

# **Retroviral targeting to tumour antigens**

**Simon Chowdhury**

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**Department of Immunology  
The Windeyer Institute of Medical Sciences  
University College London**

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## Abstract

The development of efficient, cell surface targeted retroviral vectors is critical for successful gene therapy as gene delivery to non-target cells may be harmful and would deplete the pool of viral particles. To date, the only surface-targeting strategies that have allowed efficient infection by retroviral vectors *in vivo* are those that have limited the tropism of amphotropic murine leukaemia virus (MLV-A), which can infect cells of many mammals, by modification of the envelope glycoprotein.

To this end we have explored tumour targeting of vectors based on MLV-A by modification of the retroviral surface protein (SU) backbone of the envelope chimera. The first approach used a receptor co-operation strategy to target human tumour cells by linking single chain antibodies (scFv) recognising tumour antigens (carcinoembryonic antigen (CEA) and high molecular weight melanoma-associated antigen (HMWMAA)) via proline-rich spacers to the amphotropic murine leukaemia virus surface protein. This approach showed selective targeting to both CEA and HMWMAA *in vitro*.

The second approach used a protease targeting strategy to target tumour cells expressing CEA. We fused a single-chain variable fragment (scFv) directed against CEA to the amphotropic murine leukaemia virus envelope. A proline-rich hinge and matrix metalloprotease cleavage site linked the two proteins. Following attachment to CEA, MMP cleavage of the envelope at the cell surface removed the scFv and proline-rich hinge allowing infection. This approach showed selective targeting to carcinoembryonic antigen (CEA) both *in vitro* and *in vivo* with up to 10% infection of cells within a CEA-positive tumour xenograft. No infected cells were detected after delivery of targeted vectors to CEA-negative tumour xenografts. Intraperitoneal injection of amphotropic producer cells resulted in transduction in spleen, liver and kidney, which was not detected when targeted producer cells were used. These results demonstrate the feasibility of using targeted retroviral vectors for *in vivo* gene delivery and highlight the safety benefits of targeted vectors that do not infect other host tissues.

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## **Lists of Contents, Figures and Tables**

### ***Contents***

<b>Abstract.....</b>	<b>2</b>
<b>Acknowledgements.....</b>	<b>3</b>
<b>Abbreviations.....</b>	<b>10</b>
<b>Chapter 1 - Introduction .....</b>	<b>12</b>
<b>1.1 The use of vectors in gene therapy .....</b>	<b>12</b>
<b>1.2 The use of viral vectors in gene therapy .....</b>	<b>16</b>
<b>1.3 Retroviruses.....</b>	<b>18</b>
1.3.1 Retroviral classification.....	20
1.3.2 Structure of retroviral particles.....	21
1.3.3 Retroviral genome.....	21
1.3.4 Retroviral life cycle .....	23
<b>1.4 Retroviral vectors .....</b>	<b>27</b>
<b>1.5 Safety of retroviral vectors.....</b>	<b>30</b>
1.5.1 Insertional mutagenesis.....	30
1.5.2 Gene therapy of X-linked SCID .....	31
<b>1.6 Cancer gene therapy.....</b>	<b>33</b>
1.6.1 Immuno-gene therapy .....	33
1.6.2 Tumour suppressor genes and oncogenes.....	36
1.6.3 Suicide genes .....	39
1.6.4 Anti-angiogenic genes .....	41
<b>1.7 Targeting gene delivery.....</b>	<b>41</b>
1.7.1 Retroviral envelope glycoprotein.....	43
1.7.2 Targeting with adaptor molecules.....	47
1.7.3 Targeting by envelope modification.....	48
1.7.4 Targeting by host range restriction.....	50
1.7.5 Retroviral display of cell-specific blocking domains to restrict tropism.....	51
1.7.6 Escorting viral entry.....	53
<b>1.8 Tumour antigens.....</b>	<b>53</b>
1.8.1 Carcinoembryonic antigen (CEA) .....	54
1.8.2 Targeting carcinoembryonic antigen (CEA).....	57

1.8.3 Retroviral targeting to tumour antigens by envelope modification .....	60
<b>1.9 Matrix metalloproteases.....</b>	<b>61</b>
<b>1.10 Thesis aims.....</b>	<b>63</b>
<b>Chapter 2 - Materials and Methods .....</b>	<b>64</b>
<b>2.1 Plasmid preparation and manipulation.....</b>	<b>64</b>
2.1.2 Preparation of heat shock competent E coli.....	64
2.1.2 Introduction of plasmid DNA into E. coli .....	64
2.1.3 Plasmid DNA mini-preps.....	64
2.1.4 Restriction enzyme digests .....	65
2.1.5 Isolation of DNA restriction fragments .....	65
2.1.6 Sub-cloning of DNA fragments.....	65
2.1.7 Plasmid DNA maxi-preps.....	65
2.1.7 Plasmid DNA transfer into eukaryotic cells .....	66
<b>2.2 Polymerase chain reaction .....</b>	<b>67</b>
<b>2.3 Plasmids .....</b>	<b>67</b>
<b>2.4 Cell culture .....</b>	<b>69</b>
2.4.1 Cell lines .....	69
<b>2.5 Construction of retroviral producer cell lines and infection with recombinant viruses.....</b>	<b>71</b>
2.5.1 Generation of stable producer cell lines .....	71
2.5.2 Virus harvesting and concentration .....	71
2.5.3 Viral titration.....	71
2.5.4 Inhibition of infection by single chain antibody.....	72
2.5.5 Inhibition of infection by receptor interference.....	73
2.5.6 Protease inhibition .....	73
2.5.7 Infection of mixed cell populations .....	73
<b>2.6 Protein analysis .....</b>	<b>74</b>
2.6.1 Preparation of cell lysates.....	74
2.6.2 Preparation of viral supernatants .....	74
2.6.3 Immunoblotting .....	74
2.6.4 Analysis of envelope cleavage by gelatinase A.....	76
<b>2.7 Protease activity .....</b>	<b>76</b>
<b>2.8 Detection of cell surface CEA on target cells .....</b>	<b>76</b>
<b>2.9 In vivo experiments .....</b>	<b>78</b>

