discussion

The results of the experiments performed in this section demonstrated that treatment of the polarised epithelium with the detergent polidocanol, alters the TJ integrity to allow the entry of AdV vector particles to the basolateral surface at a depth of ~8 μm. These results are in agreement with previously reported findings that demonstrated detergent treatment opens the epithelial TJ's to improve AdV-mediated gene transfer *in vivo* (Parsons *et al.*, 1998) The results represented in Figures 4.4 and 4.5 also show that detergent pre-treatment of polarised cells permits improved deposition of the AdV/Cy3 vector particles on the apical surface.

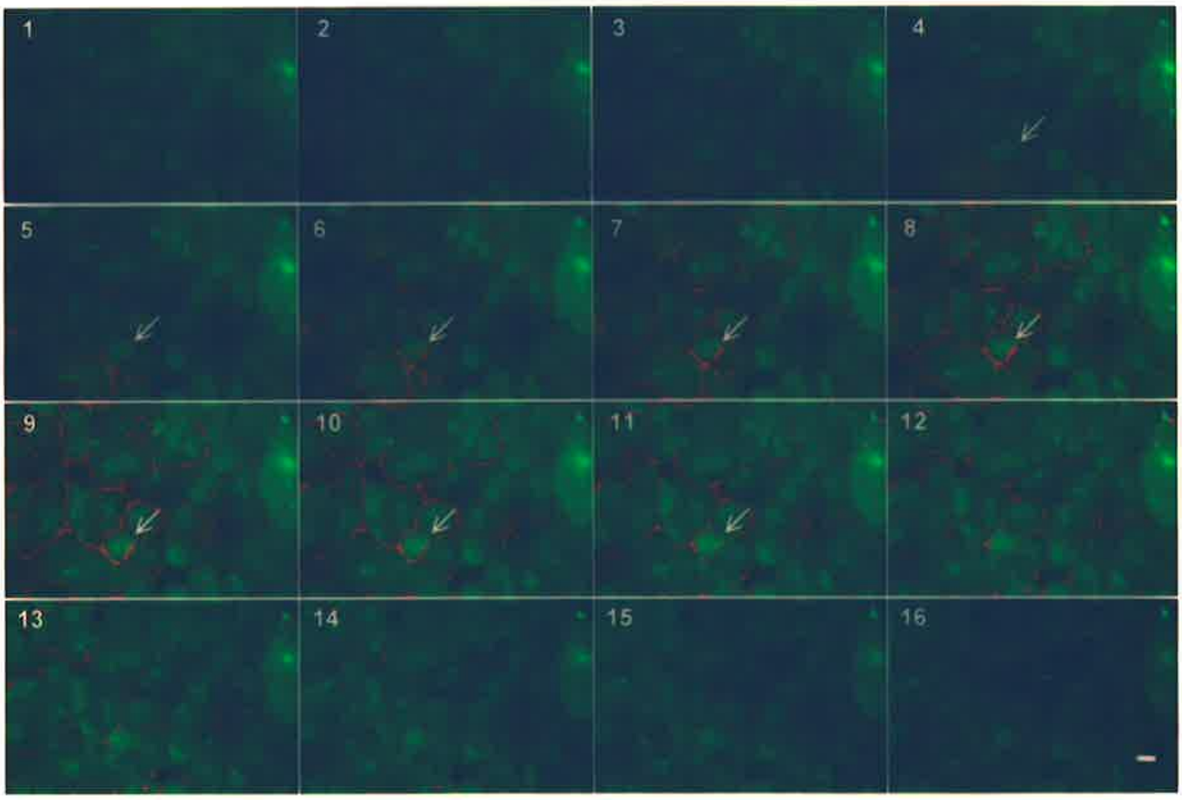

Figure 4.5: Confocal Fluorescence Analysis of the AdV/Cy3 Vector Particle Localisation in the CFDA-SE-Stained MDCK Cells Following Polidocanol Treatment.

Sixteen consecutive confocal Z-plane images (1 μm apart) were taken moving from the apical surface to the basal regions of the MDCK cells (Green) exposed to AdV/Cy3 vector particles (Red) 20 mins after polidocanol treatment. Arrow follows the entry of a specific AdV/Cy3 vector particle cluster from the apical to the basolateral cell regions. Scale bar= 10 μm.

There may be an additional reason why AdV-mediated gene transfer was improved following polidocanol treatment. Polidocanol, as a detergent, modulates TJ integrity and may destroy or alter the glycocalyx structure present on the apical surface of the MDCK cells (Pickles *et al.*, 2000). As the glycocalyx is a barrier to efficient viral-mediated gene transfer, altering its structure will significantly facilitate gene delivery.

The results from the experiments described in this section did not provide sufficient evidence as to whether modifications of the TJ's, the glycocalyx or both occurred. Nonetheless, the results do indicate that in the presence of polidocanol the barriers presented by the TJ's and the "sticky" mesh-like glycocalyx can be overcome *in vitro* to improve AdV vector particle delivery to the basolateral regions.

Leopold *et al.* (1998) used AdV/Cy3 vector particles to assess the rate of AdV vector entry into the non-polarised A549 cells, as well as its translocation to the nucleus. Some of the techniques developed by Leopold *et al.* could be applied to the current study design so as to investigate the mechanisms by which polidocanol-based airway modulation, improves AdV vector entry as well as its translocation to the nucleus *in vitro*.

CHAPTER 5

LENTIVIRUS MEDIATED GENE TRANSFER *IN VITRO*

Preface

This chapter is divided into two parts. Part A details the construction of various lentiviral vectors and the optimisation of the lentivirus vector stock production protocol. A number of technical issues that affected the use of both the β-galactosidase, LacZ, and the enhanced yellow fluorescence protein, EYFP, marker genes will also be covered.

Part B describes the LV-mediated gene transfer studies performed in immortalised epithelial cell cultures and in cultures of primary airway epithelial cells grown at air liquid interface. The effect on the efficiency of LV-mediated gene transduction of the cells in the explanted rat nasal septa, which were pre-treated with a detergent, is also described.

Part A

Technical Development

5.1 Lentiviral Vector Constructs

5.1.1 Introduction

As reviewed in Chapter 1, possibly one of the most promising strategies in developing gene therapy for CF airway disease is the identification of an integrative gene transfer vector, which can effectively deliver the CFTR gene into the quiescent cells of the airway epithelium and also result in sustained expression of the CFTR gene, so as to produce a therapeutic effect. Since the lentivirus (LV) vector derived from HIV-1 has these properties it was evaluated in this thesis project for its potential as a gene transfer vector for CF gene therapy.

This section will describe the process of cloning the LacZ marker gene, as well as the CFTR gene, into the various LV vector constructs. In addition, the helper plasmid system used for the generation of replication-defective LV vector stocks is also briefly described. Theoretically, replication-competent retrovirus (RCR) could be generated by recombination of the elements of the LV vector production system (the LV vector and packaging constructs) with each other or with endogenous viral sequences in the cell lines, which are used to produce the LV vector. As a result, special care is required to minimise the chance of RCR generation. The possibility of such recombination events occurring in the LV vector production system was minimised here by separating the *trans* packaging functions onto several different plasmids, and by reducing homology between the different constituents of the LV vector production system (Fuller and Anson, 2001).

5.1.2 Methods and Results

(a) Construction of the pBlHIVext5SV40LacZppt$^+$RRELTR vector

The pBlHIVext5SV40ppt$^+$RRELTR vector (Anson and Fuller, in preparation) contained from 5' to 3' a series of sequences including (i) the HIV-1 YU-2 5' viral long terminal repeat (LTR) and contiguous sequence extending 1150 bp into the *gag* gene, (ii) the SV40 immediate early promoter, (iii) the HIV-1 YU-2 polypurine tract (PPT) and the 25 bp sequence immediately 5' of the PPT, and (iv) the 3' LTR with the rev response element

(RRE) replacing sequences in the U3 region. This strategy rendered the LV vector self-inactive, as vital transcriptional elements in the U3 region were replaced by the RRE sequence.

The pBlHIVext5SV40EYFPppt$^+$RRELTR (LVEYFP) vector (Anson and Fuller, in preparation; Figure 5.1; i) was firstly modified by deletion of the *Sal*I site flanking the 3' end of the LV vector construct. This was done by digestion with *Sal*I, followed by blunt ending with the Klenow fragment and then re-ligation. The EYFP gene sequence was then removed by digestion with *EcoR*I and replaced with a short linker containing the *BamH*I and *Sal*I sites. Recombinants containing the linker in the correct orientation (5' *BamH*I - 3' *Sal*I) were identified by restriction with *BamH*I and *Cla*I. The LV vector construct was then restricted with *BamH*I/*Sal*I, de-phosphorylated with CIP, phenol/ chloroform extracted and precipitated.

The LacZ marker gene sequence (3.1 kb) was excised from pHR'CMVLacZ (Naldini *et al.*, 1996a) with *BamH*I and *Xho*I, gel purified and cloned into the *BamH*I and *Xho*I sites of the pBCKS (+) plasmid. This construct was designated pBCKSLacZ. The LacZ gene sequence was excised from pBCKSLacZ by digestion with *BamH*I and *Xho*I and ligated into the *BamH*I/*Sal*I restricted pBlHIVext5SV40ppt$^+$RRELTR vector construct and transformed into MC1061 cells.

Clones were analysed by restriction enzyme digestion. A recombinant of the desired type was identified and then designated pBlHIVext5SV40LacZppt$^+$RRELTR (LVLacZa) (Figure 5.1; ii).

(b) Construction of the pBCKSHIVext5cpptSV40LacZppt$^+$RRELTR vector

This vector construct is a further development of the pBlHIVext5SV40ppt$^+$RRELTR construct described above. Specifically, it contains the HIV-1 YU-2 central polypurine tract (cPPT) and termination signal sequence immediately 5' of the SV40 promoter sequence (Anson and Fuller, in preparation). This modification resulted in a 1½-fold increase of viral titre. The HIV-1 cPPT is critical for the formation of the central DNA flap, which is involved in viral genome nuclear import and has been shown to significantly improve transduction of quiescent cells by HIV-1 derived LV vectors (Zennou *et al.*, 2000). For this reason, the evaluation of this LV vector construct in gene transfer studies was considered worthwhile.

The pHR'CMVLacZ plasmid (Naldini *et al.*, 1996a) was digested with *BamH*I and *Xho*I, made blunt-ended, phenol/chloroform extracted and precipitated. The pBCKSHIV-ext5cpptSV40EYFPppt$^+$RRELTR was digested with *EcoR*I to remove the EYFP gene sequence, blunt-ended, de-phosphorylated with CIP, phenol/chloroform extracted and precipitated. The 2 digested plasmids were then ligated together and electroporated into DH10B cells.

Clones were analysed by restriction enzyme digestion. A recombinant of the desired type was identified and then designated pBCKSHIVext5cpptSV40LacZppt$^+$RRE LTR (LVLacZb) (Figure 5.1; iii).

(c) Construction of the pBCKSHIVext4cRRExtcpptSV40LacZppt$^+\Delta$LTR vector

This vector construct is a further development of the pBCKSHIVext5cpptSV40LacZppt$^+$-RRELTR construct described above. In particular, the length of the *gag* gene sequence was reduced to 550 bp and an extended RRE sequence was positioned immediately 5' of the cPPT rather than in the 3' LTR. The vector construct was then made self-inactivating by deleting the sequences between the *EcoR*V and *Pvu*II sites in the 3' LTR (Anson and Fuller, in preparation).

The pBCKSHIVext4cRRExtcpptSV40EYFPppt$^+\Delta$LTR was digested with *EcoR*I to remove the EYFP gene sequence, made blunt-ended, phenol/chloroform extracted, precipitated and then digested with *BamH*I, de-phosphorylated with CIP, phenol/chloroform extracted and precipitated. The pHR'CMVLacZ plasmid (Naldini *et al.*, 1996a) was digested with *Xho*I, blunt-ended, phenol/chloroform extracted and precipitated. It was then digested with *BamH*I and gel purified as described in section 2.2.4 (b). The 2 digested plasmids were then ligated and electroporated into DH10B cells.

Clones were analysed by restriction enzyme digestion. A recombinant of the desired type was identified and then designated pBCKSHIVext4cRRExtcpptSV40LacZ-ppt$^+\Delta$LTR (LVLacZc) (Figure 5.1; iv).

(d) Construction of the pBCKSHIVext4m2cRRExtcpptSV40CFTRppt$^+\Delta$LTR vector

This construct is similar to the pBCKSHIVext4cRRExtcpptSV40ppt$^+\Delta$LTR vector construct described above with the difference that the *gag* reading frame was blocked by mutagenesis of the ATG codons at bases 788 and 1298 of the HIV-1 YU-2 sequence to TAG stop codon sequences (Anson and Fuller, in preparation).

The pBCKSHIVext4m2cRRExtcpptSV40EYFPppt$^+\Delta$LTR was digested with *EcoR*I to remove the EYFP gene sequence, blunt-ended, de-phosphorylated with CIP, phenol/chloroform extracted and precipitated. The pCMVCFTR-936C plasmid (Gregory *et al.*, 1990) was digested with *EcoR*V and *Pst*I, phenol/chloroform extracted and precipitated. The 2 digested plasmids were ligated together and electroporated into DH10B cells.

Clones were analysed by restriction enzyme digestion. A recombinant of the desired type was identified and designated pBCKSHIVext4m2cRRExtcpptSV40CFTR-ppt$^+$ΔLTR (LVCFTR) (Figure 5.1; v).

(e) The Lentivirus Vector Packaging System

To improve the safety with which LV vector stocks can be prepared, the relevant HIV-1 reading frames, *gagpol, tat* (exon 1) and *rev* were expressed from separate plasmid constructs (Fuller and Anson, 2001). The *gagpol* sequence was expressed using a codon optimised gene sequence. Since the addition of *Tat* increased the titre of the LV vector by almost 3-fold (Fuller and Anson, 2001) the first exon of the YU-2 *tat* gene was isolated by PCR and cloned into the pcDNA3.1 expression plasmid (pcDNA3-*Tat*). Subsequently, an expression construct using a codon optimised *Tat* gene sequence, pcDNA3Tat101ml (Anson, unpublished), was also used. *Rev* was expressed from a previously described construct pCMV-*rev* (Lewis *et al.,* 1990) and was shown to be absolutely necessary for efficient LV vector production. This is most likely due to the dependence of the LV vector on the Rev/RRE system for efficient virus packaging (Fuller and Anson, 2001). The pHCMV-G plasmid (Yee *et al.*, 1994) encoding the vesicular stomatitis virus glycoprotein G was used to pseudotype the LV vector.

5.1.3 Summary

In this section a cloning strategy was utilised to facilitate blunt-ended cloning of the relatively large LacZ and CFTR gene sequences into the designated LV vector constructs. By shuttling the gene sequences of interest between plasmids expressing either chloramphenicol resistance (pBCKS) or ampicillin resistance (pBl) the need to gel purify the fragments to be cloned was negated. LV vector constructs, expressing the LacZ marker gene, were made contiguously as different LV vector constructs became available. This simply reflects the on-going development of the LV vector system.

The transduction efficiency of the LVLacZ vectors was then evaluated *in vitro* (Part B) and subsequently the gene transfer protocols were optimised in the nasal epithelium of C57Bl/6 mice (Chapter 6, Part A). The CFTR cDNA gene sequence was then cloned into the most efficient LV vector construct (pBCKSHIVext4m2cRRExtcppt-SV40ppt$^+$ΔLTR) and its transduction efficiency then evaluated in the nasal epithelium of transgenic CF mice (Chapter 6, Part B).

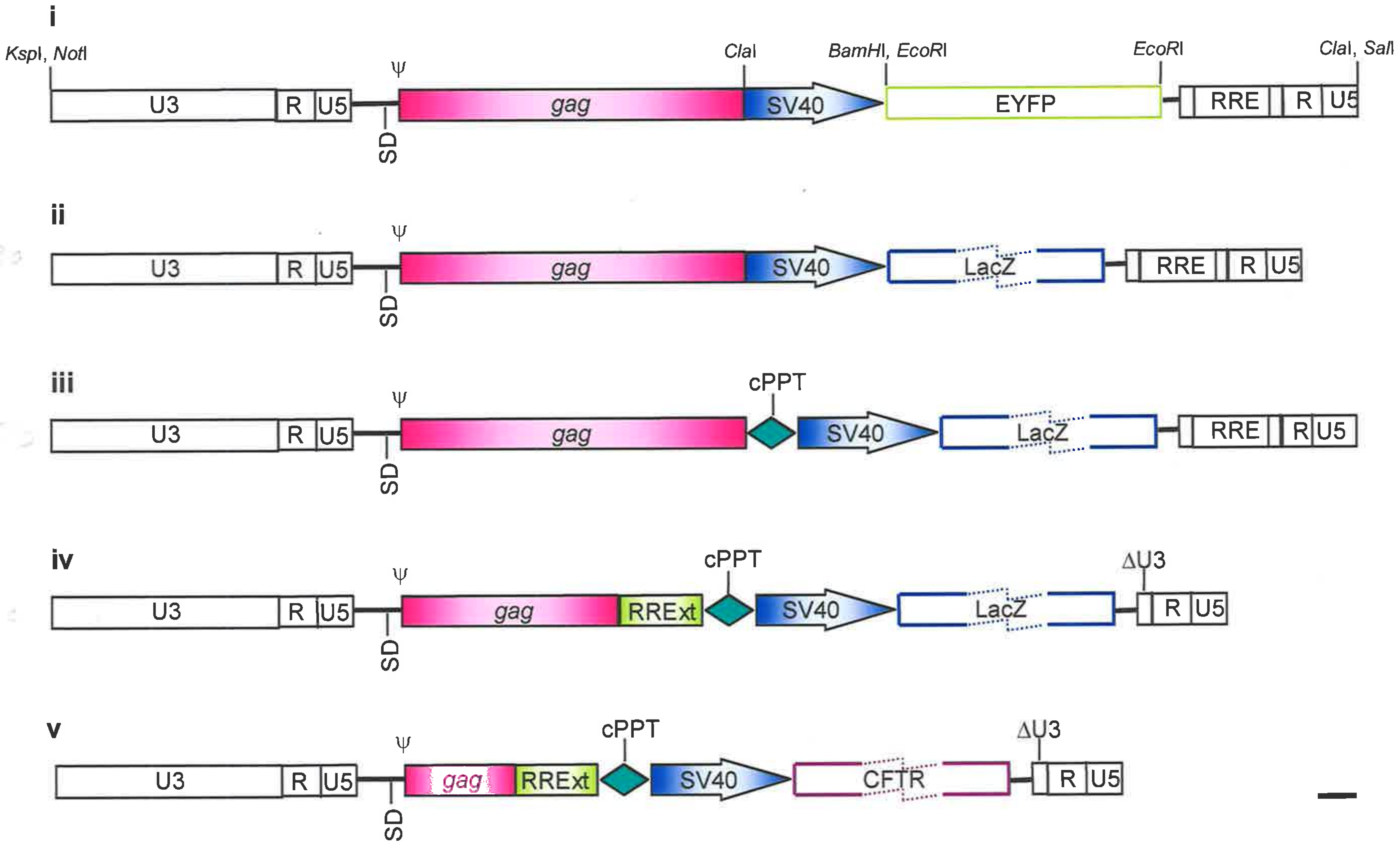
i
KspI, NotI
ψ
ClaI
BamHI, EcoRI
EcoRI
ClaI, SalI
U3
R
U5
SD
gag
SV40
EYFP
RRE
R
U5
ii
ψ
U3
R
U5
SD
gag
SV40
LacZ
RRE
R
U5
iii
ψ
cPPT
U3
R
U5
SD
gag
SV40
LacZ
RRE
R
U5
iv
ψ
cPPT
ΔU3
U3
R
U5
SD
gag
RRExt
SV40
LacZ
R
U5
v
ψ
cPPT
ΔU3
U3
R
U5
SD
gag
RRExt
SV40
CFTR
R
U5

Figure 5.1: The Structures of the Lentiviral (EYFP, LacZ, CFTR) Vector Constructs.

The various LV vector constructs (i) pBIHIVext5SV40EYFPppt$^+$RRELTR, (ii) pBIHIVext5SV40LacZ-ppt$^+$RRELTR, (iii) pBCKSHIVext5cpptSV40LacZppt$^+$RRELTR, (iv) pBCKSHIVext4cRRExtcpptSV40-LacZppt$^+$ΔLTR and (v) pBCKSHIVext4m2cRRExtcpptSV40CFTRppt$^+$ΔLTR are shown. The long terminal repeat is represented, as a white box comprised of the U3, R and U5 sequences. SD= major splice donor; ψ= packaging signal; RRE= Rev response element; cPPT= central polypurine tract; *gag*= *gag* gene sequence; SV40= simian virus 40 early promoter; EYFP= enhanced yellow fluorescence protein gene coding sequence; LacZ= β-galactosidase gene coding sequence; CFTR= cystic fibrosis transmembrane conductance regulator gene coding sequence. The pertinent restriction sites are noted in the pBIHIVext5SV40EYFPppt$^+$RRELTR vector construct only. The RRE sequence in the vector constructs (i), (ii) and (iii) was cloned between the *EcoR*V and *Pvu*II sites present in the 3' LTR replacing the HIV-1 YU-2 sequence between these sites. In the vector constructs (iv) and (v) an extended RRE sequence was positioned immediately 5' of the cPPT sequence. Scale bar= 100 bp.