2.9.1 Establishment of tumours .....	78
2.9.2 Intratumoural injections.....	78
2.9.3 Analysis of vector spreading.....	79
<b>Chapter 3 - Targeted retroviral infection by receptor cooperation .....</b>	<b>81</b>
<b>3.1 Introduction.....</b>	<b>81</b>
3.1.1 Overview.....	81
3.1.2 Targeting by host-range restriction.....	81
3.1.3 Receptor cooperation .....	82
<b>3.2 Construction of targeted envelopes .....</b>	<b>83</b>
<b>3.3 Carcinoembryonic antigen (CEA) expression.....</b>	<b>87</b>
<b>3.4 Targeted infection .....</b>	<b>90</b>
<b>3.5 Requirement of tumour antigen for infection .....</b>	<b>92</b>
<b>3.6 Requirement of Pit-2 expression for infection .....</b>	<b>95</b>
<b>3.7 Discussion .....</b>	<b>96</b>
<b>Chapter 4 - Retroviral infection by protease targeting.....</b>	<b>100</b>
<b>4.1 Introduction.....</b>	<b>100</b>
4.1.1 Overview.....	100
4.1.2 Protease targeting.....	100
<b>4.2 Construction of targeted envelope.....</b>	<b>101</b>
<b>4.3 Protease expression by target cells.....</b>	<b>107</b>
<b>4.4 Targeted infection .....</b>	<b>107</b>
<b>4.5 Requirement of tumour antigen for infection .....</b>	<b>110</b>
<b>4.6 Requirement of Pit-2 expression for infection .....</b>	<b>113</b>
<b>4.7 Protease inhibition .....</b>	<b>116</b>
<b>4.8 Infection of mixed cell populations.....</b>	<b>116</b>
<b>4.9 <i>In vivo</i> experiments .....</b>	<b>118</b>
4.9.1 Targeting of tumour xenografts .....	118
4.9.2 Analysis of vector distribution.....	122
<b>4.10 Discussion .....</b>	<b>124</b>
<b>Chapter 5 - Discussion .....</b>	<b>129</b>
<b>5.1 Targeting by envelope modification.....</b>	<b>129</b>
<b>5.2 Cell surface targets .....</b>	<b>131</b>
<b>5.3 Safety concerns.....</b>	<b>133</b>
<b>5.4 Cancer gene therapy.....</b>	<b>135</b>

**References.....140**

## *Figures*

Figure 1.1 Converting a virus into a vector .....	17
Figure 1.2 General structure of a typical retroviral particle .....	22
Figure 1.3 General organisation of a simple retrovirus genome.....	24
Figure 1.4 Replication strategy of an infectious retrovirus .....	25
Figure 1.5 Retrovirus-based vectors. ....	29
Figure 1.6 Functional domains of the retroviral envelope proteins.....	44
Figure 1.7 Molecular rearrangements of the TM structure following binding.....	46
Figure 1.8 Insertion sites into the MLV glycoprotein .....	49
Figure 1.9 The structure of IgG and its fragments.....	59
Figure 2.1 ALF envelope expression plasmid .....	68
Figure 2.2 Time course of protease expression by target cells.....	77
Figure 3.1 Construction of targeted envelopes. ....	84
Figure 3.2 Targeted envelope incorporation in retroviral particles .....	86
Figure 3.3 Detection of carcinoembryonic antigen (CEA).....	88
Figure 3.4. Surface expression of CEA. ....	89
Figure 3.5 Titers of targeted viruses. ....	91
Figure 3.6 Targeted infection requires tumour antigen .....	94
Figure 3.7 Targeted infection requires Pit-2.....	97
Figure 4.1 Construction of targeted envelope MFE23/ProMMP. ....	104
Figure 4.2 Targeted envelope incorporation in retroviral particles. ....	106
Figure 4.3 Protease expression by target cells.....	108
Figure 4.4 Titers of targeted viruses .....	109
Figure 4.5 Proposed mechanism of infection of MFE23/ProMMP.....	111
Figure 4.6 Targeted infection requires tumour antigen. ....	114
Figure 4.7 Targeted infection requires Pit-2.....	115
Figure 4.8 Targeted infection requires MMP activity.. ....	117
Figure 4.9 <i>In vivo</i> targeting.....	121
Figure 4.10 Analysis of vector spread. ....	123
Figure 4.11 Semi-quantitative analysis of proviral DNA content. ....	125
Figure 4.12 Quantitative analysis of proviral DNA.....	126

## ***Tables***

Table 1.1 Statistics of gene therapy protocols and vector usage from completed, ongoing or pending clinical trials up to 2003.....	15
Table 1.2 Main features of common gene therapy vectors.....	19
Table 2.1 Antibodies used .....	75
Table 3.1 Enhancement of receptor cooperation .....	93
Table 4.1 Enhancement of protease targeting.....	112
Table 4.2 Infection of mixed cell populations. ....	119

## Abbreviations

AAV	adeno-associated virus
AIDS	acquired immune deficiency syndrome
ALV	avian leukosis virus
bp	base pair
C	carboxy terminal domain
CA	capsid
CEA	carcinoembryonic antigen
CD	cluster of differentiation
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
eGFP	enhanced green fluorescent protein
EIAV	equine infectious anaemia virus
ELISA	enzyme-linked immunosorbent assay
Env	envelope
FACS	fluorescence-activated cell sorter
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
g	relative centrifugal force
Gag	group antigen
GAG	glycosaminoglycan
GALV	gibbon ape leukaemia virus
Gy	Gray
HIV-1	human immunodeficiency virus type 1
HIV-2	human immunodeficiency virus type 2
HMWMAA	high molecular weight melanoma associated antigen
HSV-1	herpes simplex virus type 1
IgG	immunoglobulin G
IL	interleukin
IN	integrase
iu	infectious units
kb	kilobase
kDa	kilodalton
LMH2	scFV to high molecular weight melanoma associated antigen
LTR	long terminal repeat
M	molar
MA	matrix
mCAT-1	amino acid transporter, cationic 1
mg	milligram
MFE23	single chain antibody to carcinoembryonic antigen
MHC	major histocompatibility complex
MLV	murine leukaemia virus
MMLV	Moloney murine leukaemia virus
MLV-A	amphotropic murine leukaemia virus
MLV-E	ecotropic murine leukaemia virus
MMP	matrix metalloprotease
MOI	multiplicity of infection