5.2 Optimisation of Lentivirus Vector Production in 293T Cells

5.2.1 Introduction

The majority of protocols used to generate LV vector stocks are based on transient expression of plasmids encoding the LV vector, the required HIV-1 proteins (*gagpol, tat* and *rev*) and the vesicular stomatitis virus protein G (VSV-G) envelope, to pseudotype the LV vector, (Naldini *et al.*, 1996a) in 293T cells. In an attempt to improve the production of the LV vector stocks several parameters of the LV vector production protocol were optimised. The transfection protocol, the optimal incubation time of the transfection agent with the cells, the amount of FCS present in the collection medium and the optimal collection time of the LV vector supernatant were all assessed. The LV vector production protocol was established using the following plasmids (i) the LVEYFP vector (Anson and Fuller, in preparation; Figure 5.1; i), which expresses the EYFP protein and allows for titre estimation by FACScan analysis; (ii) the HIV-1 packaging plasmid pCMVΔRnr that expresses all the HIV-1 *trans* functions with the exception of v*pr*, v*if* and e*nv* (Kafri *et al.*, 1997); and (iii) pHCMV-G (Yee *et al.*, 1994), which encodes the VSV-G envelope.

5.2.2 Methods and Results

(a) Selection of a DNA Transfection Protocol for Lentivirus Vector Production

Four different DNA transfection protocols for the production of the LV vector stocks were compared. 293T cells were plated at 1.8 x 10^6 cells per dish into 60-mm culture dishes and 24 hrs later transfected with a total of 6 μg of DNA (2 μg of each of the following plasmids, pBlHIVext5SV40EYFPppt$^+$RRELTR, pCMVΔRnr and pHCMV-G), by (i) $CaPO_4$ co-precipitation, (ii) DOTAP transfection, (iii) Fugene-6 transfection or (iv) Lipofectamine transfection, as described in section 2.2.24. Seven hrs later, the transfection medium was replaced with 4 ml of DMEM/10% (v/v) FCS and the virus collected 24 hrs later. The titre of the LV vector was then determined on A549 cells as described in section 2.2.28. The viability of the 293T cells was evaluated at the end of the 7 hr transfection period using the Trypan Blue exclusion assay as described in section 2.2.20. The results of this experiment are shown in Table 5.1.

Table 5.1: Titre of the LVEYFP Vector Produced by Four Different Transfection Protocols.

Transfection Agent	Titre x 10^5 (A549-TU/ml)	293T Cell Viability (%)
$CaPO_4$	3.9 ± 0.3 *	> 95 (n= 1)
DOTAP	1.5 ± 0.1	> 95 (n= 1)
Fugene-6	2.6 ± 0.2	> 95 (n= 1)
Lipofectamine	2.3 ± 0.2	~80 (n= 1)
Control: non-transfected cells	N/A	> 95 (n= 1)

The LV vector was generated by (i) $CaPO_4$ co-precipitation, (ii) DOTAP transfection, (iii) Fugene-6 transfection or (iv) Lipofectamine transfection of 293T cells using 2 µg of each of the following plasmids, pBIHIVext5SV40EYFPppt$^+$RRELTR, pCMVΔRnr and pHCMV-G. Seven hrs later the transfection medium was replaced with DMEM/10% (v/v) FCS. Twenty-four hrs later NIH3T3 cells were transduced with the LV vector to determine the viral titre. The $CaPO_4$ co-precipitation transfection protocol produced significantly higher viral titres compared to those attained by the DOTAP, Fugene-6 and Lipofectamine transfection protocols ($^*P< 0.001$, ANOVA, Dunnett's method, n= 6). The viability of the 293T cells was assessed at the end of transfection in one sample of cells using the Trypan Blue assay as described in section 2.2.20. Values are presented as a mean of 6 samples ± SEM, unless otherwise indicated. N/A= not applicable, TU= transducing units.

The Lipofectamine-mediated transfection protocol resulted in a higher degree of toxicity (evident as ~20% of 293T cells staining blue in the Trypan Blue exclusion assay) compared to the $CaPO_4$ co-precipitation, DOTAP or Fugene-6 transfection protocols, all of which did not result in significant cell mortality (< 5%). The results shown in Table 5.1 indicate that the $CaPO_4$ co-precipitation transfection protocol produced significantly higher viral titres compared to those attained by the DOTAP, Fugene-6 and Lipofectamine transfection protocols. As a result, the $CaPO_4$ co-precipitation transfection protocol was used for all subsequent productions of LV vector stocks.

(b) Selection of the Optimal Incubation Time of the $CaPO_4$ Co-Precipitate with the Cells

The next parameter that was assessed was the optimal incubation time of the 293T cells with the $CaPO_4$-DNA precipitate for the production of high titre LV vector stocks. These experiments were performed using the LV vector and LV packaging helper plasmid system, which was developed in our lab (Anson and Fuller, in preparation; Fuller and Anson, 2001).

293T cells were plated at 1.8 x 10^6 cells per dish into 60-mm culture dishes and 24 hrs later transfected with a total of 6 µg of DNA (1.2 µg of each of the following plasmids LVEYFP, pcDNAgagpolml, pCMV-*rev*, pcDNA3Tat and pHCMV-G) and incubated for 7, 16 or 24 hrs. The medium was then replaced with 4 ml of DMEM/10% (v/v) FCS and the cells maintained in culture for a further 24 hrs. The LV vector supernatant (4 ml) was then collected and titred on A549 cells as described in section 2.2.25. The results of this experiment are shown in Table 5.2.

Table 5.2: Titres of the LVEYFP Vector Produced in 293T Cells Using Different Incubation Times with the $CaPO_4$-DNA Complexes.

Incubation Time (hrs)	Titre x 10^5 (A549-TU/ml)
7	2.9 ± 0.3
16	3.5 ± 0.2
24	3.0 ± 0.1

The LV was generated by $CaPO_4$ co-precipitation transfection of 293T cells using 1.2 µg of each of the following plasmids: pBIHIVext5SV40EYFPppt$^+$RRELTR, pcDNAgagpolml, pCMV-*rev*, pcDNA3Tat and pHCMV-G. The transfection medium was replaced with DMEM/10% (v/v) FCS after 7, 16 or 24 hrs. Twenty-four hrs later A549 cells were transduced with the LV vector to determine the viral titre. Values are presented as a mean of 3 samples ± SEM. TU= transducing units.

There was no significant variation in the titre of the LV vector produced following the three different incubation times (7, 16 or 24 hrs) (*P*= 0.182, ANOVA, SNK); however, the low power of the test (0.05) indicated that the number of samples was insufficient to allow for statistical comparison. The three different incubation times resulted in the production of LV vector supernatants of similar viral titres. Nevertheless, as the 16 hr incubation time resulted in a slightly higher viral titre compared to the 7 and the 24 hr incubation times, it was chosen as the optimal incubation time for all subsequent LV vector stock productions.

(c) Collection of the Lentivirus Vector in DMEM Containing Varying Amounts of FCS

High titre LV vector stocks were routinely prepared in our lab using a combination of ultra-filtration and ultra-centrifugation procedures as described in section 2.2.26. However, the ultra-filtration procedure was found to be time-consuming, presumably due to the presence of 10% FCS in the collection medium. It was also observed that the subsequent ultra-centrifugation procedure resulted in the formation of a pellet that was difficult to re-suspend. Therefore, the minimum amount of FCS required in DMEM to allow for efficient production of LV vector stocks was evaluated.

293T cells were transfected with the LV vector/packaging helper plasmid system as described previously in (b). The transfection medium was then replaced with DMEM containing varying amounts of FCS (0, 2, 5 and 10% (v/v)) for 24 hrs. The LV vector supernatant was then collected and the viral titre determined on A549 cells as previously described. The results of this experiment are shown in Table 5.3.

Table 5.3: Titre of the LVEYFP Vector Collected in DMEM Containing Varying Amounts of FCS.

FCS (%)	Titre x 10^5 (A549-TU/ml)
0	1.7 ± 0.1
2	2.1 ± 0.1
5	3.1 ± 0.2 *
10	2.8 ± 0.1 *

The LV was generated by $CaPO_4$ co-precipitation transfection of 293T cells using 1.2 µg of each of the following plasmids: pBIHIVext5SV40EYFPppt$^+$RRELTR, pcDNAgagpolml, pCMV-*rev*, pcDNA3Tat and pHCMV-G. Sixteen hrs later the transfection medium was replaced with DMEM containing varying amounts of FCS. Twenty-four hrs later A549 cells were transduced with the LV vector supernatant to determine the viral titre. Collection of the LV vector in DMEM supplemented with 5 or 10% (v/v) FCS resulted in the production of a significantly higher viral titre compared to that of LV vector stocks collected in either DMEM/SF or DMEM/2% (v/v) FCS (*$P < 0.001$, ANOVA, SNK, n= 3). Values are presented as a mean of 3 samples ± SEM. TU= transducing units.

The results of this experiment demonstrated that collection of the LV vector in DMEM supplemented with 5% (v/v) FCS allowed the production of the highest titre of LV vector stocks. Furthermore, this viral titre was significantly greater than that of LV vector stocks collected in either DMEM/SF or 2% FCS (v/v) but was no different to that of the LV vector stocks collected in DMEM containing 10% (v/v) FCS. Based on the rationale of using the least amount of FCS necessary for efficient production of LV, 5% (v/v) FCS was chosen to supplement the medium for collection of LV vector stocks.

(d) Optimal Time for Collection of Lentivirus Vector Supernatant

The final parameter that was examined in this series of experiments was the optimal time of collection of the LV vector supernatant so as to obtain a high viral titre. 293T cells were transfected as previously described in (b) for 16 hrs. The medium was then aspirated and 4 ml of DMEM/5% (v/v) FCS was added and the cells further maintained in culture. The LV vector supernatant was collected at three time points (24, 48 or 72 hrs). A549 cells were then transduced with the LV vector and the viral titre determined as previously described in (b). The results of this experiment are shown in Table 5.4.

Table 5.4: Titre of the LVEYFP Vector Collected at Various Time Points Following Transfection by $CaPO_4$ Co-Precipitation.

	Collection Time (hrs)	
24	48	72
$4.0 \pm 0.2 \times 10^5$	$3.6 \pm 0.4 \times 10^5$	$2.2 \pm 0.2 \times 10^5$*

The LV was generated by $CaPO_4$ co-precipitation transfection of 293T cells using 1.2 μg of each of the following plasmids pBIHIVext5SV40EYFPppt$^+$RRELTR, pcDNAgagpolml, pCMV-*rev*, pcDNA3Tat and pHCMV-G. Sixteen hrs later the transfection medium was replaced with DMEM/5% (v/v) FCS and 24, 48 or 72 hrs later the LV vector supernatant was collected. A549 cells were transduced with the LV vector stocks to determine the viral titre. The titre of the LV vector collected at 72 hrs was significantly lower than that of the LV vector collected at either 24 or 48 hrs (*$P < 0.05$, ANOVA, SNK, n= 3); there was no difference in the titre of the LV vector collected at 24 or 48 hrs (*$P > 0.05$, ANOVA, SNK, n= 3). Values are presented as a mean of 3 samples ± SEM.

The titre of the LV vector collected at 72 hrs was significantly lower than that of the LV vector collected at 24 or 48 hrs. Since there was no significant difference in the titre of the LV vector collected at 24 or 48 hrs, the shorter time (i.e. 24 hrs) was chosen as the optimal collection time in the LV vector stock production protocol.

In summary, for optimal LV vector stock production, 293T cells were transfected by the $CaPO_4$ co-precipitation method for 16 hrs and the LV vector collected in DMEM/5% FCS (v/v) over 24 hrs. The LV vector supernatant was then filtered and concentrated as described in section 2.2.26.

The optimised protocol for LV vector production was utilised for large-scale generation of LV vector stocks essentially using the helper plasmid system developed by Fuller and Anson (2001). Specifically, five different plasmids including 14 µg of the LV vector, 3 µg of pcDNA3gagpolml (Fuller and Anson, 2001), 14 µg of pHCMV-G (Yee *et al.*, 1994), 4 µg of pCMV-*rev* (Lewis *et al.*, 1990) but 0.2 µg of pcDNA3Tat101ml (Anson, unpublished) were used in place of pcDNA3Tat; the amount of each plasmid added was optimised by Dr. D. S. Anson (Department of Chemical Pathology, WCH). Three different LVLacZ vector stocks were produced (as described in section 2.2.24, a) using the LVLacZa, LVLacZb and LVLacZc vector constructs (Figure 5.1; ii, iii and iv, respectively). The LVLacZ vector stocks were then concentrated as described in section 2.2.26 and the titre determined on NIH3T3 cells as described in section 2.2.25 (Table 5.5).

When large-scale generation of LVCFTR vector stocks was required, the optimised protocol for LV vector stock production, described previously, was utilised. The LVCFTR vector stocks were then concentrated ~1000-fold as described in section 2.2.26. To determine the titre of the LVCFTR vector stocks, NIH3T3 cells were transduced with 20 µl of either of the concentrated LVCFTR vector or the concentrated LVEYFP vector of known titre (5×10^5 NIH3T3-TU/ml) in the presence of polybrene (4 µg/ml) as described in section 2.2.25. Serial dilutions containing 1 µg, 100 and 10 ng of the DNA prepared from the LVCFTR and LVEYFP transduced cells were amplified using the primers Ext 4F and Ext 4R, which were designed to amplify a 306 bp portion of the *gag* 5' nucleotide sequence in the LV vector construct, as described in section 2.2.16 (b). The PCR products were analysed by agarose gel electrophoresis (Figure 5.2).

The titre of the LVCFTR vector stock was then estimated by quantitativcly comparing the amount of the amplified DNA present in lanes 1 - 3 with the amount of DNA prepared from cells transduced with a vector of known titre (LVEYFP) in Lanes 4 - 6. Following the comparison of the intensity of the amount of DNA present in lane 3 (10 ng of DNA prepared from LVCFTR transduced cells) to that present in lane 6 (10 ng of DNA prepared from LVEYFP transduced cells) it was evident that the titre of the LVCFTR vector was approximately 2 - 5-fold higher than that of the LVEYFP vector stocks. Therefore the titre of the concentrated LVCFTR vector was estimated to be ~1 - 2×10^6 NIH3T3-TU/ml (Table 5.5).

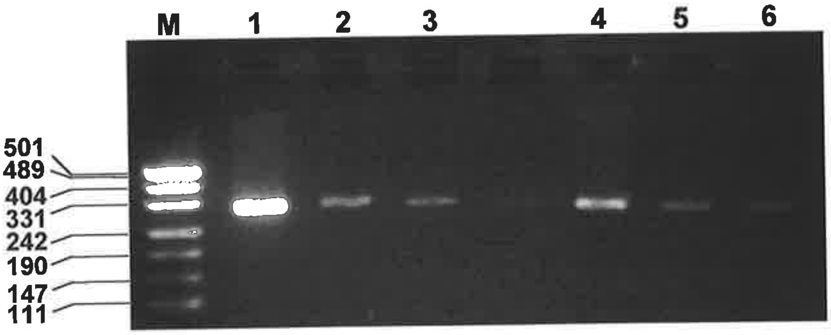

Figure 5.2: Quantitative Analysis of the Titre of the LVCFTR Vector Stock.

DNA was prepared from NIH3T3 cells transduced with either 20 µl of the LVCFTR vector (of unknown titre) or 20 µl of the LVEYFP vector containing 1 x 10^4 NIH3T3-TU. Serial dilutions containing 1 µg, 100 and 10 ng of DNA were then amplified as described in section 2.2.16 (b). The PCR products were analysed on a 2% agarose, 1 x TBE gel. M, pUC19/*Hpa*II molecular weight markers, the sizes of the relevant bands are shown to the left of the figure. Lanes 1, 2 and 3: serial dilutions (1 µg, 100 and 10 ng, respectively) of DNA from LVCFTR transduced cells; Lanes 4, 5 and 6: serial dilutions (1 µg, 100 and 10 ng, respectively) of DNA from LVEYFP transduced cells.

Table 5.5: Titre of the Various Concentrated Lentivirus Vector Stocks.

LV Vector Stocks	**Titre x 10^6 (NIH3T3-TU/ml)**
pBIHIVext5SV40LacZppt$^+$RRELTR **(LVLacZa)**	0.4
pBCKSHIVext5cpptSV40LacZppt$^+$RRELTR **(LVLacZb)**	3.0
pBCKSHIVext4cRRExtcpptSV40LacZppt$^+$ΔLTR **(LVLacZc)**	7.0
pBCKSHIVext4m2cRRExtcpptSV40CFTRppt$^+$ΔLTR **(LVCFTR)**	1.0 - 2.0

The LV was generated by $CaPO_4$ co-precipitation transfection of 293T cells with five different plasmids including 14 µg of the LV vector plasmid, 3 µg of pcDNA3gagpolml (Fuller and Anson, 2001), 14 µg of pHCMV-G (Yee *et al.*, 1994), 4 µg of pCMV-*rev* (Lewis *et al.*, 1990) and 0.2 µg of pcDNA3Tat101ml (Anson, unpublished). Sixteen hrs later the transfection medium was replaced with DMEM/5% (v/v) FCS. Twenty-four hrs later the LV vector supernatant was collected and concentrated by ultra-filtration and ultra-centrifugation. The viral titre was determined on NIH3T3 cells. Values are presented as a mean of 2 samples. TU= transducing units.

(e) Detection Assays for Replication Competent Retrovirus

The LV vector stocks were screened for replication competent retrovirus (RCR) by 2 methods. The first method involved transduction of either A549 or NIH3T3 cells, grown in 6-well tissue culture plates, with 1 μl of concentrated LV vector (expressing either LacZ or CFTR) in the presence of polybrene (4 μg/ml) for 24 hrs. The medium was then replaced with fresh growth medium. Forty-eight hrs later the supernatant (2 ml) was collected and used to transduce A549 cells (secondary transduction) in the presence of polybrene (4 μg/ml). The secondary-transduced A549 cells were then analysed for LacZ or CFTR gene expression as previously described (sections 2.2.27 and 2.2.16 (b), respectively). Detection of positive cells would be indicative of the generation of RCR. In all instances the results obtained from the secondary transduction assay were negative.

The second method of screening for RCR was by measurement of the HIV-1 p24 *gag* antigen protein that was released into the culture supernatant of primary transduced A549 cells. This was quantified by an enzyme-linked immunosorbent assay (ELISA) as described in section 2.2.29. In all cases p24 *gag* antigen protein levels declined to undetectable levels (< 10 pg/100 μl) within 7 days of transduction (Figure 5.3).

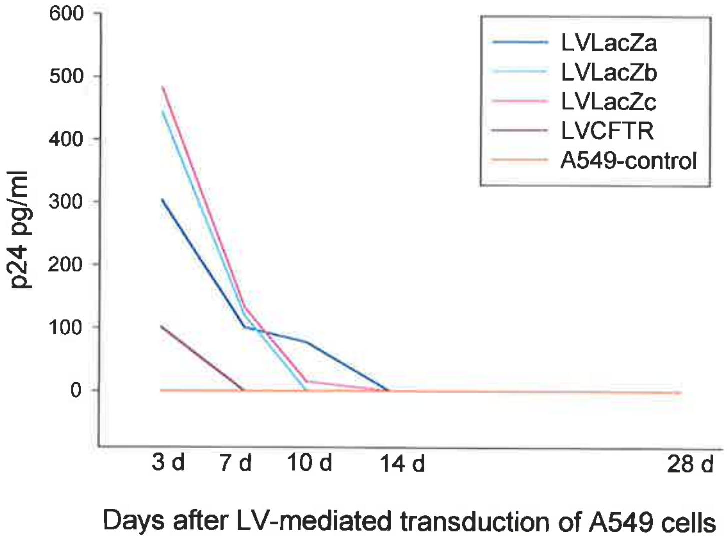

Figure 5.3: p24 *gag* Antigen Protein Values as Determined by the HIV-1 p24 ELISA.
A549 cells were transduced with the concentrated LV vector stocks in the presence of polybrene (4 μg/ml) as described in section 2.2.25. Sixteen hrs later the medium was replaced with fresh medium and the cells maintained in culture for a further 28 days. Twice a week a 1 ml sample from a confluent culture was collected and stored at minus 70 °C until all samples were collected. The samples were assayed for p24 using the HIV-1 p24 ELISA kit. The values presented are n= 1. d= days.

5.2.3 Summary

In this section four variables affecting the optimal production and concentration of the LV vector stocks were examined. Following the examination of four different transfection protocols, the $CaPO_4$ co-precipitation transfection protocol was chosen for the production of the LV vector stocks. Using this protocol, 293T cells were incubated with the $CaPO_4$-DNA precipitate for 16 hrs. The medium was then replaced with DMEM/5% (v/v) FCS. Twenty-four hrs later the LV vector supernatant was collected and concentrated ~1000-fold; initially ~10-fold by ultra-filtration and then ~100-fold by ultra-centrifugation.

Overall, the optimisation process of the $CaPO_4$ co-precipitation transfection protocol resulted in an almost 10-fold increase in the viral titre when compared to that produced by the Lipofectamine transfection protocol that had been previously used for the production of LV vector stocks in our lab.

5.3 The EYFP Marker Gene

5.3.1 Introduction

The enhanced yellow fluorescence protein, EYFP, gene is a sensitive and easy to detect marker gene. For this reason, the EYFP gene was investigated for its utility in the gene transfer studies described in this thesis project. However, as noted in Part B of this chapter the use of the EYFP gene was limited, as it caused a high degree of background fluorescence in the LV vector supernatant. Therefore, in an attempt to overcome the apparent limitation associated with the use of the EYFP marker gene, ways to minimise the background fluorescence of the LVEYFP vector stocks were explored.