mRNA	messenger ribonucleic acid
NC	nucleocapsid
nt	nucleotide
PCR	polymerase chain reaction
pg	pictogram
Pit-1	inorganic phosphate transporter 1
Pit-2	inorganic phosphate transporter 2
PIC	pre-integration complex
Pol	polymerase
poly(A)	polyadenylic acid
PR	protease
Pro	proline-rich spacer
R	repeat region
RBD	receptor binding domain
RCV	replication competent retrovirus
Rev	regulator of virion protein expression
RER	rough endoplasmic reticulum
RNA	ribonucleic acid
RNase H	ribonuclease H
RT	reverse transcriptase
RTC	reverse transcription complex
scFv	single chain antibody
SU	envelope surface subunit
TIMP	tissue inhibitor of matrix metalloproteases
TM	envelope transmembrane subunit
U3	unique 3' region of the long terminal repeat
U5	unique 5' region of the long terminal repeat
VSV	vesicular stomatitis virus
VSV-G	vesicular stomatitis virus G protein
wt	wild type



# **Chapter 1**

## **Introduction**

### **1.1 The use of vectors in gene therapy**

The aim of gene therapy is simple: insert the gene into cells where the gene product should cure or alleviate the symptoms of disease (Somia and Verma, 2000). Gene therapy was initially conceived as a treatment for diseases caused by single gene defects such as cystic fibrosis (Kay and Woo, 1994). Today its role has been expanded with acquired diseases, such as cancer, cardiovascular disease, neurodegenerative disorders and infectious disease, the subject of most gene therapy research (Thomas *et al.*, 2003) (Table 1.1). Initial enthusiasm for gene therapy has waned after successive clinical trials have failed to show efficacy (Somia and Verma, 2000). These trials highlighted important obstacles, most notably gene delivery i.e. the ability to transfer genes into a wide variety of cells, tissues and whole organs. Thus far the problem has been a failure to deliver genes efficiently, safely and to obtain sustained expression.

Gene delivery vehicles (vectors) need to address the application/disease for which they are going to be used. A property that all vectors require is that an appropriate amount of the therapeutic gene needs to be delivered to the target site without substantial toxicity.

An ideal vector should possess the following properties:

1. Efficient, easy and cheap production: high-titre preparations of vector particles should be reproducibly available. For widespread use simple production procedures are needed.
2. Safety: the vector should neither be toxic to the target cells nor induce unwanted effects including immunological reactions against the vector or its subsequent gene product. Immunological reactions may not only eliminate the

vector and/or the transduced cells but may also cause life-threatening complications such as septic shock

3. Sustained and regulated gene expression: the gene delivered should be expressed in an appropriate way. Permanent or even life-long expression is required in some diseases e.g. haemophilia or cystic fibrosis. Regulated gene expression may be required for other diseases e.g. diabetes where expression of insulin will need to be tightly regulated to blood glucose concentrations.
4. Targeting: transduction of specific cell types is highly desirable and this will be addressed later.
5. Infection of dividing and non-dividing cells: because the majority of cells in an adult human being are in a post mitotic non-dividing state, vectors should be able to transduce these cells.
6. Site-specific integration: integration into the host genome at specific site(s) would enable the repair of specific defects caused by mutations and deletions by insertion of the correct sequences.
7. Insert size: the vector should have no size limit to the genes that it can deliver.

Although no such vector is currently available, all of these properties exist, individually, in disparate delivery systems.

There are two main categories of vector: viral and non-viral. The use of non-viral vectors ranges from direct injection of DNA to mixing of DNA with polylysine or cationic lipids that allow DNA to cross the cell membrane (Niidome and Huang, 2002). Although non-viral vectors can be produced in relatively large amounts, and are likely to present fewer toxic or immunological problems, they suffer from inefficient gene transfer at present (Niidome and Huang, 2002). Furthermore, expression of the foreign gene is transient. There remains a need, in many diseases, for sustained and often high-level expression of the transgene that can only be provided by viral vectors.

**Table 1.1 Gene therapy protocols and vector usage from completed, ongoing or pending clinical trials up to 2003.**

Panels A and B show the diseases treated while panels C and D indicate the vectors used. Data obtained from the Journal of Gene Medicine clinical trials database [www.wiley.co.uk/genetherapy/clinical/](http://www.wiley.co.uk/genetherapy/clinical/)

**A. Protocols by disease**

Condition	Number of Protocols	Percentage of Protocols
Cancer	403	63.4
Monogenic disease	78	12.3
Vascular disease	51	8.0
Gene marking	49	7.7
Infectious disease	41	6.4
Healthy volunteers	2	0.3
Others	12	1.9

**B. Patients by disease**

Condition	Number of Patients	Percentage of Patients
Cancer	2392	68.5
Monogenic disease	408	11.7
Vascular disease	309	8.8
Gene marking	274	7.8
Infectious disease	86	2.5
Healthy volunteers	6	0.2
Others	19	0.5

**C. Protocols by vector**

Vector	Number of Protocols	Percentage of Protocols
Retrovirus	217	63.4
Adenovirus	171	12.3
Lipofection	77	8.0
Naked/plasmid DNA	70	7.7
Vaccinia virus	39	6.4
Adeno-associated virus	15	0.3
Herpes simplex type I	5	1.9
Others	42	6.6

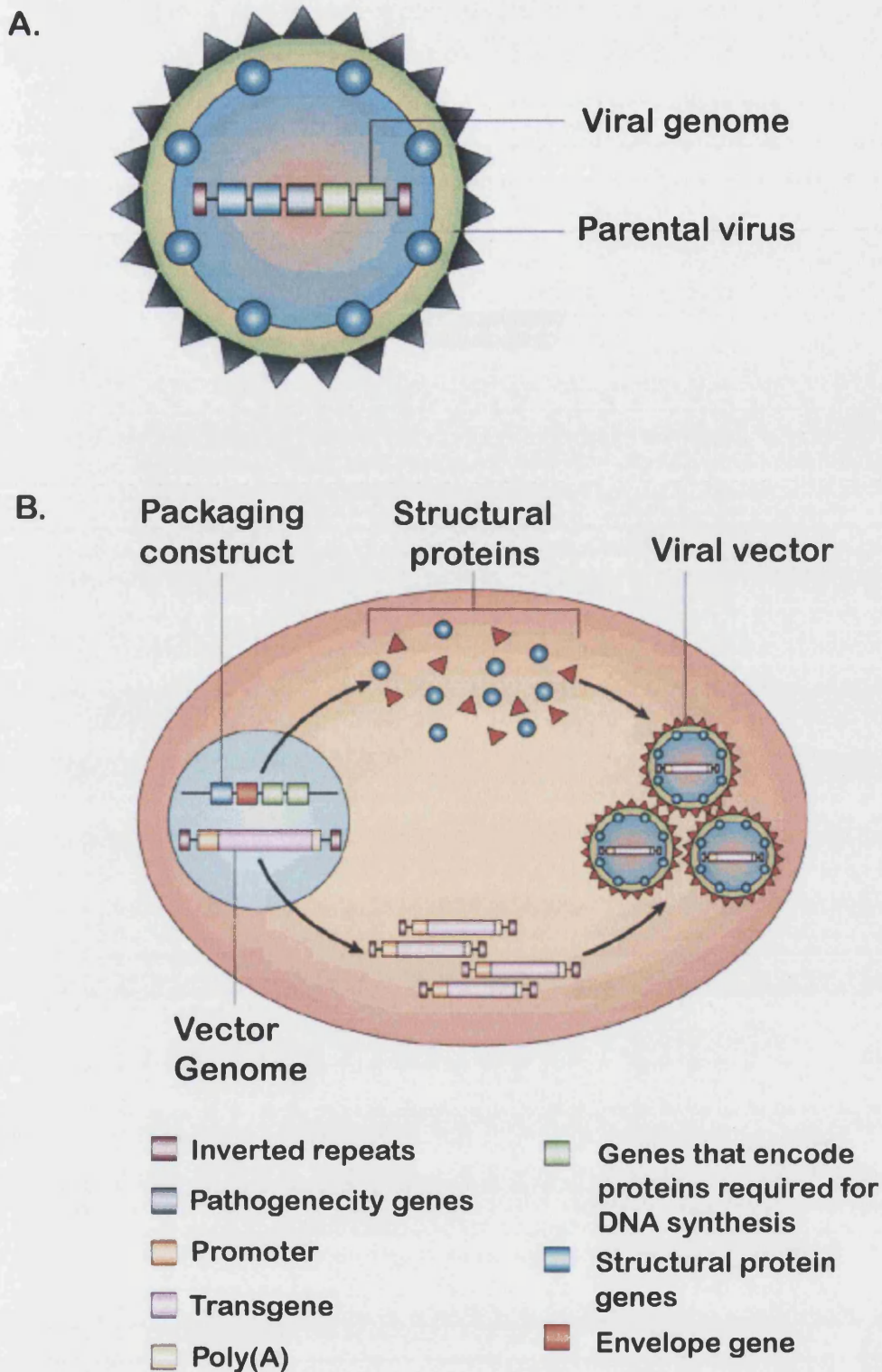
**D. Patients by vector**

Vector	Number of Patients	Percentage of Patients
Retrovirus	1755	50.2
Adenovirus	644	18.4
Lipofection	619	17.7
Naked/plasmid DNA	123	3.5
Vaccinia virus	88	2.5
Adeno-associated virus	36	1.0
Herpes simplex type I	21	0.6
Others	208	6.1

## 1.2 The use of viral vectors in gene therapy

Viruses represent highly evolved natural vectors for the transfer of foreign genetic material into cells and are thus in some ways ideally suited as vectors for gene therapy (Kay *et al.*, 2001). The viral life cycle can be divided into two distinct phases: infection and replication. Infection results in the introduction of the viral genome into the cell. This leads to an early phase of gene expression characterised by the expression of viral regulatory products, followed by a late phase when structural genes are expressed and assembly of new viral particles occurs (Kay *et al.*, 2001). Ideal virus-based vectors for most gene therapy applications harness the viral infection pathway but avoid the subsequent expression of viral genes that leads to replication and toxicity. This is achieved by deleting some or all of the coding regions from the viral genome, but leaving intact those sequences that are required *in cis* for functions such as packaging the vector genome into the virus capsid and integration of vector DNA into the host chromosome (Kay *et al.*, 2001). The therapeutic gene of interest is then cloned into the viral backbone in place of those sequences that were deleted. The deleted genes encoding proteins that are involved in replication or capsid/envelope proteins are included in separate plasmid/s to provide helper functions *in trans*. These viral genes may even be incorporated in the chromatin of producer cells to ensure their stability and limit their remobilisation. The packaging cells into which the packaging construct and vector genome are supplied produce the recombinant vector particles (Thomas *et al.*, 2003)(Figure 1.1). Such vectors are unable to replicate and thus transduction that introduces the gene of interest into the target cell is an abortive (or 'dead-end') infection.

The development of a viral vector is limited by the organisational complexity of the viral genome. The intact viral genome often ensures an appropriate balance of viral protein production by complex regulatory changes in gene expression. Importantly, *cis*-interactions between the genome and its translation products are lost in an engineered vector-packaging system. These limitations of vector design may result in inefficient packaging of vector genomes as compared to wild-type viruses, and in the release of excess defective vector particles that are incapable of gene transfer themselves and which may also interfere with the transduction of biologically active vector particles.



**Figure 1.1 Converting a virus into a vector**

A. Schematic diagram of a generic viral vector.

B. A packaging (helper) construct, containing viral genes derived from the parental virus that encode structural proteins and proteins that are required for vector genome replication, is introduced into a packaging cell line along with a construct that contains the vector genome.

Adapted from (Thomas et al., 2003).