5.3.2 Methods and Results

(a) Background Fluorescence of the Lentivirus Vector Stocks

Fluorescence microscopy analysis of the concentrated LVEYFP vector stock showed that it was highly fluorescent. In attempting to resolve the fluorescence observed in the concentrated LVEYFP vector stocks, factors that may have contributed to the presence of fluorescence were considered. Firstly, DMEM has a high riboflavin content (0.4 mg/l), which has been reported to account for more than 95% of the fluorescence of the concentrated DMEM when viewed under the fluorescence microscope (Dr B. Ludin, personal communication, Life Imaging Services, Switzerland). To minimise this fluorescence the obvious approach was to collect the LV vector in a medium formulation, which has a low riboflavin content. One such medium formulation is Medium-199, which has a riboflavin content of 0.01 mg/l, i.e. 40-fold less riboflavin content than the DMEM formulation. Although the fluorescence of the concentrated LVEYFP vector stocks was reduced when Medium-199 was used to collect the LV vector, there was also a 10-fold reduction in the viral titre. As a consequence, another approach to reduce the riboflavin content of the DMEM was explored. An Isolute lipophilic column, which binds riboflavin was utilised to extract riboflavin from the DMEM. However, this procedure proved time-consuming (almost 2 hrs to filter 500 ml of DMEM) and although the fluorescence of the concentrated LVEYFP vector stocks was reduced by more than half, it was still evident when viewed under the fluorescence microscope.

It was then observed that the concentrated LVLacZ vector stocks did not fluoresce under the fluorescence microscope. It appeared, therefore, that the fluorescence of the concentrated LVEYFP vector stocks may have been caused by the release of the, nonsecretory, EYFP protein from the 293T cells, which were used for the production of the LVEYFP vector stocks. This possibility, however, was not investigated further due to time limitations.

Since it appeared likely that the fluorescence of the concentrated LVEYFP vector stocks was due to released EYFP protein, the standard procedure for collection of the LV vector in DMEM was continued.

(b) Loss of EYFP Fluorescence Following Tissue Processing

It was unclear from the literature whether EYFP fluorescence would persist through the extensive processing, fixation and decalcification procedures, to allow sectioning of the intact mouse heads, in preparation for the quantitation of gene transfer to the nasal airway epithelium.

To evaluate the stability of EYFP fluorescence throughout the nasal airway tissue processing, NIH3T3 cells were transduced (n= 3) with the LVEYFP vector as described in section 2.2.25. Seven days later the cells were exposed to the solutions routinely used to fix and post-fix the mouse nasal airway tissues prior to histological processing. Specifically, the NIH3T3 cells were initially fixed with 2% PFA/0.5% glutaraldehyde (glutaraldehyde was typically added to preserve tissue morphology) for 2 hrs and then post-fixed with 10% NBF for 2 hrs. At this point, analysis of the EYFP positive NIH3T3 cells using a fluorescence microscope showed no marked decrease of EYFP fluorescence (Figure 5.4; a). However, after exposure of the cells to the DeCal solution, used to demineralise the nasal bone, the intensity of EYFP fluorescence was substantially reduced within 1 - 2 mins and did not recover after removal of the DeCal solution (Figure 5.4; b).

Since the processing of mouse heads involves exposure to the DeCal solution for 22 hrs, the results (Figure 5.4; b) suggest that it is more than likely that EYFP fluorescence will be destroyed following exposure to this reagent. Therefore, it became apparent that the EYFP gene was not suitable for use as a marker gene in the planned *in vivo* gene transfer studies.

5.3.3 Discussion

While the background fluorescence of the LVEYFP vector stocks was a problem in the *in vitro* gene transfer studies (described in Part B of this chapter), this may not be a limitation in the *in vivo* gene transfer studies. In these latter studies, the LV vector is typically instilled into one (right) nostril of the mouse nasal cavity. The LV vector is then cleared within a few minutes of instillation, by both aspiration and continual mucociliary clearance. Therefore, at the time of analysis, at least 7 days later, the probability of the tissue of interest being non-specifically stained with the EYFP protein is minimal.

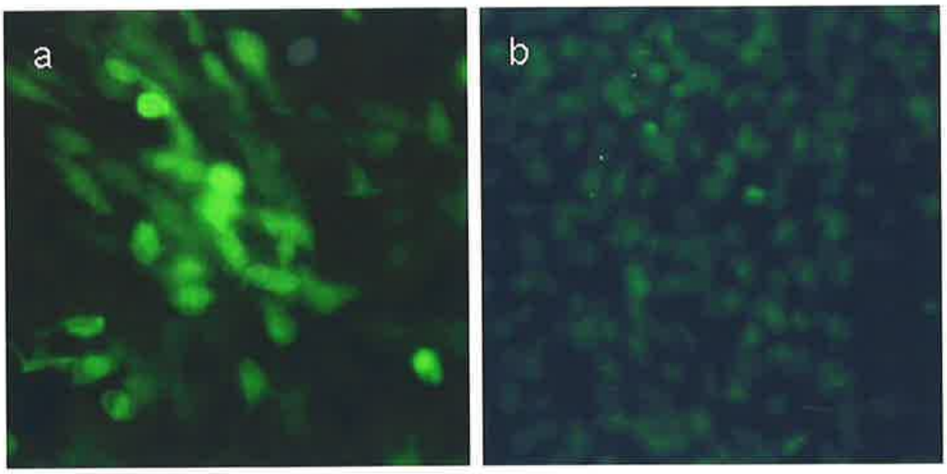

Figure 5.4: Effect of the Fixatives - used for Mouse Nasal Airway Processing - on the Fluorescence of EYFP Positive Cells.
NIH3T3 cells were transduced with the LVEYFP vector in the presence of polybrene (4 µg/ml) as described in section 2.2.25. Sixteen hrs later the transduction medium was replaced with fresh growth medium and the cells further maintained in culture. Seven days later **(a)** The NIH3T3 cells (green) were fixed with 2% PFA/0.5% glutaraldehyde for 2 hrs and then post-fixed with 10% NBF for 2 hrs. EYFP fluorescence survived exposure to this fixative. **(b)** When NIH3T3 cells were subsequently exposed to the DeCal solution, EYFP expression was deactivated, evident as loss of fluorescence. Magnification= x 200.

The results of the experiments described in this section demonstrated, however, the detrimental effect of the DeCal solution on EYFP gene expression, which prevented the use of the EYFP marker gene in the *in vivo* gene transfer studies. The use of the EYFP marker gene is also limited by the loss of EYFP gene expression following paraffin embedding and histological dehydration of tissue of interest (Dr. A. Brenner, European Institute of Oncology, Milan, Italy, personal communication). One way to overcome the destruction of EYFP fluorescence is to develop another tissue processing methodology. For example, excision of the nasal airway tissue and snap-freezing it in liquid N_2 ought to maintain EYFP fluorescence of the EYFP positive cells (Dr. F. Altruda, University of Torino, Italy, personal communication). Although this technique appears straightforward, efficient tissue sectioning will depend on the bone thickness. Furthermore, the poor tissue

preservation, inherent in frozen sections, means that the types of the positively transduced EYFP cells cannot be accurately identified using this methodology.

As a consequence of the findings of the experiments described here, the, non-fluorescent, LacZ marker gene, which is known to be stable throughout the extensive fixing and demineralising procedures employed for tissue processing (Parsons *et al.*, 1998), was used instead of the EYFP marker gene in the intact animal LV-mediated gene transfer studies.

5.4 The LacZ Marker Gene

5.4.1 Introduction

During the process of optimising the LV vector stock production protocol (section 5.1) it was found that the LVLacZ vector constructs resulted in lower viral titre (almost 10-fold) compared to that of equivalent LVEYFP vector constructs (Dr. D. S. Anson, personal communication). Unlike the EYFP marker gene sequence, the LacZ marker gene sequence is not codon optimised for mammalian cell expression. This, however, does not appear to limit expression of LacZ from the LV vector constructs. As retroviruses utilise an RNA genome, one explanation for the lower titre of LV vector stocks obtained with the LacZ marker gene is that RNA processing is occurring. Consequently, these next experiments were performed to search for evidence of aberrant RNA processing events that could be detrimental to the viral titre of the LVLacZ vector.

5.4.2 Methods and Results

(a) Northern Blot Analysis of the LVLacZ Vector Transcripts

293T cells were plated onto 60-mm culture dishes and transfected 24 hrs later using the $CaPO_4$ co-precipitation method, as described in section 5.2, with:

1. LVLacZa[1], pCMV-*rev* and pcDNA3TATml101 (*LVLacZa*)
2. LVLacZb[1], pCMV-*rev* and pcDNA3TATml101 (*LVLacZb*)
3. pCMV-*rev* and pcDNA3Tatml101 (negative control)
4. TE buffer (negative control)

Forty-eight hrs later, cytoplasmic RNA was isolated as described in section 2.2.14 and 4 μg of denatured RNA electrophoresed on a 1.2% agarose, 1 x MOPS gel for northern blot analysis as described in section 2.2.11. The membrane was then hybridised with the F4-LTR probe (bases 8341 - 9074, HIV-1 YU-2, GenBank accession number M93258) as described in section 2.2.12.

The molecular weights (MW) of the 28s (4.7 kb) and 18s (1.9 kb) ribosomal RNAs were used to plot a graph of $\log_{10}$MW versus ^{1}/mobility to allow for the extrapolation of values of the MW's of the hybridising bands, A - D, shown in Figure 5.5 (Table 5.6).

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[1] The LV vector constructs contain the HIV-1 YU-2 major splice donor site, Figure 5.6.

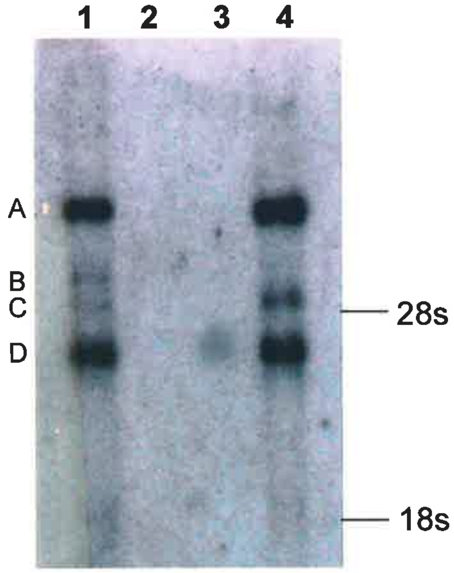


Figure 5.5: Northern Blot Analysis of Transcripts from the Lentiviral Vector Constructs. Cytoplasmic RNA was prepared from transfected cells and 4 µg analysed by northern blot analysis using a probe specific to the 3' non-coding sequence prior to the polyadenylation site. The positions of the 28s (4.7 kb) and 18s (1.9 kb) rRNA markers are shown to the right of the figure. The F4-LTR probe was designed to hybridise to spliced and un-spliced transcripts with equal efficiency. Lane 1: *LVLacZa*; lane 2: 293T control; lane 3: Tat/Rev control; lane 4: *LVLacZb*.

Table 5.6: Estimated Molecular Weights of the RNA Transcripts.

Band	Molecular Weight (kb)
A	13.8
B	6.2
C	5.0
D	3.6

The molecular weights of the hybridising bands (A - D) were extrapolated from a graph of $\log_{10}$MW versus $^{1}/$mobility plotted using the known molecular weights of the 28s (4.7 kb) and 18s (1.9 kb) ribosomal RNA.

Northern blot analysis of vector RNA from 293T cells transfected with LVLacZa/b vector(s), pcDNA3Tatml101 and pCMV-*rev* suggested that RNA processing was occurring in the vector, as RNA species smaller than the predicted size of the LV vector (5.1 kb) were observed.

The larger transcript band A (~13.8 kb), which was present in lanes 1 and 4 most likely represents read-through of the polyadenylation site in the 3' LTR of the LV vector, which may then result in polyadenylation at the 5' LTR after complete transcription of the plasmid. In lane 1 (*LVLacZa*), four RNA transcripts were present. It is unclear, however, whether the LV vector transcript is represented by band B or band C (of MW's ~6.2 and ~5.0 kb, respectively). In Lane 4 (*LVLacZb*) only three RNA transcripts, corresponding to bands A, C and D were seen; band C was significantly more abundant in Lane 4 than in Lane 1. Since the LVLacZb vector is more efficient than the LVLacZa vector, band C most likely corresponds to genomic RNA. It should be noted that the lower intensity of bands B and C suggests that both these transcripts represent less abundant RNA species, as the probe used here (F4-LTR) was designed to hybridise to spliced and un-spliced transcripts with equal efficiency. The smaller band D present in Lanes 1 and 4 represents a spliced transcript resulting from splicing between the HIV-1 major SD site and a cryptic SA site in the SV40 promoter (Dr. D. S. Anson, personal communication).

To identify possible cryptic SA sites in the LacZ gene sequence the area of interest was amplified from 1st strand cDNA by RT-PCR and the sequence then analysed.

(b) Sequence Analysis of the RNA Transcripts

Using 10 μg of the RNA template from samples 1 and 2 (*LVLacZa* and *LVLacZb*, respectively) 1st strand cDNA was synthesised as described in section 2.2.15. The cDNA templates were then amplified as described in section 2.2.16 (c) using the sense strand primer (MSD236; section 2.1.15), labelled as 4 in Figure 5.6, and one of three anti-sense primers, LacZa, LacZb and LacZc, corresponding to the complement of bases 1559 - 1580, 2098 - 2119 and 2638 - 2659 in the LacZ gene sequence (ECLACZ, GenBank accession number V00296), shown as 1, 2 and 3, respectively, in Figure 5.6.

From the size of the PCR products shown in Figure 5.7 it appears as though splicing is occurring from the HIV-1 major SD site to at least three cryptic SA sites in the LacZ gene sequence. The sizes of these PCR products suggested that splicing was occurring to cryptic SA sites at approximately (i) 0.75 kb (lane 1 and 4, ~0.8 kb; lane 2, 1.3 kb), (ii) 1.7 kb (lane 2 and 5, 0.45 kb) and (iii) 2.3 kb (lane 3 and 6, 0.4 kb) from the 5' end of the LacZ gene sequence. Analysis of the PCR products with the 'BigDYE Terminator Cycle Sequencing Ready Reaction Kit' confirmed the presence of at least 2 SA sites. The sequences of these products were then aligned to the LacZ marker gene sequence using the Best-Fit program (Australian National Genome Information System, WebAngis: http://www.angis.org.au).

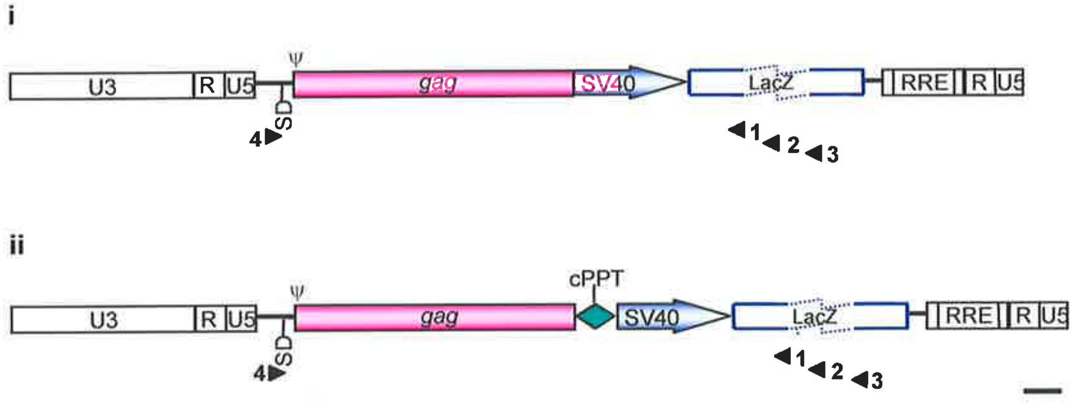


Figure 5.6: Positions of the Sense and Anti-Sense Primers Used to Amplify the cDNA Templates from the Lentiviral Vector Constructs *LVLacZa* and *LVLacZb*.

Positions of the anti-sense primers designed at positions **1** (bases 1559 - 1580), **2** (bases 2098 - 2119) and **3** (bases 2638 - 2659) in the LacZ gene (not to scale) sequence (GenBank accession number V00296) and the sense primer designed at position **4** (located 37 bp 5' of the HIV-1 major splice donor site). Scale bar= 100 bp.

The PCR products were then analysed by agarose gel electrophoresis (Figure 5.7).

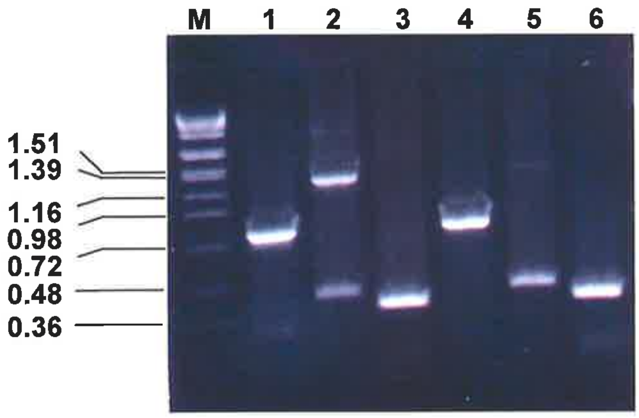


Figure 5.7: RT-PCR Analysis of Transcripts from the Lentiviral Vector Constructs.

1st strand cDNA was synthesised from total cytoplasmic RNA prepared from 293T cells, which were transfected with plasmids encoding LVLacZa or LVLacZb, Tat and Rev. PCR analysis was then performed with the MSD236 primer and either the LacZa, LacZb or LacZc primer. M, SPP1/*EcoR*I molecular weight markers, the sizes of the relevant bands are shown to the left of the figure. Lanes 1 - 3: LVLacZa construct, LacZa, LacZb, LacZc primers, respectively; Lanes 4 - 6: LVLacZb construct, LacZa, LacZb, LacZc primers, respectively.

The sequence analysis confirmed the presence of cryptic SA sites at bases 785 and 2300 in the LacZ marker gene sequence (Figure 5.8). For technical reasons the smaller band present in lanes 2 and 5 could not be isolated in sufficient quantity to allow sequencing, and therefore the position of the third cryptic SA site was not identified. Subsequent analysis confirmed the presence of a fourth cryptic SA site at base 1939 in the LacZ marker gene sequence (Dr. D. S. Anson, personal communication) (Figure 5.8).

(c) Identification of the Lariat Consensus Sequences Associated with the LacZ Cryptic Splice Acceptor Sites

The 3' splice site is typically identified by the lariat consensus sequence ($Py_{80}NPy_{80}Py_{87}Pu_{75}A\ Py_{95}$, YNYYRAY) that lies between 18 - 40 bp 5' of the splice acceptor (SA) site (AG consensus) and a stretch of pyrimidine (Y) residues that immediately precede the SA site (Lewin, 1994, page 917). As a consequence, a search for lariat consensus sequences was performed. For the first cryptic SA site only one close match (6/7, TACCTAC) for a lariat consensus sequence at bases 757 - 763 was found (Figure 5.9). Similarly, for the second cryptic SA site only one close match (6/7, CATCGAA) for a lariat consensus sequence at bases 1905 - 1911 was found (Figure 5.9). For the third SA site 2 possible matches for a lariat consensus sequence were found. These were at bases 2266 - 2272 (6/7, TGGCAAT), and at bases 2271 - 2277 (6/7, ATTTAAC) (Figure 5.9).

5.4.3 Discussion

The use of the LacZ marker gene in the LV vector is especially important for *in vivo* gene transfer development, as it allows for the precise quantification of gene transfer by counts of LacZ positive cells in the mouse nasal airway tissue. The X-gal reaction that stains the LacZ positive cells survives the effects of the decalcification process (unlike the EYFP marker gene, section 5.3) that is required when working with mouse nasal airways. Importantly, the X-gal staining does not interfere with the histological stains utilised to display cell morphology (e.g haematoxylin/eosin). Therefore it allows for the precise identification of the type of LacZ positive cells using standard histological staining procedures, as described in section 2.2.35.

Figure 5.8: Sequence Analysis of the LacZ Gene Splicing in the LVLacZ Vectors.

Splice-Acceptor Site at LacZ Base 785

```
                              280        SD       300
HIV-1 YU-2                 AGGGGCGGCGACTGGTGAGTACGCCAAAAAATT
                           ||||||||||||||
Spliced sequence           AGGGGCGGCGACTGGGTGAAACGCAGGTCGCCA
                                         |||||||||||||||||||
LacZ marker gene sequence  TTTCTTTATGGCAGGGTGAAACGCAGGTCGCCA
                                780    SA            800
```

Splice-Acceptor Site at LacZ Base 1939

```
                              280        SD       300
HIV-1 YU-2                 AGGGGCGGCGACTGGTGAGTACGCCAAAAAATT
                           ||||||||||||||
Spliced sequence           AGGGGCGGCGACTGCGATAACGAGCTCCTGCAC
                                         |||||||||||||||||||
LacZ marker gene sequence  CTGTTCCGTCATAGCGATAACGAGCTCCTGCAC
                           1930        SA      1950
```

Splice-Acceptor Site at LacZ Base 2300

```
                              280        SD       300
HIV-1 YU-2                 AGGGGCGGCGACTGGTGAGTACGCCAAAAAATT
                           ||||||||||||||
Spliced sequence           AGGGGCGGCGACTGATGTGGATTGGCGATAAAA
                                         |||||||||||||||||||
LacZ marker gene sequence  GCTTTCTTTCACAGATGTGGATTGGCGATAAAA
                                       SA     2310      2320
```

Nucleotide numbering for HIV-1 YU-2 is as for HIVYU2X (GenBank accession number M93258) and for LacZ is as for ECLACZ (GenBank accession number V00296). The splice donor (SD) and splice acceptor (SA) dinucleotide consensus sequences are shown in bold.

The identification of at least three SA sites in the LacZ marker gene sequence provides one possible reason for the low titres of the LVLacZ vector stocks. Splicing will decrease the level of genomic RNA available for packaging, therefore reducing the titre of the LVLacZ vector stocks. It is possible to overcome splicing by mutating either the SA consensus sequences in the LacZ marker gene or the SD consensus sequence in HIV-1. However, as HIV-1 also has cryptic SD sites (Purcell and Martin, 1993) this approach may prove more difficult than mutating the SA consensus sequences in the LacZ marker gene. The northern blot analysis (section 5.4.2, a) suggested that inefficient poly-adenylation is also a problem, thus providing another reason for the low titre of the

LVLacZ vector stocks. One way to overcome inefficient polyadenylation is by using a heterologous polyadenylation sequence. The feasibility of this approach has already been successfully demonstrated (Iwakuma *et al.*, 1999).

Figure 5.9: Identification of the Lariat Consensus Sequences Associated with the Cryptic LacZ Splice-Acceptor Sites.

Splice-Acceptor Site at LacZ Base 785

```
      750        760       770       780    SA 790       800
CGGCGAGTTGCGTGACTACCTACGGGTAACAGTTTCTTTATGGCAGGGTGAAACGCAGGT
                |||| ||         ||||||| | ||||
                YNYYRAY       YYYYYYYYYYYYNCAG
```

Splice-Acceptor Site at LacZ Base 1939

```
       1900      1910      1920      1930        SA      1950
TTTATCCGGGCAAACCATCGAAGTGACCAGCGAATACCTGTTCCGTCATAGCGATAACGAG
               ||||||               ||| |||| ||||||
               YNYYRAY             YYYYYYYYYYYYNCAG
```

Splice-Acceptor at LacZ Base 2300

(a)

```
      2260      2270       2280     2290         SA     2310
CTGGGTAATAAGCGTTGGCAATTTAACCGCCAGTCAGGCTTTCTTTCACAGATGTGGATT
               || ||||                ||||||||||||||
               YNYYRAY             YYYYYYYYYYYYNCAG
```

(b)

```
      2260      2270       2280     2290         SA     2310
CTGGGTAATAAGCGTTGGCAATTTAACCGCCAGTCAGGCTTTCTTTCACAGATGTGGATT
                     ||||||           |||||||||||||
                    YNYYRAY        YYYYYYYYYYYYNCAG
```

Nucleotide numbering for HIV-1 YU-2 is as for HIVYU2X (GenBank accession number M93258) and for LacZ is as for ECLACZ (GenBank accession number V00296). R= purine (A, G); Y= pyrimidine (T, C); N= (R, Y). The splice acceptor (SA) dinucleotide consensus sequences are shown in bold.

Part B

Lentivirus-Mediated Gene Transfer

5.5 Lentivirus-Mediated Gene Transfer to Immortalised Cells

5.5.1 Introduction

It has been reported that LPC treatment of the mouse nasal airway epithelium prior to AdV vector instillation, as well as co-administration of LPC with the AdV vector, significantly improves gene transfer *in vivo* (Dr. D. W. Parsons, personal communication). To determine whether LPC could similarly enhance LV-mediated gene transfer *in vivo*, its use was first investigated *in vitro*. Specifically, the possibility of improving gene transfer by LPC pre-treatment of the airway epithelium or by co-administration of LPC with the LV vector, was explored.

One significant difference between the AdV and the LV vectors is that unlike the AdV capsid that is protein-based, the LV envelope is lipid-based. Therefore, the effect of LPC on the viability of the viral titre of the LV vector was also investigated.

5.5.2 Methods and Results

(a) Lentivirus-Mediated Transduction of Epithelial Cells Following Detergent Treatment

The results of Chapter 4 demonstrated that treatment of polarised cells with polidocanol prior to AdV vector exposure significantly improved AdV-mediated gene transfer *in vitro*. LPC, a detergent-like component of pulmonary surfactant, has been shown to also improve AdV-mediated gene transfer (Parsons *et al.,* 1999). Therefore both these detergents were evaluated for their ability to improve LV-mediated gene transfer *in vitro*.

To determine whether detergent treatment of the cells improved LV-mediated gene transfer in a similar way to AdV-mediated gene transfer, polarised cells (MDCK and 16HBE) and non-polarised cells (1HAE, CFPAC-1 and A549) were treated with either polidocanol or LPC prior to exposure to the LVEYFP vector.

Each cell line was plated onto 12-well polycarbonate Transwell support membranes at a density of 3 x 10^5 cells per well in 0.5 ml of growth medium and allowed to grow to confluence. Formation of tight junctions was monitored in MDCK and 16HBE cells by measuring their transepithelial resistance (TR) as described in section 3.1.2. Once 1HAE, CFPAC-1 and A549 cells had reached confluence, and the MDCK and 16HBE cells had formed TJ's, they were treated with either the dose of polidocanol, which resulted in the highest AdV-mediated gene transfer *in vitro* (section 4.2), or with LPC at a 10-fold lower dose (Table 5.7).

Table 5.7: Doses and Incubation Times of the Detergents Used to Pre-Treat the Cells to Improve Lentivirus-Mediated Gene Transfer.

Cell lines	Polidocanol (%)	LPC (%)	Incubation time (mins)
MDCK	0.10	0.010	2
16HBE	0.05	0.005	3
1HAE	0.01	0.001	15
CFPAC-1	0.01	0.001	15
A549	0.01	0.001	15

The use of a reduced dose of LPC was based on *in vivo* findings that a 10-fold lower concentration of LPC resulted in the same levels of AdV-mediated gene transfer to those obtained following polidocanol treatment (Parsons *et al.*, 1999).

One hr after the detergent treatment the cells were transduced with 500 µl of LVEYFP containing 2 x 10^5 NIH3T3-TU in the presence of polybrene (4 µg/ml). Sixteen hrs later the LVEYFP vector was removed and 500 µl of DMEM/10% (v/v) FCS were added to the cells. Seven days later the cells were harvested and LV-mediated EYFP transduction assessed by FACScan analysis (Figure 5.10).

The results shown in Figure 5.10 indicate that both polidocanol and LPC pre-treatment of the MDCK cells significantly improved LV-mediated gene transfer (~3-fold) compared to the PBS-treated (control) cells ($P< 0.001$, ANOVA, Dunnett's method, n= 4). A modest improvement in LV-mediated gene transfer was also evident in the 16HBE cells pre-treated with polidocanol, compared to those treated with either LPC or PBS ($P= 0.011$, ANOVA, Dunnett's method, n= 4).

For the non-polarised cell lines (1HAE, CFPAC-1 and A549) treatment with polidocanol or LPC did not significantly alter the level of LV-mediated gene transfer

[A549 (*P*= 0.273); 1HAE (*P*= 0.442); CFPAC-1 (*P*= 0.523); ANOVA, Dunnett's method, n= 4); however, the low power of the tests [A549 (0.05); 1HAE (0.05); CFPAC-1 (0.05)] indicates that the number of samples was insufficient to allow statistical analysis.

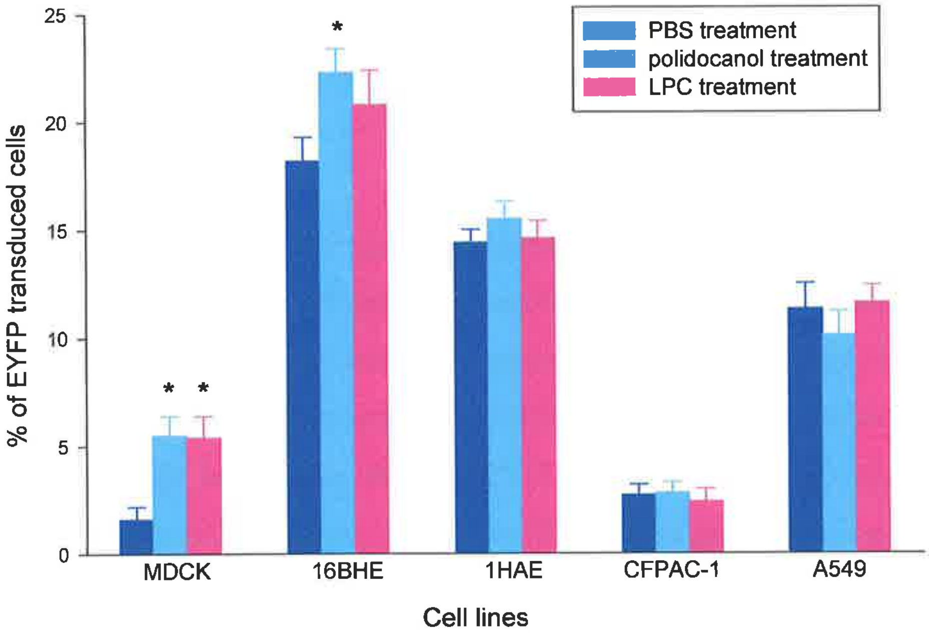


Figure 5.10: Lentivirus-Mediated EYFP Transfer After Detergent Treatment of Immortalised Cell Lines.

Confluent cultures of MDCK and 16HBE cells (polarised), and 1HAE, CFPAC-1 and A549 cells (non-polarised) were exposed to the polidocanol treatment that resulted in the highest AdV-mediated gene transfer *in vitro* (section 4.2), or to LPC at a 10-fold lower dose. Values are presented as the mean of 4 samples ± SEM. The SEM is represented in the graph as an error bar. * *P*< 0.001, ANOVA, Dunnett's method.

The improvement in LV-mediated gene transfer following polidocanol treatment was not the same as that obtained in similar AdV-mediated gene transfer studies described in section 4.2. For MDCK cells, polidocanol treatment resulted in a substantially higher (> 50-fold) improvement in AdV-mediated gene transfer compared to PBS-treated cells (Figure 4.3; b). The same treatment regimen, however, resulted in only a ~4-fold improvement in LV-mediated gene transfer compared to PBS-treated cells (Figure 5.10). For 16HBE cells, polidocanol treatment resulted in a ~2-fold improvement in AdV-mediated gene transfer compared to PBS-treated cells (Figure 4.3; b), whereas the same treatment regimen resulted in a modest improvement in LV-mediated gene transfer compared to PBS-treated cells (Figure 5.10).

LPC, like polidocanol, has been shown to open epithelial TJ's *in vivo* (Parsons *et al.*, 1999). This suggests that the increase in the level of LV-mediated gene transfer to the polarised cells (MDCK and 16HBE) following LPC treatment is also due to the opening of the TJ's, which allows for increased accessibility of the LV vector particles to the basolateral cell surface where their relevant (VSV-G) receptors are located (Fuller *et al.*, 1984).

(b) Effect of LPC Treatment on Lentivirus Vector Viability

This experiment was performed to determine whether the simultaneous instillation of LPC with the LV vector would have a detrimental effect on viral titre viability. One ml of LVEYFP vector containing 5 x 10^5 NIH3T3-TU was mixed with 10 μl of 1% LPC (final concentration: 0.01%), incubated at 37 °C and 150 μl sampled after 0.5, 2, 4 and 6 hrs (n= 3). As a control, 1 ml of LVEYFP vector was mixed with 10 μl of PBS, incubated at 37 °C and samples collected at the same time points (n= 3). The titre of the LV vector supernatant collected at each time point was then determined on A549 cells as described in section 2.2.25. The results of this experiment are shown in Figure 5.11.

Two findings on LV vector viability are apparent from this experiment. First, the titre of the LV vector that was not treated with LPC was stable for at least 6 hrs, i.e. to the termination of the experiment, at 37 °C. Second, incubation of LPC with the LV vector from 0.5 to 6 hrs resulted in a significant reduction of viral titre compared to the titre of the PBS-treated (control) LV vector at all time points ($P < 0.001$, ANOVA, SNK).

As shown in Figure 5.11, co-incubation of LPC with the LV vector for 0.5 hr reduced the titre of the LV by more than half. This finding suggests that co-instillation of the LV vector with 0.01% LPC is possible since this dose of LPC does not completely destroy the viability of the vector titre.

Although the incubation of the LPC with the LV vector *in vitro* resulted in a significant decrease in viral titre, it is more than likely that in an *in vivo* setting the presence of LPC in the LV vector solution may not have such a detrimental effect on viral titre. As the LPC/LV solution will be cleared from the nasal airway within a few minutes, the reduction, if any, in LV vector viability may remain acceptable for effective gene transfer. Nonetheless there is clearly a need to look more closely at the immediate effect of various doses of LPC on LV vector viability at incubation time periods of less than 30 mins (i.e. 1, 5, 10 mins).

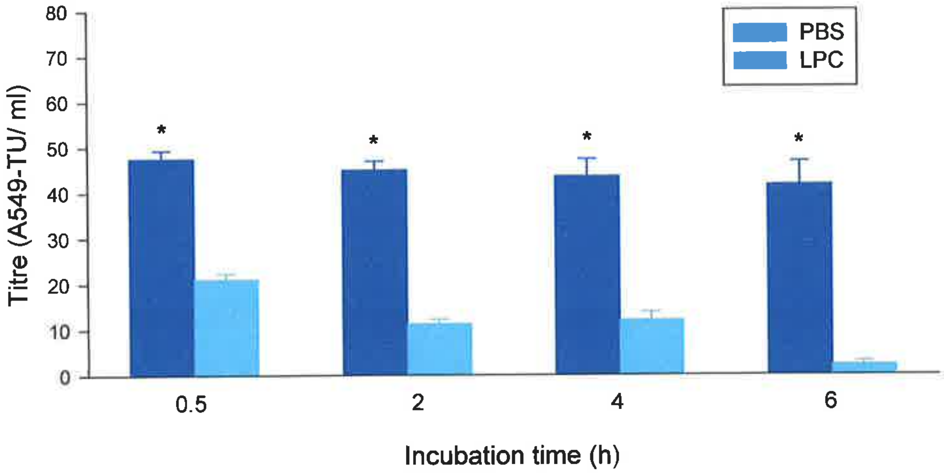


Figure 5.11: Effect of LPC on the Viability of the Lentivirus Vector.

A 1 ml sample of the LVEYFP vector was treated with LPC (final concentration: 0.01%) and incubated at 37 °C for 0.5, 2, 4 and 6 hrs. As a control, 1 ml of LVEYFP vector was mixed with 10 µl of PBS and incubated at 37 °C for the same time points. The titre of the LV vector samples was determined on A549 cells. Values are presented as the mean of 3 samples ± SEM. The SEM is represented in the graph as an error bar. * $P< 0.001$, ANOVA, SNK.

5.6 Lentivirus-Mediated Gene Transfer to Primary Airway Epithelial Cells

5.6.1 Introduction

As discussed in Chapter 3 the cultures of primary RTE cells and cultures of explanted rat nasal septa grown at ALI provide reasonable models of the intact human airway epithelium. In these culture systems most of the airway surface barriers most likely to affect LV-mediated gene transfer *in vivo* i.e. the overlaying mucus layer, the MCC mechanisms co-ordinated by the cilia, the glycocalyx located on the apical surface and the tight junctions (McCray, 2001) are represented. Therefore, in an attempt to establish the initial dosage parameters to be used in experiments designed to optimise the efficiency of LV-mediated gene transfer to the mouse nasal airway epithelium, the ability of the LV vector to transduce airway epithelial cells was evaluated in these *in vitro* culture systems.

5.6.2 Methods and Results

(a) Lentivirus-Mediated Gene Transfer to the Rat Tracheal Epithelial Cells

(i) LVEYFP Trials:

The results from the gene transfer studies using MDCK and 16HBE cells showed that the LV vector efficiently transduced immortalised polarised epithelial cells. As a result, the LV vector was further tested *in vitro* to determine whether it could also transduce primary airway epithelial cells.

Airway epithelial cells were isolated from the rat trachea, as described in section 2.2.31, and grown on 12-well collagen-coated Transwell support membranes. Although these cultures did not differentiate to form polarised airway epithelium, they were nonetheless used for the initial assessment of the ability of the LVEYFP vector to transduce primary airway epithelial cells. The RTE cells were grown at ALI to ~80% confluence, as described in section 2.2.31 and transduced with 500 μl of the LVEYFP vector (Figure 5.1; i) containing 2.5 x 10^5 NIH3T3-TU in the presence of polybrene (4 μg/ml) for 16 hrs (n= 3). During this time the medium present in the bottom reservoir was replaced with an equivalent volume of perfluorochemical (PFC) liquid. This prevented any loss of the viral supernatant from the Transwell support membrane to the bottom reservoir. As the PFC liquid is biologically inert it did not mix with the LV vector solution and therefore maintained the volume of the LV vector supernatant present on the apical surface of the cells constant. Sixteen hrs later the PFC liquid and the LV vector were aspirated. The cells were then washed twice with PBS (37 °C) and 0.5 ml of low-protein medium was added to the cells, and 1 ml of low-protein medium was added to the bottom reservoir. The cells were maintained in culture for 7 days to allow for marker gene expression to develop.

The cells were then examined under a fluorescence microscope for EYFP expression. While fluorescent cells were clearly present, the background fluorescence of the support membrane, presumably due to EYFP protein binding to the collagen, which was used to coat the Transwells, precluded accurate analysis of these positively transduced cells. The experiment was therefore repeated using the LVLacZa vector.

(ii) LVLacZ Trials:

The RTE cells were transduced with 500 μl of the LVLacZa vector (Figure 5.1; ii) containing 2 x 10^5 NIH3T3-TU in the presence of polybrene (4 μg/ml) using the same protocols described in (i). Seven days later the cells were fixed and exposed to the X-gal solution to reveal LacZ marker gene expression.

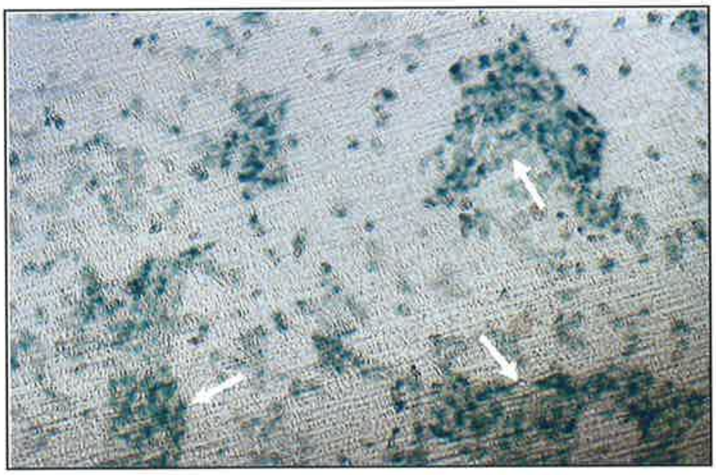


Figure 5.12: Example of LacZ Positive RTE Cells Following LV-Mediated LacZ Gene Transfer.

Semi-confluent cultures of RTE cells grown on collagen coated Transwell support membranes were transduced with 500 μl of LVLacZ vector. LacZ positive cells stained dark blue (arrows). The image appears "out of focus" due to the scattering of transmitted light caused by the Transwell support membrane. Representative images of n= 3 samples. Magnification: x 40.

The results of this experiment showed that more than 90% of the RTE cells were transduced (Figure 5.12), demonstrating that the LV vector could successfully transduce primary airway epithelial cells.

(b) Lentivirus-Mediated Gene Transfer to Explanted Rat Nasal Septa

(i) LVEYFP Trials:

Nasal septa encompassing the anterior non-cartilaginous central region were excised from rats as described in section 2.2.32, and placed onto a polycarbonate Transwell support membrane. This region of septum is primarily populated with ciliated epithelium, characteristic of airway tissue.

The nasal septa were transduced 7 days after excision with either the LVEYFP vector (Figure 5.1; i) or with a carrier solution (PBS). The medium from the membrane support was carefully aspirated and 150 µl of LVEYFP vector containing 7.5×10^4 NIH3T3-TU in the presence of polybrene (4 µg/ml) were added to the apical surface of the nasal septum for 16 hrs (n= 3). As a control, nasal septa were exposed to 150 µl of PBS (n= 3).

To minimise equilibration between the LV vector supernatant (or the PBS) and the medium present in the bottom reservoir, this medium was replaced with an equivalent volume of PFC liquid, as described in section a (i), and the cultures maintained at 35 °C. Sixteen hrs later the PFC liquid and the LV vector supernatant were aspirated. The nasal septa were then washed twice with PBS (35 °C) and 150 µl of low-protein medium was added to the septum and 0.5 ml of low-protein medium was added to the bottom reservoir. The nasal septa were maintained in culture for a further 7 days to allow EYFP gene expression to develop.

No fluorescence was seen in the nasal septa treated with PBS alone. Examination of the nasal septa transduced with the LV vector showed that the nasal cartilage, as well as the support membrane, were highly fluorescent. The excessive non-specific fluorescence again prevented quantitative analysis of the EYFP positive cells. Therefore, these gene transfer studies were repeated using the LVLacZa vector.