After production in a packaging cell line, the recombinant vector particles are purified and quantified. The relative concentration of vectors is measured as a titre expressed as the concentration of viral particles that are capable of transduction. This is usually expressed as the number of transducing units per millilitre. The transducing particles usually represent a small percentage of the total particles and can vary between different preparations. Quantification may vary as a result of different methods used in different laboratories. Thus there is a need for standardised methods to determine the specific activity of vectors. Particle titre and an infectious or transducing titre are both important as impurities and variations in infectious activity can influence efficacy, toxicity and immunogenicity.

The number of different viruses that are under development as gene-therapy vectors is steadily increasing, but there are at present five main classes of clinically applicable viral vectors. These can be subdivided into those which integrate into the hosts chromosomal DNA (oncoretroviruses and lentiviruses) and those that are non-integrating (adenoviruses, adeno-associated viruses (AAVs) and herpes simplex-1 viruses (HSV-1s)). Each of these vectors have a different set of properties that make it suitable for some applications but not for others. Their particular characteristics compared to their non-viral counterparts are shown in Table 1.2.

Oncoretrovirus vectors were the first class of viral vector to be developed and have been important in the technical and conceptual development of viral vectors as a whole (Somia and Verma, 2000). Retroviral vectors have, so far, been the most widely used in clinical trials (Table 1.1).

### **1.3 Retroviruses**

The *Retroviridae* comprise a diverse family of enveloped animal viruses with single stranded positive sense diploid RNA genomes. Whilst retroviruses have been identified in a wide range of vertebrate hosts (Herniou *et al.*, 1998) they all share certain similarities with regards to their RNA genome, viral structure, mode of entry and replication.

Vector	Retrovirus	Lentivirus	Adenovirus	AAV	HSV-1	Vaccinia	Liposomes	Naked DNA
Insert size	~8kb	~8kb	~35kb	<4kb	>20kb	>25kb	>20kb	>20kb
Titre/ml	$10^7$	$10^7$	$10^{11}$	$10^9$	$10^{10}$	N/A	N/A	N/A
Integration	Yes	Yes	No	Yes/No	No	No	No	No
Sustained gene expression	Variable	Variable	Transient	Variable	Transient	Transient	Transient	?
In vivo delivery	Poor	Poor	High	High	High	High	Variable	?
Transduction of non-dividing cells	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Immune problems	Few	Few	Extensive	Few	Few	Few	Few	Few
Pre-existing host immunity	Unlikely	Unlikely. Possible in HIV+VE	Yes	Yes	Yes	Yes	?	Yes
Safety problems	Insertional mutagenesis	Insertional mutagenesis	Toxicity	None	Toxicity	Toxicity	None	None

**Table 1.2 Main features of common gene therapy vectors**

Adapted from (Somia and Verma, 2000) (N/A: not applicable, ?: not known)



Reverse transcriptase is an RNA-dependent DNA polymerase enzyme first identified in 1970 in murine and avian RNA tumour viruses (Baltimore, 1970; Temin and Mizutani, 1970). Subsequently, these viruses were named 'retro'viruses as they replicate by converting their viral genomic RNA into double stranded DNA. The DNA copy of the viral genome then integrates into the host chromosomal DNA as a provirus, which is subsequently transcribed and translated by host cellular mechanisms to provide the necessary proteins for packaging full length RNA transcripts into progeny virions.

### 1.3.1 Retroviral classification

Initial retroviral classification based on electron microscopy divided retroviruses into four morphological groups termed as types A, B, C and D. The A-type particle has an electron-lucent centre with one or two concentric electron-dense rings. This morphology is commonly termed 'immature' as a similar morphology is seen in mutant retroviruses unable to proteolytically process their internal proteins. B-type particles (e.g. Mouse Mammary Tumour Virus (MMTV)) have large, dense, round cores with characteristic 'spiky' surface glycoproteins. C-type particles comprise avian and mammalian retroviruses and have dense cores which appear centrally located in the virion. D-type particles (e.g. Mason Pfizer Monkey Virus (MPMV)) have cylindrical cores and bear short surface spikes (de Harven, 1974; Coffin, 1992). A, B and D-type particles assemble within the cell cytoplasm forming stable structures (often termed intracytoplasmic A-type particles (ICAPs)) that are transported to the plasma membrane where they acquire their envelopes upon budding. In contrast, C-type particle assembly occurs at the plasma membrane with the concurrent formation of immature viral cores and envelope acquisition.

Retroviruses have also been grouped according to their biological activity and disease association into three groups: *oncoviruses* implicated in tumours and immunodeficiency; *lentiviruses* causing slow chronically progressing disease with extended latency periods between initial infection and disease manifestation; and *spumaviruses* or foamy viruses, which cause vacuolation of cells in culture but with, as yet, no known disease association (Teich, 1984).