(ii) LVLacZ Trials:

In an attempt to determine whether detergent treatment improved LV-mediated gene transfer to the nasal airway epithelium, groups of septa (n= 3) were exposed to 150 µl of either 0.1% LPC, 1% polidocanol or PBS (as control treatment) for 10 mins. The septa were then washed twice with PBS (35 °C) and subsequently transduced with 150 μl of LVLacZa vector (Figure 5.1; ii) containing 7.5 x 10^4 NIH3T3-TU in the presence of polybrene (4 μg/ml) as described in section (i). Seven days later the nasal septa were fixed and stained with X-gal solution to reveal LacZ marker gene expression.

Results from the microscopic *en face* examination of the nasal septa showed that pre-treatment with 0.1% LPC results in a qualitatively higher degree of LacZ gene transfer compared to both pre-treatment with 1% polidocanol and PBS (negative control) (Figure 5.13). Similar to the results of the AdV- and LV-mediated gene transfer studies performed in immortalised epithelial cell lines (i.e. MDCK and16 HBE) (Figure 4.3; b and Figure 5.10, respectively), the results shown here also suggest that detergent treatment prior to LV-mediated gene transduction is not necessary for gene transfer to occur.

The nasal septa were then fixed, sectioned and stained with Saf-O and also H/E to allow for the quantification of the number and the determination of the type of LacZ positive cells, respectively. However, microscopic examination of both the Saf-O and H/E stained nasal septum cross-sections showed that significant degeneration of the nasal septum tissue had occurred, which precluded the quantification and accurate determination of the number and types of LacZ positive cells.

5.6.3 Discussion

The series of the gene transfer experiments described in this section demonstrate that the LV vector can efficiently transduce the airway epithelial cells in two different *in vitro* culture systems. In addition, results from the gene transfer experiments utilising the rat nasal septum culture system showed that gene transfer was improved following detergent treatment with either polidocanol or LPC.

The improved gene transfer observed in the LPC-treated nasal septa could be due to the modulation of the TJ integrity (Parsons *et al.*, 1999). However, there are other potential mechanisms by which LPC could act to modify the airway epithelium surface of the nasal septum. The mucolytic properties of LPC could solubilise airway mucus by reducing its viscosity and elasticity (Martin *et al.*, 1978); LPC should also reduce MCC activity since it is able to reduce cilial beat (Merkus *et al.*, 1993). In pilot studies LPC has improved surrogate vector particle deposition onto the airway epithelium *in vivo* (Parsons *et al.*, 2000a). While there are likely to be other biological actions of LPC (Prokazova *et al.*, 1998) that may be relevant to gene transfer, the direct airway surface effects described here would each be expected to contribute to improved gene transfer *in vitro*.

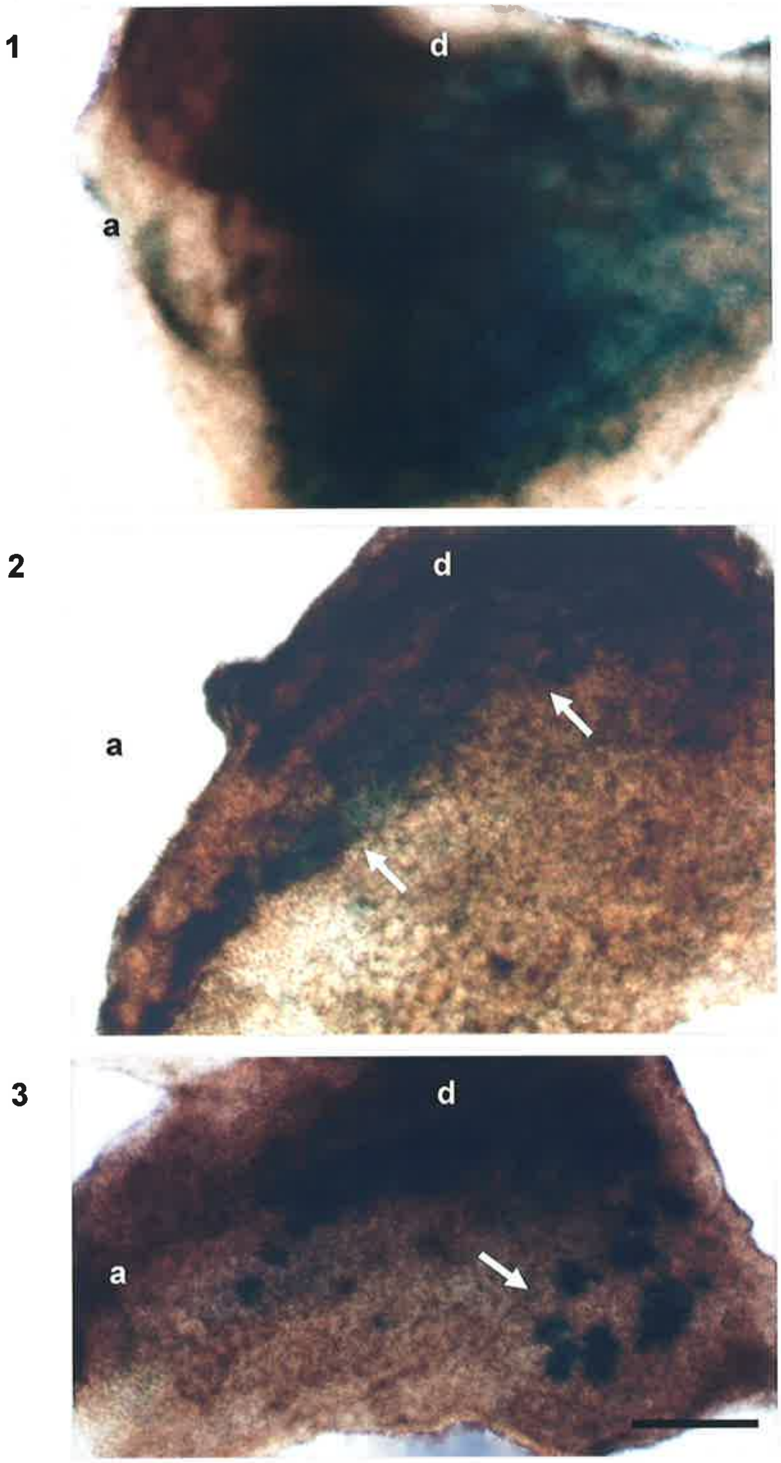


Figure 5.13: Examples of LV-Mediated LacZ Transfer to Explanted Rat Nasal Septa Following Detergent Treatment.

Rat nasal septa grown on polycarbonate Transwell support membranes were transduced with the LVLacZa vector. LacZ positive cells appear as dark blue spots against a beige background (septum) (arrows). The image appears "out of focus" due to the scattering of transmitted light caused by the Transwell support membrane and thickness of the septum tissue. **(1)** 0.1% LPC-, **(2)** 1% polidocanol- and **(3)** PBS- treated nasal septa. a: anterior, d: dorsal. Scale bar= 1 mm.

In particular, these may improve both the retention of the gene transfer vector particles after deposition and also subsequent access to the basolateral cell surface where the VSV-G viral receptors are located (Johnson *et al.*, 2000).

As described in Chapter 3 the two *in vitro* culture systems that were utilised in these gene transfer experiments were limited in their ability to model the intact human airway epithelium. Therefore, the difficulties associated with each culture system combined with the encouraging results from these *in vitro* gene transfer experiments suggested that *in vivo* studies using this LV vector system would be worthwhile.

CHAPTER 6

LENTIVIRUS-MEDIATED GENE TRANSFER *IN VIVO*

Preface

Chapter 6 is divided into two parts. Part A will focus on the LV-mediated gene transfer of the LacZ marker gene into the nasal airway epithelium of C57Bl/6 mice. Several LV vector constructs expressing the LacZ marker gene were utilised in these studies, with the changes in the LV vector constructs used simply reflecting the ongoing development of the LV vector system. The LV-mediated gene transfer protocols were then optimised and the persistence of LacZ gene expression was also examined.

Part B examines the potential use of LV-mediated airway gene transfer for CF gene therapy. The ability of the LV vector carrying the therapeutic CFTR gene to correct the electrophysiological defect, which is present in the nasal airway epithelium of *cftr*$^{\mathrm{tm1Unc}}$ mice, was analysed using the transepithelial potential difference assay.

Part A

Lentivirus-Mediated LacZ Gene Transfer

Introduction

The *in vitro* LV-mediated LacZ gene transfer studies in cultures of both immortalised and primary airway epithelial cells provided valuable information on the ability of detergents to improve the level of LV-mediated LacZ gene transduction (Chapter 5, section 5.5). Since these culture systems were limited in their ability to mimic the intact human airway epithelium, the findings of the *in vitro* gene transfer studies could not be assumed to accurately represent the efficiency of LV-mediated gene transduction to the intact airway epithelium.

The improvement of LV-mediated gene transfer *in vitro* following polidocanol and LPC treatment (sections 5.6 and 5.7) indicated that evaluation of detergent treatment of the mouse nasal airway epithelium prior to LV vector instillation was the next logical step in the development and testing of an effective airway gene transfer vector.

6.1 Pilot Study of Lentivirus-Mediated LacZ Gene Transfer to the Mouse Nasal Airway Epithelium

6.1.1 Introduction

In this preliminary study two detergents were tested to determine whether either detergent, when used as an airway pre-treatment, would facilitate LV-mediated LacZ gene transduction of mouse nasal airway epithelium.

6.1.2 Methods and Results

Groups of C57Bl/6 mice (n= 3) were exposed to 4 µl of either 1% polidocanol, 0.1% LPC or carrier solution (PBS), 1 hr prior to the instillation of 20 µl of the LVLacZa vector (Figure 5.1; ii) preparation as described in section 2.2.33. The LVLacZa vector preparation (20 µl) contained 8 x 10^3 NIH3T3-TU and 4 µg/ml of polybrene.

Polidocanol and LPC were used at similar percentage concentrations due to their comparable molecular weights (MW) ($MW_{polidocanol}$, 583; MW_{LPC}, ~540).

Seven days later, LacZ gene expression in the nasal airway epithelium was assessed as described in section 2.2.34.

The LV-treated mice did not display any behavioural signs of distress nor was significant weight loss observed following either anaesthesia or the LV vector instillation procedures. Typical weight loss following those procedures requiring anaesthesia was expected to be transient and approximately 10% of pre-anaesthesia weight. This expectation was based on the weight loss of untreated (control) animals anaesthetised for the same time as the treated mice (Figure 6.1).

Seven days after pre-treatment and LV vector instillation the treated mice were sacrificed. Stereo-microscope *en face* examination of the grossly-sectioned blocks of the X-gal stained head, prior to paraffin embedding and histological processing, revealed a small number of dark-blue stained cells in the nasal airway epithelium of mice treated with 1% polidocanol. The distribution of these LacZ positive cells remained unilateral and was noted only at Level 16 (data not shown). In contrast, in the nasal airways of mice that were pre-treated with either 0.1% LPC or PBS, no LacZ positive cells were observed. The light green artefactual staining, which often appeared when X-gal staining procedures were utilised, was distributed bilaterally in all tissue samples and was not apparent in the nasal cross-sections (H/E or Saf-O) when these were viewed *via* a compound microscope. LacZ positive cells were not seen in the olfactory regions of the nasal airway.

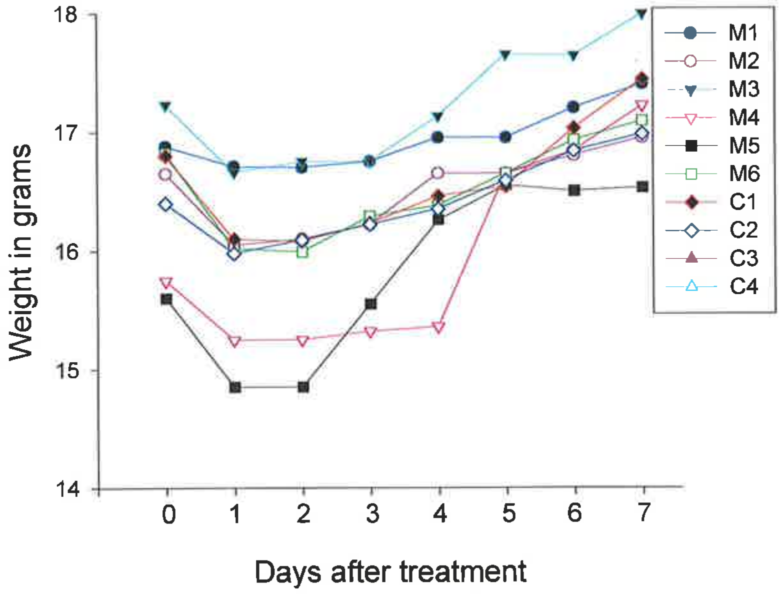


Figure 6.1: Effect of Anaesthesia and Exposure to the Detergent/LV Vector Solutions on Mouse Weight.

Mouse weights (M1-6 and C1-4) were measured prior to anaesthesia and then monitored up to the termination of the experiment (day 7). Mice coded M1/2 were exposed to 1% polidocanol 1 hr prior to LVLacZa vector instillation; M3/4 were exposed to 0.1% LPC 1 hr prior to LVLacZa vector instillation; M5/6 were exposed to PBS 1 hr prior to LVLacZa vector instillation. Mice C1-4 were only anaesthetised to monitor normal weight loss following anaesthesia.

The total number of LacZ positive cells present in the nasal airway epithelium of mice treated with 1% polidocanol was determined in the Saf-O stained cross-sections as described in section 2.2.34 and was found to be 5 ± 3 (n= 3).

The H/E stained cross-sections of mice pre-treated with polidocanol revealed signs of ciliated epithelial cell re-generation. Specifically, the apical surface of the ciliated cells displayed shorter cilia in some regions of the treated nasal airway compared to the cilia of ciliated cells in adjacent regions not affected by polidocanol.

6.1.3 Discussion

The results of this pilot study demonstrate that polidocanol treatment allowed LacZ gene transfer to occur *in vivo*. The efficiency of LV-mediated LacZ gene transfer, however, was low when compared to the significant improvement of AdV-mediated LacZ gene transfer obtained by Parsons and colleagues (1998) using a similar pre-treatment protocol. In their study an AdVLacZ vector was combined with polidocanol pre-treatment of the mouse nasal airway to produce high levels of LacZ gene transfer.

Despite the low efficiency of LacZ gene transfer seen in the current study the results of these experiments show that pre-treatment of the airway epithelium with a detergent allows LV-mediated LacZ gene transfer to occur. Since the LV-treated mice did not display any behavioural signs of distress nor was significant weight loss observed during the 7 day study, it is unlikely that significant immune or inflammatory reactions were evoked in response to either polidocanol administration or to its combination with the LVLacZa vector.

The absence of LV-mediated LacZ gene transfer following LPC treatment of the nasal airway epithelium may have been due to the lower dose (0.1%) used, compared to that of polidocanol (1%). Although this lower dose of LPC can effect the same levels of AdV-mediated gene transfer as those obtained using 1% polidocanol (Parsons *et al.*, 1999), this was not apparent in this study.

As the doses of polidocanol and LPC used in this study were not comparable, it became apparent that a direct comparison of the efficiency of LV-mediated LacZ gene transfer to the mouse nasal airway epithelium treated with an identical dose of either polidocanol or LPC was warranted.

6.2 Efficiency of Lentivirus-Mediated LacZ Gene Transfer following Polidocanol versus LPC Conditioning of the Nasal Airway Epithelium Surface

6.2.1 Introduction

To determine the most effective detergent treatment regimen, the effect of treatment with two doses of polidocanol or LPC, on the level of LacZ gene transduction was assessed here. The LVLacZb vector construct evaluated in this experiment contained the central polyypurine tract (cPPT) sequence, which has been reported to improve LV-mediated gene transduction of quiescent cells (Zennou *et al.*, 2000). In contrast, the LVLacZa vector construct used in the previous experiment did not contain the cPPT sequence.

6.2.2 Methods and Results

Groups of mice (n= 3) were exposed to either polidocanol (1% or 0.1%), or LPC (1% or 0.1%), 1 hr prior to the instillation of 20 µl of either the LVLacZb vector (Figure 5.1; iii) preparation containing 6 x 10^4 NIH3T3-TU, or the carrier solution (PBS). To assess the effectiveness of polybrene on LV-mediated LacZ gene transfer, a further group of mice pre-treated with 1% LPC were exposed to the LVLacZb vector preparation containing 4 µg/ml of polybrene.

The LV-treated mice again did not present any behavioural signs of distress nor was significant weight loss observed following anaesthesia or the LV vector instillation procedures. Seven days later LacZ gene transduction was assessed as previously described (section 6.1.2).

En face examination of the grossly-sectioned blocks of the X-gal stained head showed that the highest levels of gene transfer were obtained in mice pre-treated with the higher (1%) dose of LPC (Figure 6.2; a). LacZ positive cells were present as scattered, punctate blue-stained cells and were only found ipsilaterally, whereas a diffuse light green artefactual staining was distributed bilaterally (apparent in Figure 6.2; b, c). No LacZ positive cells were seen in the PBS pre-treated (control) mice. In addition no LacZ positive cells were present in the olfactory regions of the nasal airway. The nasal airway epithelium blocks were sectioned and then counterstained with Saf-O. Quantitative

determination of LacZ positive cells was restricted to areas of respiratory and transitional epithelium, both of which contain ciliated cells.

The results of this study clearly demonstrated that only when the LVLacZb vector was combined with a detergent pre-treatment of the nasal airway was LacZ gene transduction noted. The 1% dose of LPC, delivered 1 hr prior to the instillation of the LVLacZb vector preparation, resulted in significantly higher gene transfer compared to the 0.1% dose of LPC (Figure 6.2) and to both doses of polidocanol (0.1% and 1%) ($P < 0.05$, ANOVA, SNK, n= 3). Furthermore, treatment of the nasal airway with 1% LPC produced a 4-fold increase in the level of LV-mediated LacZ gene transduction compared to the pre-treatment with 1% polidocanol (Figure 6.3).

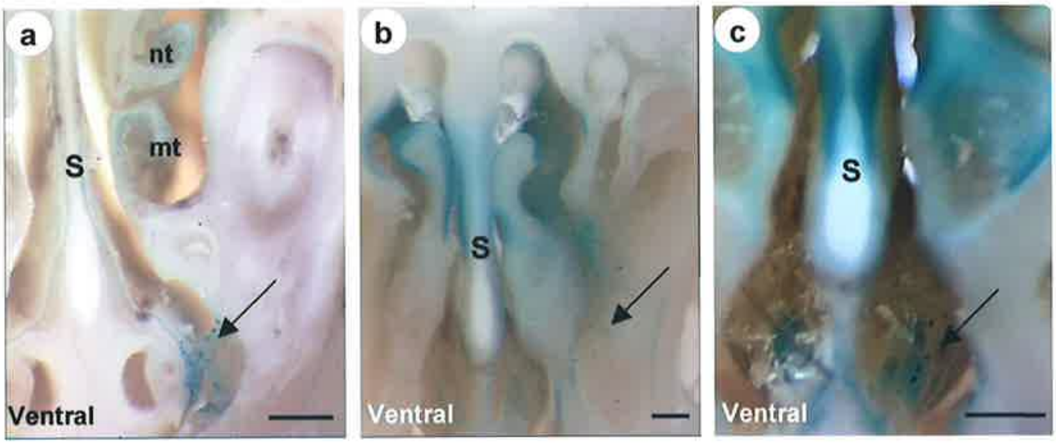


Figure 6.2: LacZ Gene Transfer After a Single Instillation of the LVLacZb Vector into the Detergent-Treated Mouse Nasal Airway.

(a) *En face* view of the 1% LPC-treated nasal airway epithelium followed by exposure to the LVLacZb vector preparation. **(b)** *En face* view of the 1% LPC-treated nasal airway epithelium followed by exposure to the LVLacZb vector preparation in the presence of polybrene (4 μg/ml). **(c)** *En face* view of the 1% polidocanol-treated nasal airway epithelium followed by exposure to the LVLacZb vector preparation. The un-dosed left nasal airway displays no LacZ positive cells, whilst the treated right side shows scattered LacZ positive cells (arrows). Sections are similar to that of Level 16 (Mery *et al.*, 1994). The contrast between the patchy punctate blue staining of the LacZ positive cells and the diffuse blue-green background stain, produced by X-gal processing in un-sectioned tissue, is apparent in sections **(b)** and **(c)**. S= septum, nt= nasoturbinate, mt= maxilloturbinate. Scale bar= 1 mm.

Another interesting finding was that the inclusion of polybrene (PB) in the LVLacZb vector preparation resulted in a significant decrease (4-fold) in the level of LacZ gene transfer, compared to that obtained with the LVLacZb vector, which did not contain PB ($P= 0.002$, Student t-test, n= 3; Figure 6.3).

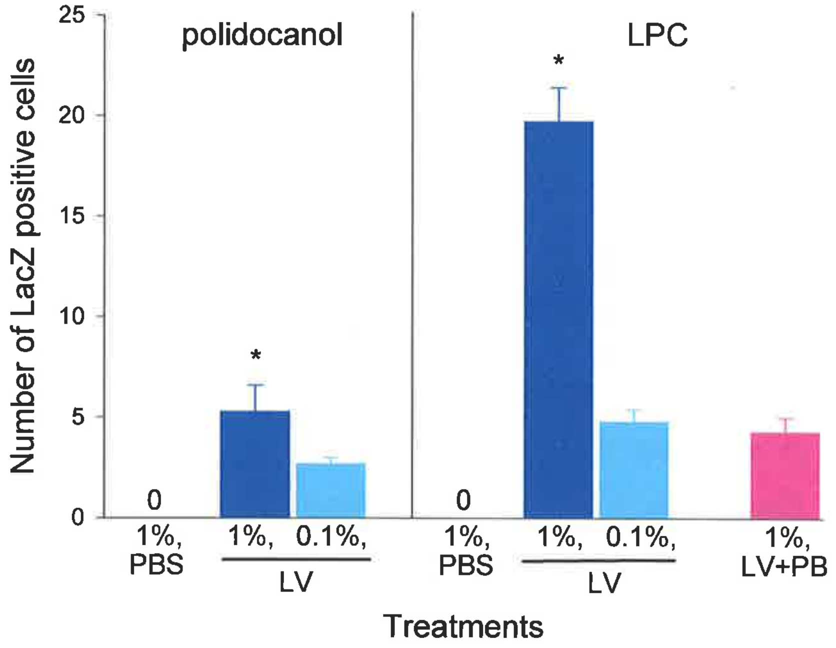


Figure 6.3: Quantitation of *In Vivo* LVLacZb-Mediated Gene Transfer.