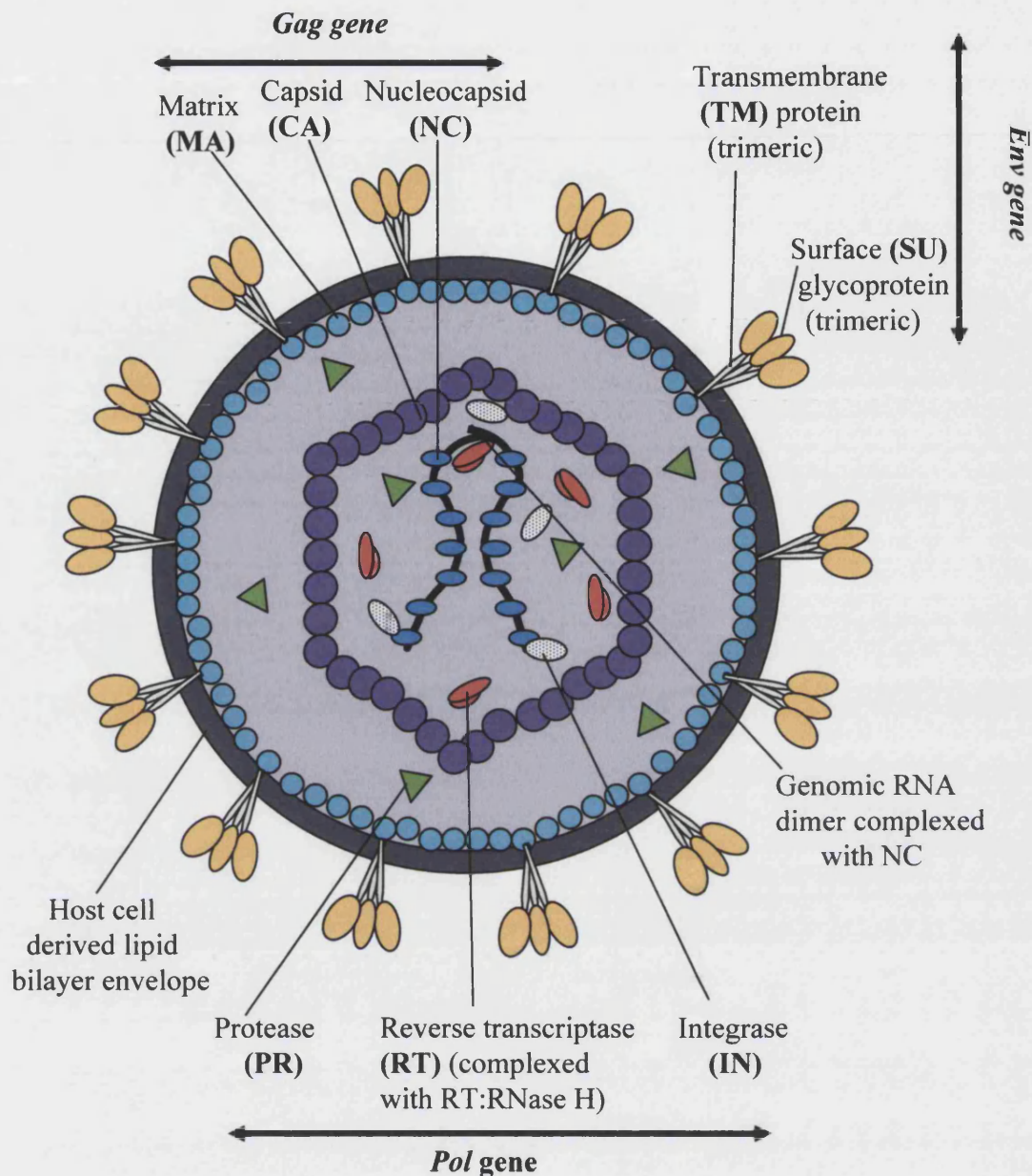
Recent retroviral classification, based on the genetic similarity of genome sequences between the different retroviral groups, has differentiated seven distinct retroviral genera. These are the spumaviruses, the lentiviruses, the HTLV-related viruses (deltaretroviruses), the mammalian B-type and D-type viruses (betaretroviruses), the mammalian C-type viruses (gammaretroviruses), the avian C-type viruses (alpharetroviruses) and the epsilonretroviruses (e.g. Walleye Dermal Sarcoma Virus) (Hunter and Stoye, 2000).

### **1.3.2 Structure of retroviral particles.**

Most retroviruses are 80-130 nm in diameter with a similar structural morphology. The virion core consists of two molecules of genomic RNA that are associated with the nucleocapsid (NC) protein (NC-RNA complex) and a tRNA molecule, which primes the start of reverse transcription. The NC-RNA complex is surrounded by the capsid (CA) protein containing copies of three retroviral enzymes: reverse transcriptase (RT), protease (PR) and integrase (IN), which are all required in the early stages of infection. The matrix (MA) protein surrounds the capsid proteins or 'core', and lies just below the envelope that is derived from the host cell membrane. The retroviral envelope is studded with envelope glycoproteins arranged in oligomeric complexes that contain three or four heterodimers composed of a transmembrane, TM, component associated with a surface unit, SU glycoprotein (Vogt, 1997) (Figure 1.2). In certain retroviruses (e.g. ASLV and MLV) envelope oligomeric complexes consist of trimers of SU-TM heterodimers (Einfeld and Hunter, 1988; Kamps *et al.*, 1991), while in others (e.g. HIV and SIV), dimer, trimer and tetrameric forms of envelope proteins have been described (Doms *et al.*, 1991).

### **1.3.3 Retroviral genome.**

The full length RNA genome varies between 7-13 kb with two identical molecules carried in a single virion. Conceptually it is easier to discuss the retroviral genome organisation in terms of the DNA that is integrated into cellular DNA, as this places the promoter, the RNA start site and the polyadenylation site in the same position as found in typical host cells.



**Figure 1.2 General structure of a typical retroviral particle**

The schematic model of a typical mature retrovirus depicts virion components common to all retroviruses. The virus is bound by a host cell derived lipid bilayer envelope into which trimeric envelope glycoproteins (SU-TM) are embedded. The matrix (MA) surrounds the 'core' proteins comprised of capsid (CA) and nucleocapsid (NC) proteins and the linear, single stranded, positive-sense RNA genome which is packaged as a dimer. Viral enzymes reverse transcriptase (RT-RNase-H), integrase (IN), protease (PR) are packaged within the capsid.

Adapted from (Swanstorm and Wills, 1997).

The retrovirus genome is organised with protein coding regions flanked by regulatory domains known as long terminal repeats (LTRs) (Figure 1.3). LTRs are identical sequences which can be divided into three elements: U3, derived from unique sequences to the 3' end of viral RNA; R derived from repeat sequences identical to both ends of viral RNA; and U5, derived from sequences unique to the 5' end of viral RNA. The LTRs are generated during reverse transcription and as a result, proviral DNA is longer than genomic viral RNA. The LTRs contain sequences important in the control of viral transcription and post-transcriptional modifications, such as promoters, multiple enhancers and polyadenylation signals (Vogt, 1997).

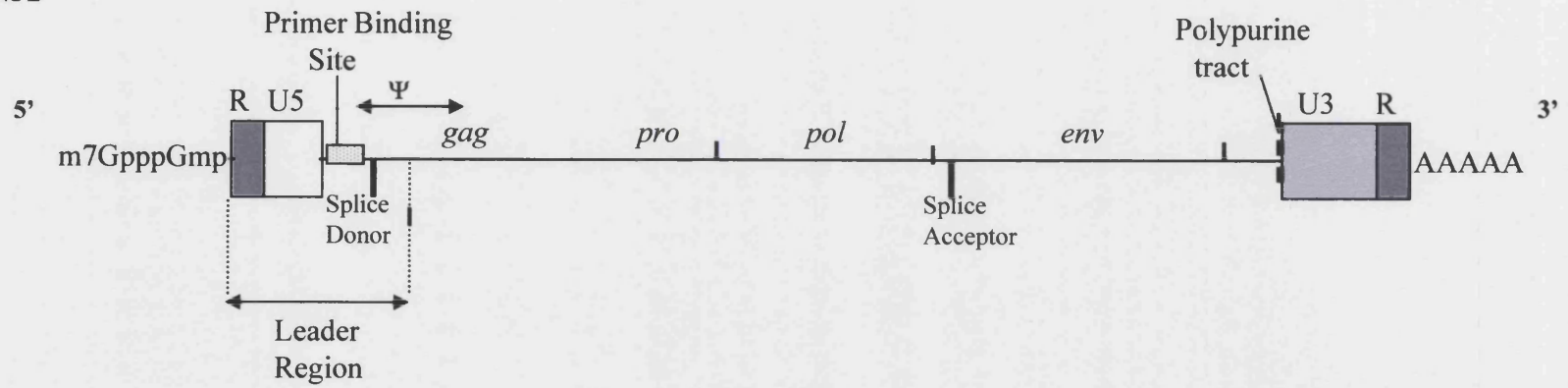
The essential viral genes *gag*, *pro*, *pol* and *env* occupy the body of the DNA (Figure 1.3). They are present in all retroviruses and for 'simple' retroviruses, such as gammaretroviruses, they are the only genes, whereas 'complex' retroviruses, such as lentiviruses and spumaviruses, express other accessory genes required to co-ordinate and regulate viral replication. The *gag* gene encodes the internal structural protein of the virus. Gag is proteolytically processed into the mature proteins MA (matrix), CA (capsid), NC (nucleocapsid) and sometimes others whose function is uncertain e.g. p12 for MLV. The *pro* gene encodes the viral protease (PR) that acts late in assembly of viral particles by cleaving the proteins encoded by *gag*, *pro*, *pol* and sometimes *env*. *pol* encodes the enzymes reverse transcriptase, which has both DNA polymerase and RNase H activity, and integrase, which mediates replication of the genome. *env* encodes the surface unit glycoprotein and transmembrane protein of the virus, which form a complex that determines viral envelope tropism that will be discussed later.

#### 1.3.4 Retroviral Life Cycle.

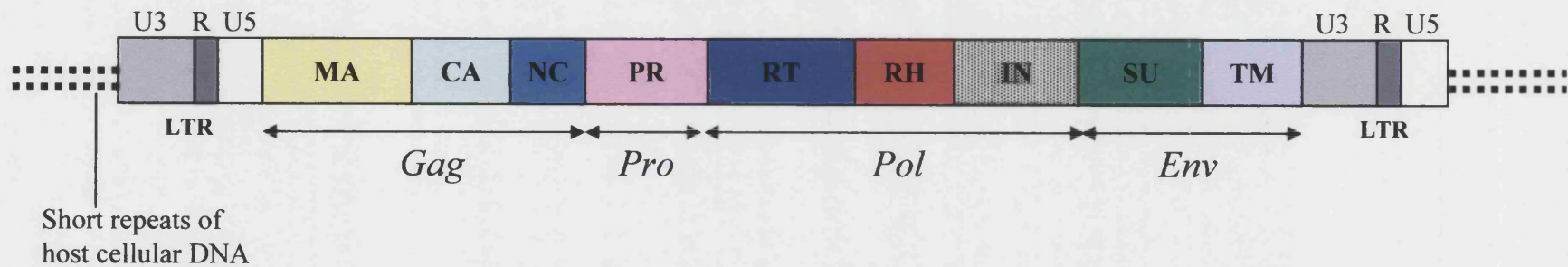
Knowledge of the retroviral life cycle is crucial in development of retroviral vectors (Figure 1.4). It can be divided into two phases. The first involving attachment, entry, reverse transcription and integration with the host DNA and the second involving synthesis of viral proteins and genomic RNA, assembly and budding of infectious virions from the host cell.

The first step is attachment to the target cell. The viral envelope glycoprotein dictates the host range of the virus through its interaction with the receptors on the target cells.