C57Bl/6 mice (n= 3) were pre-treated with either detergent (polidocanol or LPC) or PBS and dosed with 20 µl of the LVLacZb vector preparation. Data are presented as the mean ± SEM of the total number of LacZ positive cells counted in 3 standard Saf-O counterstained cross-sections. PB= polybrene. * $P < 0.05$, ANOVA, SNK.

The H/E counterstained cross-sections of the mouse nasal airway pre-treated with the higher doses (1%) of either LPC or polidocanol again revealed signs of epithelial cell re-generation, as found previously in section 6.1.2.

6.2.3 Discussion

The key finding of this experiment was that detergent (polidocanol and LPC) pre-treatment of the mouse nasal airway epithelium facilitated LV-mediated LacZ gene transduction. Comparative studies between polidocanol and LPC treatment of the airway epithelium revealed that LPC was the more effective conditioning agent. When LPC was used to condition the airway epithelium 1 hr prior to the instillation of a VSV-G pseudotyped LVLacZb vector it resulted in a significantly higher LacZ gene expression compared to either pre-treatment with a lower dose of LPC (0.1%) or polidocanol (0.1% and 1%).

The additional sequence of the cPPT, which has been shown to significantly improve gene transduction *in vivo* (Zennou *et al.*, 2000), in the LVLacZb vector construct did not appear to result in significantly higher LacZ gene transduction, even though its viral titre was double that of the LVLacZa vector. In particular, the number of LacZ positive cells observed following transduction of the 1% polidocanol-conditioned airway, using either the LVLacZa vector (section 6.1.2) or the LVLacZb vector was not significantly different (P> 0.0.5, Students t-test).

The results of this experiment also suggest that the widely used enhancer for retroviral vectors, polybrene (Coelen *et al.*, 1983), inhibits LV-mediated gene transfer when used *in vivo*. This finding highlights the need to cautiously interpret results generated from *in vitro* gene transfer experiments prior to commencing *in vivo* gene transfer studies. Based on the findings of the experiments described in this section, airway conditioning with 1% LPC and polybrene-free LV vector preparations were used in all subsequent gene transfer studies.

6.3 Efficiency of Lentivirus-Mediated LacZ Gene Transfer Following 0.1% versus 1% LPC Conditioning of the Nasal Airway Epithelium Surface

6.3.1 Introduction

This experiment was designed to determine which LPC dosing regimen results in the highest level of LV-mediated LacZ gene transfer. Pre-treatment and co-treatment regimens (Table 6.1) were assessed for their effect on the level of LV-mediated LacZ gene transduction. Additional appropriate negative controls were also included to exclude non-specific effects and also β-gal pseudo-transduction.

6.3.2 Methods and Results

Groups of mice (n= 3) were exposed to LPC (1% or 0.1%) prior to the instillation of 20 μl of the LVLacZb vector preparation containing 6 x 10^4 NIH3T3-TU. Mice (n= 3) were transduced with 20 μl of the LVEYFP vector containing 5 x 10^5 NIH3T3-TU to assess any non-specific effects as a result of LV vector exposure. In addition, another group of mice (n= 3) was transduced with 20 μl of pcDNALacZ "virus". This virus was prepared as described in section 2.2.24 (a) with the difference being that the LVLacZ vector plasmid was substituted with a plasmid expression construct for β-gal (pcDNA3.1LacZ) based on pcDNA3.1 (Invitrogen). This provided a control for pseudo-transduction of β-gal expressed in the 293T cells, which were used for LV vector production, in the absence of LVLacZ-mediated gene transfer. Seven days later LacZ gene transduction was assessed as previously described (section 6.1.2).

Qualitative *en face* examination of the blocks of X-gal stained nasal airway epithelium revealed the presence of LacZ positive cells in all mouse airways conditioned with LPC prior to LV vector instillation. Consistent with the findings of the previous experiment (section 6.2), the highest level of LacZ gene transfer was evident in the nasal airway epithelium of mice pre-conditioned with 1% LPC 1 hr prior to the instillation of the LVLacZb vector preparation.

The total number of LacZ positive cells in the 3 standard cross-sections counterstained with Saf-O was then determined (Table 6.1) as previously described (section 6.1.2). LacZ positive cells were not seen in the olfactory regions of the nasal airway.

Table 6.1: Quantitation of *In Vivo* LVLacZb-Mediated Gene Transfer.

Group	LPC (%)	Time interval	Viral solution	Number of LacZ positive cells ± SEM
		Controls		
a	1	1 hr	PBS	0
b	1	1 hr	pcDNALacZ "virus"[1]	0
c	1	1 hr	LVEYFP	0
		Treatments		
d	0.1	1 hr	LVLacZb	12.0 ± 0.7
e	1	1 hr	LVLacZb	22.0 ± 1.4
f	1	~1 min	LVLacZb	0
g	1	Co-administration	LVLacZb	0

The values presented here are the mean ± SEM of the total number of LacZ positive cells counted in 3 standard Saf-O stained cross-sections (as described in section 2.2.34) of n= 3 mice. [1]Pseudo-transduction control.

The results show that the conditioning of the airway epithelium surface with 1% LPC 1 hr prior to the instillation of the LVLacZb vector preparation produced a significantly higher number of LacZ positive cells (22 ± 1.4), compared to that seen in mice conditioned with the 0.1% dose of LPC (12 ± 0.7) ($P< 0.05$, Students t-test). No LacZ positive cells were evident in the nasal airway epithelium of mice conditioned with 1% LPC immediately prior to LVLacZb vector instillation (Group f), or following the instillation of the LVLacZb vector that had been mixed with LPC immediately before delivery (Group g).

LacZ positive cells were not observed in the Saf-O counterstained cross-sections from any of the mice of the control groups (Groups a, b and c), confirming that up-regulation of endogenous β-gal or pseudo-transduction of β-gal had not occurred as a result of the instillation of the LV vector or exposure to LPC treatment.

6.3.3 Discussion

Only two LPC dosing time points were examined in this experiment, but the results do suggest that there is a time-dependence between the instillation of the LVLacZ vector preparation and airway conditioning with LPC. This was indicated by maximal LacZ gene transfer 1 hr after treatment with LPC (1%).

The absence of LV-mediated LacZ gene transduction found after co-administration of the LV vector with the 1% dose of LPC may be due to the detrimental surfactant action of LPC on the viability of the LV vector titre. The results from section 5.5.2 (b) showed that incubation of the LV vector with LPC (to a concentration of 0.01%) for 30 mins reduced the viral titre by more than half. Although in this present study the incubation time of the LV vector with the LPC (i.e. the period between mixing the LPC and virus, and its instillation) is probably less than one min, the dose of LPC used here was 100-fold higher (1%) than that used in the experiment described in section 5.5.2 (b). Therefore, it is more than likely that this dose of LPC destroyed the titre of the LVLacZb vector, thus accounting for the complete absence of LV-mediated LacZ transduction seen here.

Interestingly, LacZ gene transfer was not evident when the LVLacZb vector was instilled immediately following LPC treatment. Although LPC has been shown to permeabilise the airway epithelium TJ integrity (Parsons *et al.*, 1999), the opening of the TJ's may require a minimum time to take effect. This may be one reason why LacZ gene transduction was not apparent when the LVLacZb vector was added immediately after airway conditioning. Clearly a more detailed analysis of this dose/time-dependent relationship of LPC on LV-mediated LacZ gene transduction and on the LV vector itself, is warranted. Due to time limitations, however, further investigations were precluded.

6.4 Persistence of Lentivirus-Mediated LacZ Gene Transduction to the Nasal Airway Epithelium

6.4.1 Introduction

The level of persistence of gene expression will be a critical determinant for the utility of a specific gene transfer vector to correct the CFTR defect present in the CF airway epithelium. In this vein, the next parameter assessed here was the persistence of LacZ gene expression following successful LV-mediated gene transduction in the nasal airway epithelium. The LVLacZb vector (featured in sections 6.2 and 6.3) and the LVLacZc vector, used in this present study, contain essentially the same HIV-1 sequence elements but differ slightly in the arrangement of these sequences. The most notable difference was the shorter length of *gag* gene sequence in the latter vector (Figure 5.1; iv).

6.4.2 Methods and Results

The right nostril of 9 mice was exposed to 1% LPC 1 hr prior to the instillation of 20 μl of the LVLacZc vector (Figure 5.1; iv) preparation containing 1.4 x 10^5 NIH3T3-TU.

Three LV-treated mice were subsequently sacrificed at 7, 28 and 92 days and the mouse heads analysed for LacZ gene expression as described in section 6.1.2. Qualitative *en face* examination of the grossly-sectioned blocks of the X-gal stained nasal airway epithelium at each post-treatment point (7, 28 and 92 days) revealed substantially higher numbers of LacZ positive cells compared to those present in the previous experiments (sections 6.2 and 6.3). The distribution of the LacZ positive cells at each time-point remained ipsilateral along the treated nostril (Figure 6.4; a, c, e) with high numbers of LacZ positive cells located as far posteriorly as the nasopharyngeal meatus (Figure 6.4; b, d, f). Anatomically the two nasal airways coalesce into one nasopharyngeal airway at this posterior level of the nose. It was, therefore, interesting to note that LacZ positive cells remained localised to only the dosed side of the head. LacZ positive cells were not seen in the olfactory regions of the nasal airway.

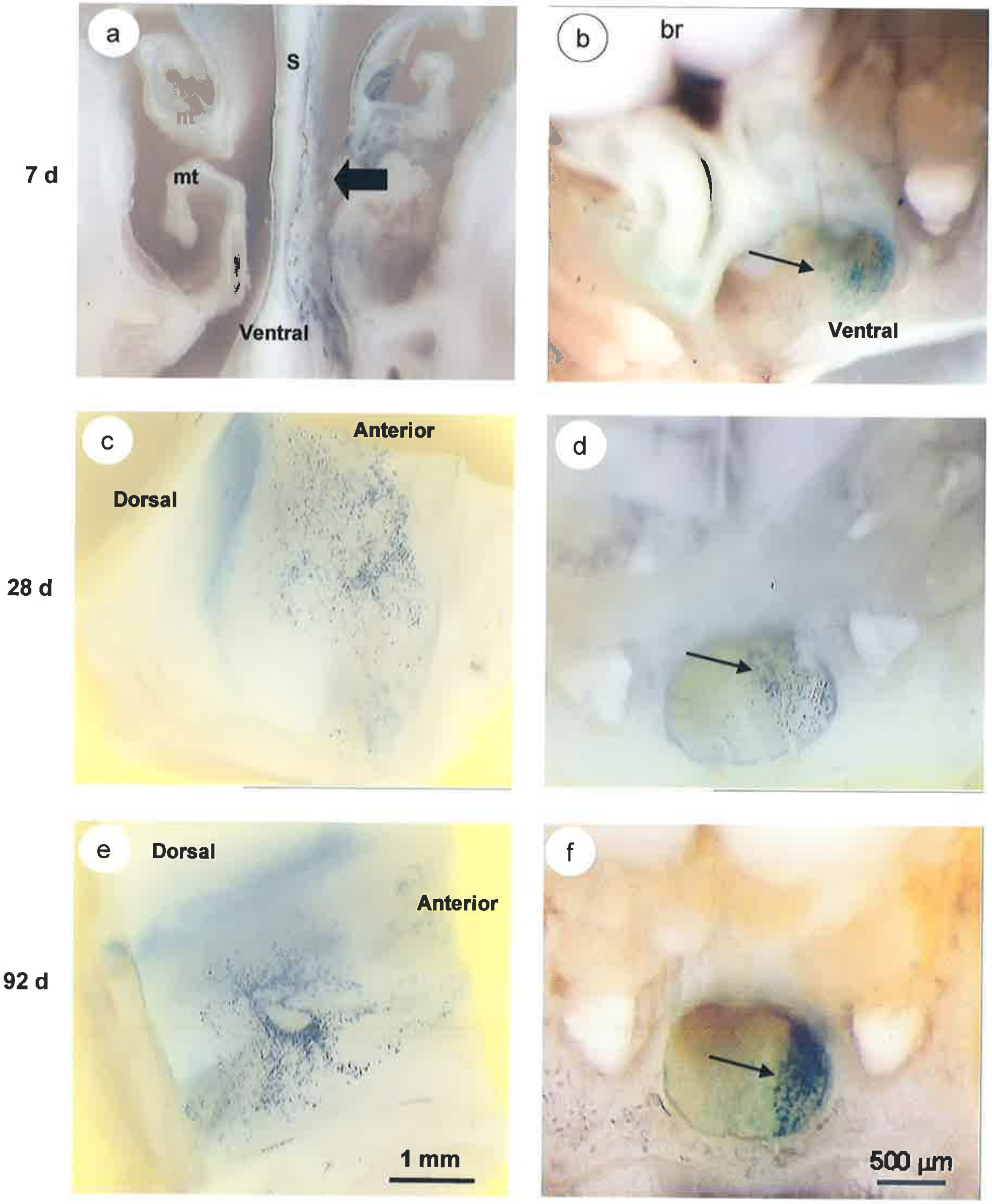

7 d
a
s
mt
mt
Ventral
b
br
Ventral
28 d
c
Anterior
Dorsal
d
92 d
e
Dorsal
Anterior
1 mm
f
500 µm

Figure 6.4: LacZ Gene Transfer After a Single Instillation of the LVLacZc Vector into the LPC-Conditioned Mouse Nasal Airway.

(a): 7 days. *En face* anteriorly directed view of septum (s) and turbinates (nasoturbinate (nt), maxilloturbinate (mt)) at 7 days. The un-dosed (left) nasal airway displayed no LacZ positive cells or regions, while the treated (right) side showed scattered LacZ positive cells along the vertical face of the septum and in some faces of the nasal turbinates. The thick arrow shows direction of view of septum face in panels c and e. Note the lack of LacZ positive cells in the un-treated nasal airway. Section is similar to that of Level 16 (Mery *et al.*, 1994). **(b)**: 7 days. Situated below the brain (br) at the posterior of the nasal cavity, moderate LacZ cell staining is present in the nasopharyngeal meatus on only the ipsilateral (right) portion of this airway, corresponding to the dosed nostril (arrow). **(c)**: 28 days. View of the septum wall as indicated by arrow in **(a)**. The remaining nasal airway of the ispilateral (dosed) nostril has been cut away to allow this view. The contrast of the patchy punctate blue staining of LacZ positive cells with the diffuse blue-green background stain, characteristic of X-gal processing in un-sectioned tissue (here, in the olfactory region), is apparent. **(d)**: 28 days. LacZ positive cells in the nasopharyngeal meatus (arrow) again remain ipsilateral. **(e)**: 92 days. View of the X-gal processed septum and left nostril (the curve of the contralateral dorsal olfactory region is visible here outlined by the diffuse background X-gal stain. Scale bar applies to **(a)**, **(c)** and **(e)**. **(f)**: 92 days. Strongly expressing LacZ positive cells present in the ipsilateral half of the nasopharyngeal meatus (arrow) reveal the persistence of LacZ gene expression for 92 days after the single dose of the LVLacZc vector preparation. Scale bar applies to **(b)**, **(d)** and **(f)**. d: days.

LacZ positive cells were observed in all regions of the nasal airway with the exception of the olfactory and squamous regions (Figure 6.4). When the number of LacZ positive cells was quantified in Saf-O counterstained cross-sections (Figure 6.5; a, b, c) the number of LacZ positive cells observed at days 7, 28 and 92 post-treatment was maintained at similar levels (Figure 6.6) confirming the persistence of LacZ gene expression produced by LV-mediated gene transfer.

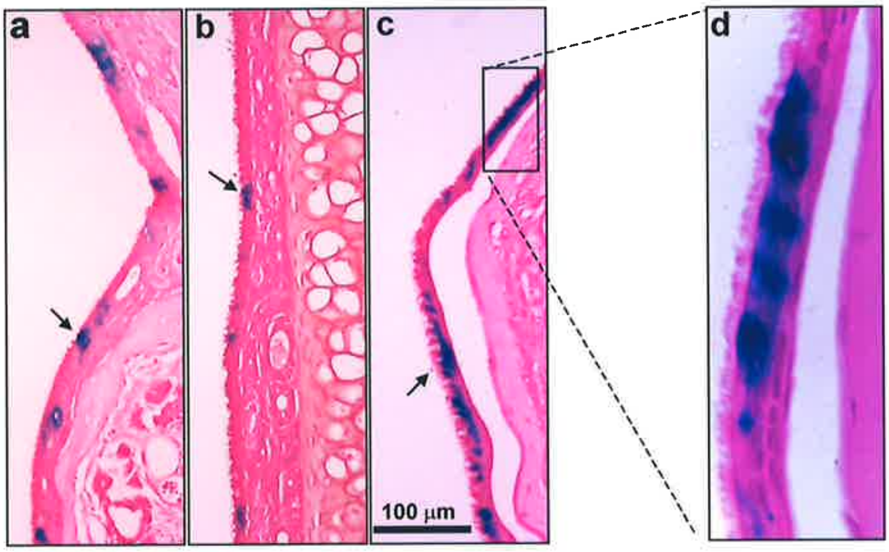


Figure 6.5: Details of Anterior Nasal LacZ Gene Expression After LV-Mediated Gene Transfer.

LacZ gene transfer into the ciliated airway epithelium of the nasal septum was limited to the right treated nostril. Saf-O stained cross-sections at **(a)** 7 days, **(b)** 28 days and **(c)** 92 days after exposure to the LVLacZc vector show LacZ gene expression as individual blue darkly-stained cells or groups of LacZ positive cells (arrows). The type of LacZ positive cells present in the Saf-O stained cross-sections was determined in adjacent H/E stained sections. For example, the cells present in the defined area of section **(c)** when examined at x 1000 (oil immersion) were identified as ciliated in the H/E stained section **(d)** (magnification x 400). Scale bar applies to **(a)**, **(b)** and **(c)**.

On days 7, 28 and 92 post-treatment the level of LV-mediated LacZ gene transfer did not differ significantly suggesting that LV-mediated gene transfer had persisted. Furthermore, the number of LacZ positive cells observed at 92 days displayed a slight (but not statistically significant) increase (Figure 6.6); although statistical power (0.05) was too low for conclusive analysis.

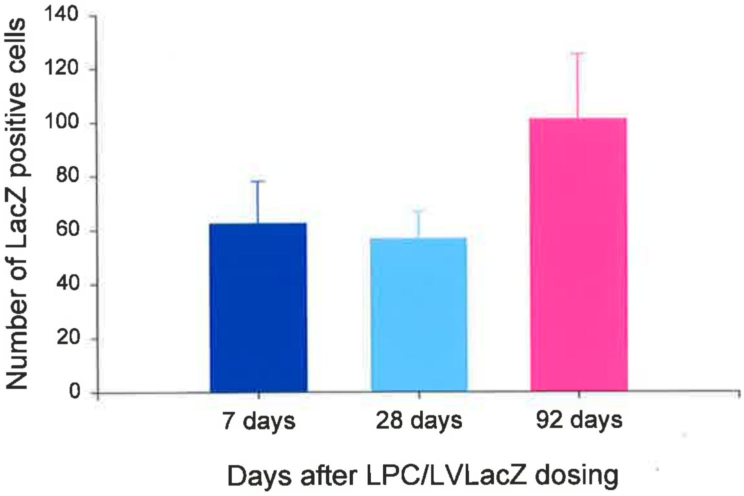


Figure 6.6: Quantitation of *In Vivo* LVLacZc-Mediated Gene Transfer.
The total number of LacZ positive cells over 3 standard nasal airway cross-sections supports the qualitative impression of the persistence of LacZ gene expression for at least 92 days after dosing (Figure 6.5, a, b, c). The apparent increase in transduction at day 92 was not significant (P= 0.64, ANOVA, SNK, n= 3), although the statistical power (0.05) was too low for conclusive analysis. Values represent the mean of the total number of LacZ positive cells in n= 3 mice ± SEM.

It is important that the appropriate airway epithelial cell(s) are transduced when developing gene transfer protocols for CF gene therapy (Parsons *et al.*, 2000b). Hence the types of positively transduced cells were determined in H/E stained sections (Figure 6.5; d). LacZ positive cells were found to be predominantly ciliated or non-ciliated; but smaller numbers of positively transduced secretory cells (predominantly goblet) and basal cells were also seen. The numbers of each cell type showed significant changes over the duration of the experiment (Figure 6.7). Of note was that LacZ positive secretory cells were only observed at 92 days post-treatment.

6.4.3 Discussion

The number of LacZ positive cells at day 92 showed an increase, although this was not significant (Figure 6.6). As the cell turnover time of rodent airway epithelium is thought to be in the order of ~3 months (Borthwick *et al.*, 2001), the total number of LacZ positive cells observed should have dropped substantially by 92 days, unless airway progenitor cells had been successfully transduced. In support of the belief that progenitor cells were transduced, it must be noted that although LacZ positive secretory cells were not seen at

days 7 or 28, they were present at day 92 (Figure 6.7). This suggests that outgrowth and differentiation of positively transduced progenitor cells into secretory cells may have occurred between days 28 and 92. Given the current rudimentary understanding of stem cell identity and physiology in the intact airway epithelium the reason for the changes in the numbers of LacZ positive ciliated, non-ciliated and basal cells across the 3 assessment time-points could not be addressed here. Presumably, the changes observed represented a dynamic balance between the turnover of mature cells and their replacement by outgrowth and differentiation of various progenitor populations, each of which would display a different initial transduction efficiency.

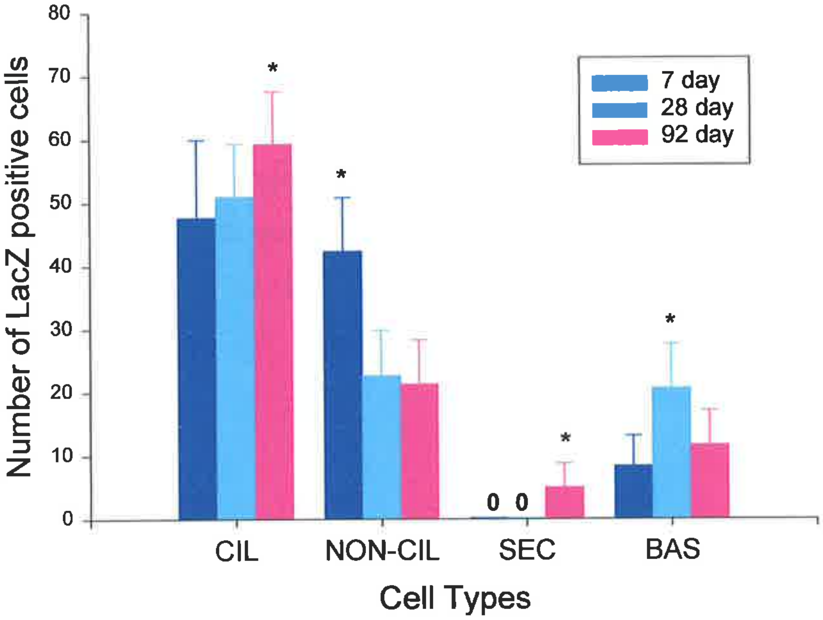


Figure 6.7: Types of LacZ Positive Cells in the Mouse Nasal Airway Epithelium.

The significance of changes in the proportion of positively transduced cells of each cell type over the 3 assessment time points was individually examined using logistic regression analysis. For each cell type the proportion of LacZ positive cells altered significantly during the assessment period (* Ciliated P= 0.01, Non-ciliated P< 0.001, Secretory P< 0.001 and Basal P< 0.001). Statistically, the significance of the results for the Secretory cells should be regarded as approximate, given the zero counts on days 7 and 28. CIL: Ciliated; NON-CIL: Non-ciliated, SEC: Secretory, BAS: Basal.

As reviewed in Chapter 1 (section 1.3.7, b) endogenous LPC is rapidly converted in cell systems (Besterman and Domanico, 1992) and lung alveoli (Seidner *et al.*, 1988) to the ubiquitous and non-toxic dipalmitoylphosphatidylcholine (DPPC), a primary component of biological membranes. Accordingly, there may be some capacity for exogenous LPC to be similarly converted *in vivo*, providing a measure of active post effect detoxification that is not a feature of the other airway barrier modulation reagents (e.g. SO_2, EDTA, etc.) reported to date.

Ideally, a pre-treatment/conditioning agent should produce a transient and tolerable perturbation of the barrier function(s) of the ciliated airway epithelium. The histological injury that was observed immediately after dosing with 1% LPC alone, i.e. limited areas of deciliation or exfoliation, was found anteriorly close to the dosed site (Dr. D. W. Parsons, personal communication) and this same LPC exposure prior to LV vector instillation was associated with effective LacZ gene expression (Figure 6.4). Persisting LacZ gene expression was also found in the nasal airway epithelium as far posterior as the nasopharyngeal meatus (Figure 6.4; b, d, f) a site where LPC-induced cell injury was not observed. Therefore it appears that even more dilute concentrations of LPC reaching this region will also permit LV-mediated gene transfer. This finding is consistent with data showing that milder, non-injurious, LPC-based airway modulation also improves the efficacy of other gene transfer vectors (i.e. adenovirus vectors) (Parsons *et al.*, 1999).

This observation supports the contention that a more detailed analysis of the LPC-based airway modulation protocol was warranted. Due to time limitations, however, further investigations were precluded.

Part B

Lentivirus-Mediated CFTR Gene Transfer

6.5 Recovery of Airway CFTR Function in *cftr*^{tm1Unc} Mice after Single Dose Lentivirus-Mediated CFTR Gene Transfer

6.5.1 Introduction

On-going expression of functional CFTR in CF airways is the primary goal in the efforts to develop a gene therapy cure for CF lung disease. The greater than 3-month persistence of LV-mediated LacZ gene expression found after only a single LVLacZ vector dose (section 6.4) provides strong evidence in support of the LV-mediated CFTR gene transfer studies. As a logical step in gene therapy development this protocol was tested in *cftr*^{tm1Unc} mice.

The LVCFTR vector used here was essentially the same as the LVLacZc vector with the difference being that the CFTR cDNA sequence replaced the LacZ marker gene sequence.

6.5.2 Methods and Results

Six *cftr*^{tm1Unc} mice were exposed to a 4 µl bolus of 1% LPC 1 hr prior to the instillation of 20 µl of the LVCFTR vector (Figure 5.1; v) preparation containing ~4 x 10^4 NIH3T3-TU. As a control for the effect of LPC alone, 3 *cftr*^{tm1Unc} mice were exposed to 1% LPC 1 hr prior to the instillation of the vector carrier solution (PBS). Successful LV-mediated CFTR gene transduction was measured as a decrease in the ΔPD values. More negative ΔPD values reflect functional correction of the CFTR-mediated Cl^- secretion defect (Parsons *et al.*, 1998).

Untreated *cftr*^{tm1Unc} mice exhibited a ΔPD value of +9.5 ± 1.2 mV (n= 6) (Figure 6.8; a) and treatment of the *cftr*^{tm1Unc} mouse nasal airway epithelium with LPC prior to the instillation of PBS (control) did not significantly alter these values (ΔPD= +8 ± 3.2 mV) when examined 7 days after treatment ($P > 0.05$, ANOVA, SNK). Furthermore, the maintenance of the TPD basal values of the nasal airway epithelium of these latter mice indicated that any detergent-induced injury that may have occurred to the airway epithelium was fully resolved electrophysiologically within 7 days.

Recovery of Airway CFTR Function

(a) Seven Days Post-Treatment

The exposure of the LPC-conditioned *cftr*$^{\text{tm1Unc}}$ mouse nasal airway epithelium to the LVCFTR vector resulted in functional expression of CFTR. In particular, the mean ΔPD values (+2.5 ± 2.2 mV) were more negative (but not significantly different) compared to those of untreated *cftr*$^{\text{tm1Unc}}$ mice (+9.5 ± 1.2 mV; *P*< 0.05, ANOVA, SNK, n= 6) (Figure 6.8; a). This change represented a 31% functional correction towards the mean ΔPD value of heterozygote CF colony mice (-16.5 ± 2 mV, n= 8).

(b) Forty-Six Days Post-Treatment

At this time point CFTR gene expression was re-assessed in the 3 surviving LV-treated *cftr*$^{\text{tm1Unc}}$ mice. The analysis of the TPD values showed that functional recovery of CFTR activity had reached 54% of the mean heterozygote ΔPD value, a statistically significant improvement (ΔPD= -4.5 ± 3.1 mV, *P*< 0.05 ANOVA, Dunnett's method, n= 3) (Figure 6.8; a) from the untreated *cftr*$^{\text{tm1Unc}}$ mouse ΔPD value (+9.5 ± 1.2 mV).

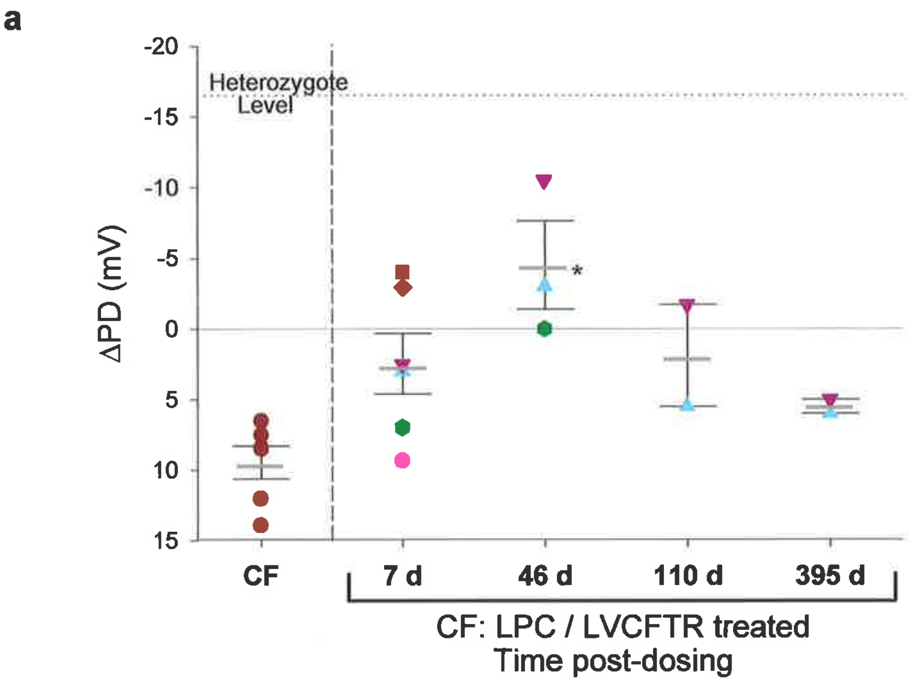
The smaller number of animals available for assessment of CFTR gene transfer was due to technical problems associated with the TPD measurement technique and anaesthesia in CF mice at 7 days; the ensuing low sample size limited the level of statistical analysis of this and later time points.

(c) One Hundred and Ten Days Post-Treatment

CFTR gene expression persisted in one of the two surviving LV-treated *cftr*$^{\text{tm1Unc}}$ mice (ΔPD= -1.7 mV; 43% of mean heterozygote ΔPD values), whilst in the other mouse the ΔPD (ΔPD= +5.5 mV; 15% of mean heterozygote ΔPD values) had waned to near the ΔPD values of untreated *cftr*$^{\text{tm1Unc}}$ mice (+9.5 ± 1.2 mV, n= 6) (Figure 6.8; a).

(d) Three Hundred and Ninety-Five Days (Thirteen Months) Post-Treatment

At the final time point of this experiment CFTR gene expression in the two surviving LV-treated *cftr*$^{\text{tm1Unc}}$ mice had declined further (ΔPD= +5.0 mV and ΔPD= +6.0 mV; 17% and 13% of mean heterozygote ΔPD values, respectively) (Figure 6.8; a); but their ΔPD values were marginally outside the range of the ΔPD values of untreated *cftr*$^{\text{tm1Unc}}$ mice (+6.5 to +13.9).


a
-20
-15
-10
-5
0
5
10
15
ΔPD (mV)
Heterozygote Level
*
CF
7 d
46 d
110 d
395 d
CF: LPC / LVCFTR treated
Time post-dosing

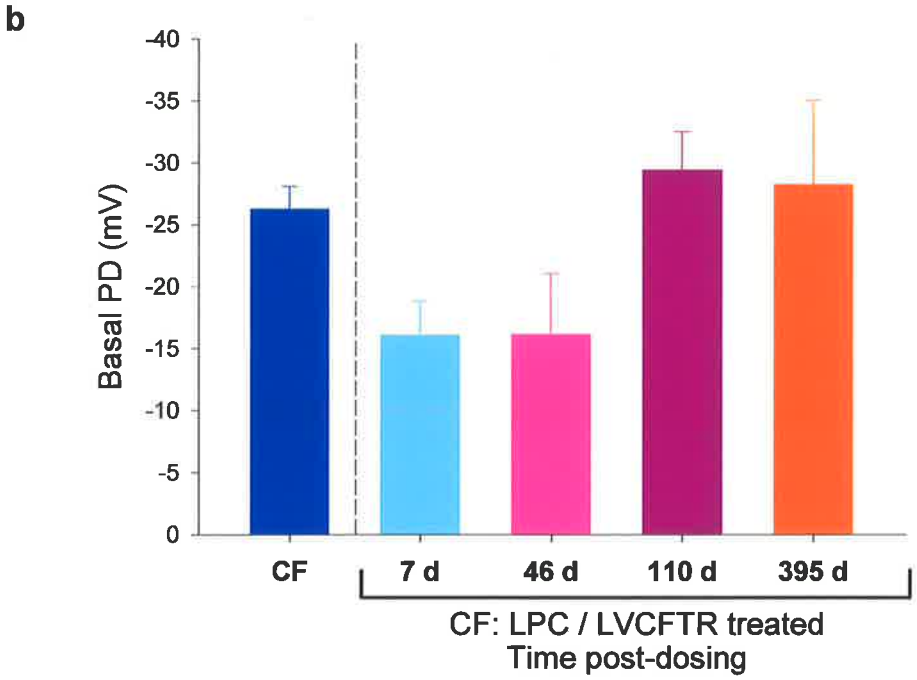

b
-40
-35
-30
-25
-20
-15
-10
-5
0
Basal PD (mV)
CF
7 d
46 d
110 d
395 d
CF: LPC / LVCFTR treated
Time post-dosing

Figure 6.8:

(a) Effect of LVCFTR Vector Instillation on the ΔPD of the Mouse Nasal Airway Epithelium.

Between 7 and 395 days the mean change in ΔPD (horizontal grey bars: mean with SEM) as well as individual time-linked ΔPD values (symbols ■, ◆, ▼, ▲, ⬢, ●) are shown. Significant partial recovery of the CFTR electrophysiological function compared to the mean ΔPD of untreated $cftr^{tm1Unc}$ mice, n= 6) was present at 46 days (* $P< 0.05$, ANOVA, Dunnett's method, n= 3). At 110 days the ΔPD of one mouse remained high (▼), but by 395 days the ΔPD for both remaining LV-treated $cftr^{tm1Unc}$ mice in this study had waned to near untreated $cftr^{tm1Unc}$ mouse ΔPD mean values. Time series repeated measures comparisons could not be performed due to animal losses during the course of this study. CF= cystic fibrosis; d= days.

(b) Effect of LVCFTR Vector Instillation on Sodium Hyperabsorption.

ANOVA analysis indicated that there was a significant difference between the five treatment groups ($P= 0.03$) but subsequent multiple comparisons against the untreated $cftr^{tm1Unc}$ mouse control group (Dunnett's method) did not identify the source of the significant TPD reduction(s). The low statistical power (0.58) did not allow conclusive analysis.

The influence of LVCFTR vector instillation on the Na^+ hyperabsorption defect, that is a secondary feature of the CF airway dysfunction, was not specifically addressed in these studies *via* comparison of airway TPD in amiloride-supplemented basal and low Cl^- solutions (see also section 3.4.2, b). The apparent reductions in basal TPD values present at days 7 and 46 (Figure 6.8; b) after LVCFTR vector exposure did not reach statistical significance.

To evaluate any non-specific effects following LV vector exposure, additional negative controls such as mice transduced with a LV vector carrying a marker gene (i.e. LacZ or EYFP) would have been appropriate. The limited number of *cftr*$^{\mathrm{tm1Unc}}$ mice available at the time of experimentation, however, obviated these controls from the experimental protocols.

6.5.3 Discussion

The key finding of this study was that exposure to a single dose of a LV vector carrying the CFTR gene could produce extended electrophysiological recovery of CFTR function in *cftr*$^{\mathrm{tm1Unc}}$ mouse nasal airway epithelium. This recovery, however, began to decline by 110 days and had almost disappeared by 395 days (Figure 6.8; a). Several factors may have contributed to this apparent difference in persistence in expression of the LacZ and CFTR genes. First, the measurement of nasal TPD in mice has inherent technical limitations (Parsons *et al.*, 2000b) and it is possible that these limitations may have contributed to the variability observed in the TPD values at each assessment time point. In particular, at each TPD assessment point, the cannula tip may not have sampled from precisely the same area of airway epithelium. Due to the complexity of the mouse anterior nasal anatomy (Parsons *et al.*, 2000b); the relative positional changes in nasal anatomy that accompanies growth; and the inherent variability in nasal cannula insertion procedures, it is necessary that larger numbers of mice are tested in future studies in order to overcome this source of variability. Second, differences in the completeness of sampling of gene expression may be important. LacZ gene expression provides a visible and unambiguous assessment applicable to both the entire nasal airway (Figure 6.4) and to standard samples of airway (Figure 6.5), whilst measurement of CFTR gene expression samples from a restricted area of airway epithelium under the TPD cannula tip.

The correction of the Na^+ absorption impairment, which is characteristic of CF airway dysfunction, as a result of LVCFTR gene transfer was not specifically examined in this study. Nevertheless, the reduction (non-significant) in the mean basal TPD values, an index of Na^+ channel activity, apparent at days 7 and 46 following LVCFTR vector instillation suggested that the LV-mediated CFTR transfer can alter the basal TPD. The basal TPD values present at the later time points were, however, clearly no different to those present in untreated *cftr*^{tm1Unc} mice. Additional studies that include appropriate TPD comparisons using amiloride-supplemented/free solutions are indicated, to both improve statistical power and to directly examine how CF airway Na^+ hyperabsorption is altered by LVCFTR vector exposure.

The reasons for the discrepancy in the gene expression persistence produced by the LVLacZ and LVCFTR vectors are not known. To resolve this discrepancy the cell types require CFTR correction and the cells that produce the electrophysiologically measured changes in epithelial TPD need to be identified. It is also vital to determine the association between the ΔPD value, the level of CFTR expression per cell and the percentage of cells expressing vector-delivered CFTR in the intact human airway epithelium. Understanding this relationship should provide key information to help direct the development of more efficient airway gene transfer vectors. Detailed, longer-term gene transfer studies using both the LacZ marker gene and the CFTR therapeutic gene to resolve these issues are clearly warranted.

This first demonstration of lentivirus-mediated *in vivo* recovery of CFTR function in CF airway epithelium illustrates the potential of combining a pre-conditioning of the airway epithelium surface with a simple and brief LV vector exposure to produce therapeutic gene expression in the CF airway. Furthermore the simplicity of this transduction protocol should facilitate further development towards clinical application.

CHAPTER 7

DISCUSSION

Preface

Chapter 7 is divided into two parts. Part A gives a general overview of the work described in this thesis and reviews areas of this work that should be investigated in more depth. Part B provides a general overview of the current status of CF gene therapy.

Part A

7.1 Overview of the Work Described in this Thesis

The research described in this thesis represents the first demonstration of *in vivo* recovery of CFTR function in CF airway epithelium using an LV vector. The key finding of this study was that exposure to a single dose of a LV vector carrying the CFTR gene could produce extended (at least 110 days) electrophysiological recovery of CFTR function in CF mouse nasal airway. Furthermore, the use of LPC conditioning, which enhanced gene transfer, appeared not to be associated with any adverse effects. This simple transduction protocol offers an optimistic outlook for successful clinical application in the future.

The experimental work of this thesis began with the evaluation of various cell culture systems in Chapter 3 in an attempt to identify a model of the intact human airway epithelium suitable to develop and assess LV-mediated gene transfer. Immortalised cell lines, primary cultures of airway epithelial cells and cultures of explanted nasal septa were each evaluated. Due to limitations, none of these cultures provided a suitable and reliable model of the intact human airway epithelium. In particular, whilst immortalised epithelial cell lines are easy to culture and provide an infinite resource, they do not differentiate into ciliated cells, goblet cells, they do not produce an overlaying mucus layer etc, all of which are defining characteristics of the intact airway epithelium. In contrast, air liquid interface cultures of primary airway epithelial cells provide a much more relevant model of the intact airway epithelium.

The technique of growing airway epithelial cells at air liquid interface was not reliably established during this thesis project, preventing its general use in the gene transfer development studies. As an alternative, cultures of explanted rat nasal septa were evaluated. These cultures appeared to provide a more reliable model of the intact airway epithelium. It should be noted that controlled application of solutions, viral or detergent to the apical surface of the tissue was difficult. As a consequence, the generally positive and encouraging results of the *in vitro* gene transfer studies (Chapter 5) could not be considered reliable enough to use in the planned LV vector development and delivery studies. Furthermore, this culture system was not amenable to the direct quantification of gene transfer, as discussed in section 5.6.2. Studies therefore moved directly into the evaluation of LV-mediated gene transfer to the nasal airway of C57Bl/6 mice using the LacZ marker gene, prior to examining the therapeutic potential of the developed gene transfer protocol in the nasal airway of $cftr^{tm1Unc}$ mice using the CFTR gene.

The hypothesis that detergent-mediated disruption of the epithelial tight-junction (TJ) integrity improves viral particle access to the basolateral cell surface was supported by the sub-TJ localisation of the fluorescence-labelled AdV vector particles in polarised MDCK cells following treatment with polidocanol, as demonstrated in Chapter 4.

Driven by the need to develop more successful gene transfer vectors for CFTR gene delivery to the airways of CF patients, the LV vector based on HIV-1, was identified as a suitable candidate. The construction and incremental development of this LV vector system has been described in Chapter 5 (section 5.1). As high viral titre LV vector stocks will be necessary for pulmonary gene therapy applications, due to (i) the extensive epithelium surface area that must be targeted and (ii) the vector losses if nebulisation is used for airway delivery, a number of parameters of the LV vector production protocol were investigated. The LV vector production protocol that was developed employed calcium-phosphate transfection of 293T cells, collection of the viral supernatant in DMEM/5% (v/v) FCS and viral stock concentration using a combination of ultra-filtration and ultra-centrifugation procedures.

An unexpected difficulty associated with the use of the EYFP marker gene was described in section 5.3; the de-mineralising solution (DeCal) used for nasal airway processing was found to destroy EYFP fluorescence, preventing the planned use of EYFP as a marker gene in gene transfer studies *in vivo*. However, when an alternate marker gene, LacZ, was used the titre of the resulting LV vector was almost 10-fold lower than that of the equivalent LVEYFP vector (Dr. D. S. Anson, personal communication). Since retroviruses utilise an RNA genome, one explanation for the lower titre of the LVLacZ vector was that RNA processing may have occurred; indeed, at least three cryptic splice-acceptor sites were identified in the LacZ gene sequence (at LacZ positions 785, 1939 and 2300 - section 5.4).

The *in vitro* gene transfer studies described in Chapter 5 made use of both the EYFP, since the DeCal solution was not required in cell culture processing and LacZ marker genes. These studies examined firstly, whether pre-treatment of polarised cells could improve LV-mediated gene transduction and secondly, whether the LV vector could transduce primary airway epithelial cells. The increase in the level of LVEYFP-mediated gene transfer into polarised MDCK and 16HBE cells following either polidocanol or LPC treatment supported the notion that LPC, like polidocanol, improved gene transfer by permeabilising the epithelial TJ's. In addition, it was shown that the LVLacZ vector could transduce both primary rat tracheal epithelial cells and cells in the respiratory epithelium of the explanted rat nasal septa.

Chapter 6 described the application of the LV vector in the *in vivo* gene transfer studies. Initial experiments made use of the LacZ marker gene to assess whether treatment of the epithelium with a detergent improved the efficiency of LV-mediated gene transfer. Several LV vector constructs expressing the LacZ marker gene were utilised in these studies. The main difference between the LVLacZa and both the LVLacZb and LVLacZc vectors was the absence of the central polypurine tract (cPPT) sequence from the LVLacZa vector. Although the cPPT sequence has been shown to improve transduction of quiescent cells by LV-based vectors (Zennou *et al.*, 2000), this was not apparent in these *in vivo* gene transfer studies.

The results of the *in vivo* LacZ gene transfer studies showed that pre-treatment of the intact nasal airway epithelium with either of the two detergents (polidocanol and LPC) allowed for LV-mediated LacZ gene transduction to occur; LPC (1%) was the more effective conditioning agent. Furthermore, LV-mediated LacZ gene expression persisted for at least 3 months after the single LVLacZc vector instillation. Interestingly, there was a (non-significant) trend towards an increase in the number of LacZ positive cells with time, which combined with the late appearance of LacZ positive secretory cells, suggested that airway progenitor cells may have been successfully transduced.

The persistence of LV-mediated LacZ gene expression found after the single LV vector instillation was encouraging and suggested that the evaluation of LV-mediated CFTR gene transfer in *cftr*$^{\mathrm{tm1Unc}}$ mice was worthwhile. The LVCFTR vector used in these studies was essentially the same as the LVLacZc vector but with the CFTR cDNA sequence replacing the LacZ marker gene sequence. The most important finding of these studies was that a single dose of the LVCFTR vector could produce significant electrophysiological recovery of CFTR function. However, this recovery began to decline by 110 days.

The reasons for the discrepancy in gene expression persistence produced by the LVLacZ and LVCFTR vectors are not known. In order to resolve this discrepancy the cells requiring CFTR correction and the cells that produce the electrophysiologically measured changes in epithelial TPD need to be identified. Furthermore, determining the link between the ΔPD value, the level of CFTR expression per cell and the percentage of cells expressing vector-delivered CFTR should provide key information to help direct the development of more effective airway gene transfer vectors for CF gene therapy.

7.2 Future Directions

Due to time limitations other experimental approaches designed to improve LV-mediated gene transduction could not be addressed as part of this thesis project. Nonetheless, these approaches must be carefully addressed in future studies if the encouraging findings described in this thesis are to be developed to a stage where this LV vector system could be considered for use in a clinical setting.

(a) LPC Dosing Regimen

The role and use of LPC for airway surface conditioning warrants more detailed investigation and development. Future studies should aim to clearly define the most effective dose of LPC and its temporal relationship to the instillation of the LV vector preparation, so as to achieve maximal gene transfer *in vivo*.

The potential for un-desirable side effects caused by the use of LPC should also be examined in future studies. In particular, a more detailed investigation of the inflammatory implications of the LPC-mediated opening of the epithelial TJ's is warranted. In the gene transfer studies described in this thesis project the treated mice showed no signs of behavioural distress, nor was unusual weight change ever observed. This suggests that immune or inflammatory responses were not evoked in response to the instillation of LPC, the LV vector or to the combination of both. Preliminary findings from studies elsewhere designed to examine the presence of inflammatory cells (neutrophils, macrophages, lymphocytes, etc.) in nasal and bronchoalveolar lavage fluid samples from LPC-dosed mice, suggest that LPC treatment does not induce significant inflammation (Dr. D. W. Parsons, personal communication).

More than 15 types of LPC exist and these are derived from various sources, including bovine brain, liver and heart. The form of LPC used in this thesis project (L-α-lysophosphatidylcholine Type I; Figure 1.3, b) was derived from egg yolk and contained a mixture of palmitic and stearic acids. Since the length and the composition of the fatty acid chain affects the detergent properties of LPC, it will be important to identify the exact LPC variant that is optimal for efficient LV-mediated gene transfer *in vivo*. Once identified this LPC variant may have a faster or a slower duration of activity compared to that of the LPC form used in this thesis project. It may also act differently on both the structure of the cell membrane and the TJ integrity and therefore be associated with more or less injury to the airway epithelium. These effects, however, may be intrinsically linked to the efficiency of gene transfer.

Although the *in vivo* gene transfer studies described in this thesis project were performed in the nasal airway, it is clear that the LV-based gene delivery protocol must ultimately be focused towards gene delivery to the conducting airways. The two obvious methods for LPC (or vector) administration into the conducting airways, which could be employed in a clinical setting, are bolus instillation *via* a bronchoscope or nebulisation. Instillation of the LPC (or vector) *via* a bronchoscope would allow the delivery of the solution to a specified area in the lung. This method of instillation, however, can expose the alveolar space to respiratory debris, thus causing alveolar contamination and subsequently generating of an inflammatory response (Joseph *et al.*, 2001). In contrast, nebulisation is a relatively simple technique and more convenient in a clinical setting than the liquid instillation approach. The disadvantage of this technique, however, is that the administration of the solution cannot be localised to a specified area. As a consequence, the oesophagus, the mouth and the nasal airways are also exposed to LPC. In addition, the efficiency of this technique depends heavily on the breathing pattern of the individual patient.

Another method of administration, which has been recently described, relies on delivering small volumes of liquid as large droplets allowing for the instillation of solutions to a specific site in the airways (Cipolla *et al.*, 2000). This method appears to allow precise deposition of the solution to a specific area in the airways without causing alveolar contamination.

The efficiency and appropriateness of any of the administration routes described above would need to be carefully evaluated in the lungs of large animal models and subsequently in the lungs of patients with pulmonary disease prior to its application in CF gene therapy clinical trials.

(b) The Lentivirus Gene Transfer Vector System

The optimised LV vector construct evaluated in the LacZ and CFTR gene transfer studies (described in Chapter 6) contained the cPPT sequence known to be essential for efficient gene transduction *in vivo* (Zennou *et al.*, 2000). Other viral sequences have also been shown to improve transduction efficiency and gene expression *in vivo*. Some examples of these sequences are the matrix attachment regions from a variety of genes (Park and Kay, 2001), and the 'Spleen Focus Forming' virus and the 'Woodchuck' hepatitis virus post-transcriptional regulatory elements (Parsley *et al.*, 2002). The effect of some or all of these sequences on the transduction efficiency and gene expression of the LV vector constructs should be directly evaluated in future studies.

The LV vector used in this thesis project was pseudotyped with VSV-G as this allowed for efficient concentration of the vector stocks. One approach that has recently received attention is pseudotyping the LV vector with glycoproteins from enveloped viruses, which are characterised by a natural tropism for the airway epithelium. This pseudotyping approach may enhance the efficiency of gene transduction, allowing for the instillation of a lower number of viral vector particles to the area of interest, hence reducing inflammation or other side-effects associated with the application of higher doses of viral vector preparations. Examples of some glycoproteins from enveloped viruses that have been used to pseudotype LV vectors for delivery to the airway epithelial cells are the (i) Ebola Zaire virus (Kobinger *et al.*, 2001); (ii) the measles virus (Sinn *et al.*, 2002); and (iii) the paramyxovirus and orthomyxovirus (Kobayashi *et al.*, 2002).

Due to time limitations it was not possible to evaluate whether repetitive LV vector exposure would have improved the degree of gene transfer in mice. Up to date there have been no reported cases of an immune response towards a single LV vector administration in animals, which suggests that repeated administration of the LV vector is possible. As LV vectors do not express any virus-derived genes they are unlikely to invoke a vector-specific immune response, which may subsequently destroy the positively transduced cells. It is apparent, however, that a more detailed investigation on the possible accumulative effect of vector re-administration on immune responses is warranted.

Prior to using LV vectors in gene therapy clinical trials, it is imperative that they are shown to be safe. The known risks associated with LV-based gene transfer include the (i) possibility of oncogene activation by promoter insertion, (ii) germline transmission of vector sequences and (iii) emergence of replication competent retrovirus (RCR). By using self-inactivating LV vectors, as was the vector used in this thesis project, the possibility of oncogene activation by promoter insertion was reduced. In theory, it is possible that multiple recombination events between the LV vector and either the packaging helper plasmid system or the endogenous viral sequences present in the cells, used to produce the LV vector, may result in the emergence of an RCR. The contamination of a vector stock, prepared for human gene therapy applications, with RCR may cause harm to the recipient(s). Therefore it is vital to develop a sensitive assay to detect the presence of RCR in large volume LV vector preparations. It should be noted here that the LV vector system that was used in this thesis project was designed in such a way (section 5.1; Fuller and Anson, 2001; Anson and Fuller, in preparation) as to minimise the possibility of RCR emergence.

(c) Animal Models to Assess the Efficiency of Lentivirus Delivery to the Airway Epithelium

The *in vivo* gene transfer studies described in this thesis project were performed in the mouse nasal airway epithelium. This animal model was used here, as its nasal airway epithelium most closely resembles the human conducting airways with respect to the types of cells present (Grubb and Boucher, 1999). However, it is still not known how comparable the mouse nasal airway epithelium is to the human lung epithelium. Specifically, the TJ permeabilities and the composition of the ASL layer are probably the most important factors that will determine its suitability as a model of the human lung. In future, gene transfer studies in mice (or in larger animal models) should include a focus on exploring LV vector delivery to the conducting airways since this is where CF lung disease commences.

Although there have been efforts to induce lung disease in the CF mouse models (Stotland *et al.*, 2000), these attempts have not been successful. Similar to pancreatic insufficient CF patients, the *cftr*$^{\mathrm{tm1unc}}$ mouse is characterised by obstructive gut disease but it does not exhibit the spontaneous lung disease seen in most CF patients. The *cftr*$^{\mathrm{tm1unc}}$ mouse nasal airways, however, exhibit the ion transport impairment displayed across the human CF lung (Grubb and Boucher, 1999), making it an excellent model tissue to assess CF gene therapy.

Although some may argue that the *cftr*$^{\mathrm{tm1Unc}}$ mouse model used in this project was not ideal, primarily due to problems with its survival rate, there is currently no animal that accurately models the CF lung disease present in CF patients. As a result, some groups have focused their attention to generating large CF animal models including ferrets, sheep and pigs (Van Heeckeren, 2000), although to date there has been no reported success.

(d) Assessment of CFTR Gene Transfer

In the LVCFTR gene transfer studies performed here only the ΔPD assay was utilised to evaluate successful CFTR gene delivery to the airway epithelium, due to time and technical limitations. To provide the widest physiological validation of successful CFTR gene transfer, CFTR gene expression in future studies should be analysed using an assay that allows full assessment of physiological correction. For example, using the ΔPD assay in which methods to examine both the Cl^- and Na^+ channel correction have been incorporated. The ΔPD assay should also be combined with assays that can quantify and localise CFTR gene expression. For instance, quantification of the particular cell types transduced, and the level of CFTR gene expression, can be determined by immunohistochemistry analysis of CFTR expression in tissue sections. Likewise, epi-fluorescence microscopy of the LV-treated airway epithelium using the halide sensitive fluorescent marker SPQ (6-methoxy-*N*-(3-sulphopropyl)quinolinium) (Stern *et al.*, 1995) could also be used to quantify CFTR gene expression. A recent report by Ziady *et al.* (2002) suggests that CFTR gene transfer in CF airways reverses the down-regulation of nitric oxide synthase-2. Therefore, increased nitric oxide synthase-2 levels in the airway epithelium may be a valid indicator of successful CFTR gene transfer.

As briefly reviewed in Chapter 1 (section 1.2.4) there is controversy surrounding the generation of CF airway disease. The "high-salt" hypothesis group propose that the ASL layer has a high salt content, which inactivates the salt-sensitive defensins. Tarran *et al.* (2001) recently showed, however, that the Cl^- concentration in the ASL layer in both normal and CF mice was similar. In view of these findings, the phenotypic features of the CF airway i.e. the reduced ASL depth and goblet cell hyperplasia (Knowles and Boucher, 2002) could also be examined to assess the efficiency of CFTR gene transfer rather than quantifying the NaCl levels in the ASL. Methods to assay the ASL depth and to quantify the number of goblet cells present in the nasal airway epithelium have been described (Tarran *et al.*, 2001). Although the correction of goblet cell hyperplasia may be take some time, primarily due to the normal cell turnover rate of the airway epithelium (~ 3 months), the depth of the ASL layer should normalise once the electrophysiological defect has been corrected in the relevant cells.

In conclusion, the ΔPD assay allows for the evaluation of the effect of CFTR gene transfer on the correction of the electrophysiological defect present in CF airways. However, the results obtained from the ΔPD assay cannot be used to directly evaluate correction of CF airway disease. As a result of recent advances in ASL sampling and goblet cell quantification techniques, it is now possible to directly assess the effect of CFTR gene transfer on the phenotypic correction of CF lung pathophysiology.

Part B

Gene Therapy for Cystic Fibrosis Airway Disease

The discovery of the CFTR gene in 1989 was greeted by scientists and the CF community with great enthusiasm. CF is a monogenic disease and its lung pathophysiology is the primary cause of morbidity and mortality. CF lung disease takes many years to establish and the infective component quickly becomes resistant to antibiotic therapies. It often includes pathologies not directly related to CFTR dysfunction, such as airway wall damage subsequent to chronic bacterial infection. Thus, gene therapy is now considered to be the only curative option for CF airway disease. Since the lung is large and easily accessible, and the cells that need to be corrected by gene therapy lie on or near to the apical surface, it is a good candidate for gene therapy.

Gene therapy clinical trials for CF airway disease were initiated in 1993 and many scientists and clinicians were optimistic about the early results of both pre-clinical animal studies and Phase I clinical trials. However, the disappointing results from subsequent clinical trials tempered this enthusiasm. It became apparent that the viral gene transfer vectors (i.e. AdV vectors) used in the initial clinical trials only conferred transient expression of the CFTR gene and also produced unacceptably high immune and inflammatory responses in CF subjects. The use of non-viral gene transfer vectors (e.g. cationic lipids) in gene therapy clinical trials has been equally disappointing. Nonetheless, the Phase I clinical trials that have been completed have provided scientists and clinicians with important information about the difficulties associated with CF gene therapy.

The death of US teenager Jesse Gelsinger in a gene therapy clinical trial for the treatment of ornithine transcarbamylase deficiency in 1999 brought into focus some of the risks associated with gene therapy. Jesse Gelsinger was one of the subjects that received a high dose (10^{13} pfu) of a third-generation E1/E4 deleted AdV vector. The route chosen for AdV vector administration in this clinical trial was *via* an intravascular injection rather than the less invasive methods used for airway delivery (liquid instillation or nebulisation). Unfortunately he died as a result of a large immune and inflammatory response to this dose (Lehrman, 1999). It could be argued, however, that his death was as a consequence of inadequate protocol and clinical trial design and review. Jesse's death fostered negative worldwide media comments towards all gene therapies. Encouraging results from other gene therapy trials, however, such as the recent demonstration that MLV-mediated gene transfer resulted in full correction of the SCID-X1 phenotype in two treated patients (Cavazzana-Calvo *et al.*, 2000), has helped reassure the wider community that effective gene therapy can be both successful and safe.

The need to use integrating gene transfer vectors, which provide persistence of gene expression and do not induce immune and inflammatory responses, for CF gene therapy is now apparent. Of the viral gene transfer vectors currently available, LV vectors have particular advantages in that they can transduce both quiescent and dividing cells, provide stable and sustained gene expression and as LV vectors do not express any virus-derived genes they are unlikely to induce a significant host immune response (Amado and Chen, 1999). It is important, however, that the safety of LV vectors is adequately addressed prior to their use in human volunteers.

Attention has also been directed towards understanding and manipulating the various airway barriers, which prevent the efficient delivery of gene transfer vectors (as described in section 1.3.4). In Johnson's study (2000) LV-mediated gene transfer in mouse or rat nasal airway epithelium only occurred following pre-treatment with SO_2, an approach that is not feasible for clinical applications but which accords with the general approach described in this thesis project. Pre-treatment of rabbit tracheal epithelium with 12 mM EGTA has been shown to significantly improve viral-mediated gene transfer *in vivo* (Wang *et al.*, 1999; Wang *et al.*, 2000). In Wang's studies, however, viral- (feline immunodeficieny virus, retrovirus or AdV) mediated gene transfer only occurred after the trachea had been filled with a low dose of EGTA (12 µM) for 30- 60 mins, followed by a 45 min incubation with the viral solution (Wang *et al.*, 1999; Wang *et al.*, 2000). Although these results contribute significantly to our understanding of the role airway barriers play in viral gene transfer, this dosing regimen would be impracticable for use in CF patients. In contrast to the low dose of EGTA used in Wang's study (1999, 2000), Chu and colleagues (2001) used higher doses (> 0.1 M) of EGTA to treat the mouse airways (trachea or lung) 30 mins prior to the delivery of an AdV vector. Although the efficiency of gene transfer was substantially enhanced, the feasibility of using such high concentrations of EGTA in a clinical setting is inconceivable. Based on the *in vivo* findings described in this thesis project it appears feasible that conditioning the lung airways of CF patients with the "natural" biological detergent LPC may safely facilitate successful LV-mediated CFTR gene transfer.

Even if the issue of delivery to the airway epithelium is resolved, longevity of gene transfer is pertinent, as permanent expression of functional CFTR is essential to maintain correction of the electrophysiological defect. Successful transduction of progenitor/stem cells using an integrating vector, such as the LV vector, would be expected to result in the generation of a stable population of gene corrected airway epithelial cells over long time-frames. The identity of the progenitor/stem cells in the airways that need to be corrected to restore CFTR electrophysiological function still remains unknown.

The amount of CFTR expression necessary to restore CFTR function in the defective airway epithelial cells is also not known. Heterozygotes for any CF gene mutation do not exhibit symptomologies or deficits typical of CF, indicating that 50% of the normal level of CFTR gene expression is sufficient to prevent the lung disease associated with CF (Korst *et al.*, 1995; Dorin *et al.*, 1996). Furthermore, some CF gene mutations (e.g. A455E) that are associated with ~ 5% CFTR functionality, cause only mild airway disease (Gan *et al.*, 1995). This suggests that restoring CFTR expression to ~5% (rather than 50%) may suffice to cure/prevent CF airway disease.

The exact relationship between CFTR expression and the resolution of CF airway disease remains to be determined. Recovery of CFTR function alone, in CF patients with chronic lung disease, is unlikely to produce immediate restoration of lung function. Gene therapy for CF lung disease therefore needs to be targeted to the early childhood period prior to the acquisition of lung infections. Genetic neonatal screening for CF, which is now routinely performed in many hospitals, allows for early detection and the possibility of successful gene therapy application prior to the acquisition of lung disease. Before gene therapy can be "prescribed" for CF patients, parents, researchers and clinicians must be satisfied with the safety profile of both the gene transfer vector(s) and of any airway-conditioning reagent(s) used.

For the last 10 years scientists have endeavoured to find a cure for CF airway disease. Although the search has been elusive thus far, progress in gene therapy provides a more optimistic outlook for the future. The enormous potential of gene therapy techniques and the lack of promising alternatives for a cure, means that major efforts and resources will continue to be applied to overcome the current problems of achieving successful gene transfer. The demonstration of persisting *in vivo* CFTR gene transfer after a simple dosing protocol offers hope that the promise of LV gene therapy can indeed be translated into a safe and effective treatment of CF lung disease. The simplicity of the LV-mediated transduction protocol described in this thesis project, which utilises brief single exposures to LPC and the LV vector and produces gene expression that lasts for several months, should facilitate further development towards clinical application of safe and effective airway gene therapy for CF.

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