## RNA



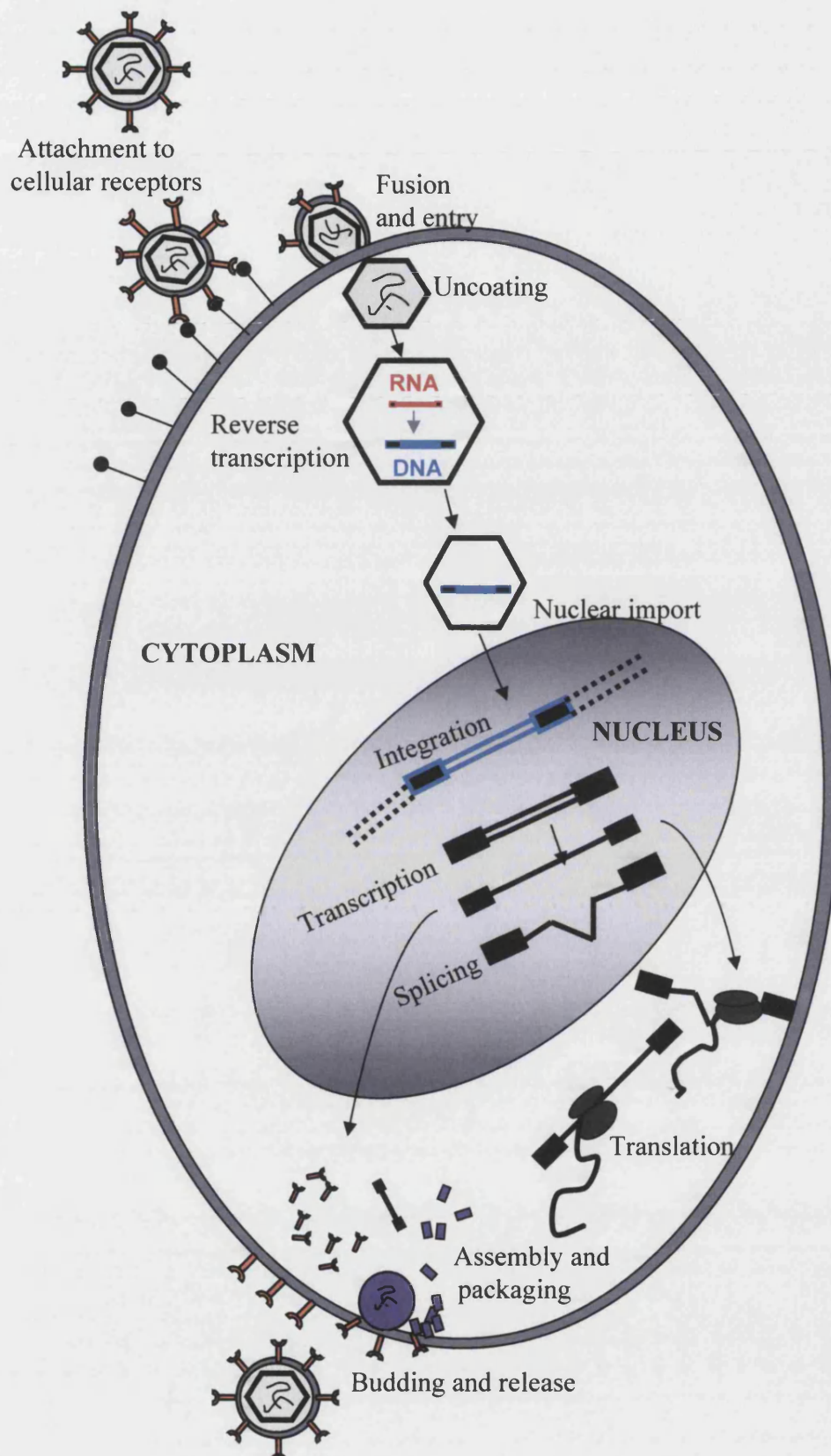
## DNA



**Figure 1.3 General organisation of a simple retrovirus genome**

Adapted from (Vogt,1997).





**Figure 1.4 Replication strategy of an infectious retrovirus**

Attachment of the envelope SU binding domain to a specific receptor triggers conformational changes within the SU. This leads to exposure of the hydrophobic 'fusion peptide' within the TM domain that mediates fusion of cellular and viral membranes and results in viral core entry into the cell cytoplasm (Hunter, 1997).

In the cytoplasm viral genomic RNA (as part of a nucleoprotein complex) is reverse transcribed by activated RT (Hunter, 1997). Once DNA synthesis is complete viral integrase cleaves the terminal two nucleotides from each 3' end of the linear double stranded viral DNA, creating recessed 3'-OH groups. In simple retroviruses like MLV, entry of the viral nucleoprotein complex into the nucleus occurs during mitosis when the nuclear membrane is dissociated (Roe *et al.*, 1993). However, in lentiviruses, nuclear entry can also occur during interphase by active transport through the nuclear pore allowing the infection of non-dividing cells (Weinberg *et al.*, 1991).

Binding of viral DNA with host DNA is initiated by integrase, which uses the 3'-OH groups at the end of the viral DNA (in the integrase-viral complex) to attack the phosphodiester bonds on the host chromosomal DNA. Once in the host chromosome integration is completed by cellular enzymes, which remove mismatched bases, repair single strand gaps and ligate host/viral DNA (Lee and Craigie, 1994).

The provirus is transcribed by the host cell machinery as a cellular gene. Viral mRNA, like its cellular counterpart, is 5' capped and 3' polyadenylated ensuring its stable export to the cytoplasm. *env* genes are translated from mRNA that has been spliced by cellular spliceosomes, while unspliced mRNAs are used either for *gag* and *pol* translation or incorporated into new virions as genomic RNA.

Retroviral proteins can be translated as a large Gag-Pro-Pol fusion polyprotein precursor or as a single Gag polyprotein or as an Env polyprotein. In MLV a read-through mechanism occurs to encode *gag* and *pol* in the same reading frame and thus produce a Gag-Pro-Pol fusion protein (Yoshinaka *et al.*, 1985). This translational mechanism ensures that correct amounts of proteins are packaged into mature virions, typically 2000 copies of Gag and 20-50 copies of Pol.

Retroviral assembly involves interactions between viral proteins, genomic proteins and host cell tRNA. Genomic RNA encapsidation and dimerisation, tRNA packaging, viral protein and RNA transport to an assembly site at the host cell plasma membrane and envelope acquisition during budding are events common to all retroviruses. In C type retroviruses (e.g. MLV) and lentiviruses, Gag-Pro-Pol core polyproteins and genomic RNA migrate to the plasma membrane, directed by the Gag MA domain, where they assemble before budding. Retroviral RNA contains cis-acting signals that allow it to be encapsidated by Gag polyproteins during virus assembly. This process requires interactions between the packaging signal, Psi ( $\psi$ ) or the encapsidation signal (E) and the Gag polyprotein (Berkowitz *et al.*, 1996).

The final stages in the retroviral lifecycle involve the maturation of viral particles. In most retroviruses the core particles have an 'immature' morphology irrespective of their assembly pathway. Gag and Gag-Pro-Pol polyprotein precursors are cleaved by viral protease to initiate maturation (Vogt, 1997). The timing of this protease-mediated cleavage is crucial and usually occurs at late assembly or just after budding. Premature processing results in intracytoplasmic accumulation of mature viral protein forms, which may interfere with normal particle assembly. Cleavage is critical in the production of infectious viruses, as demonstrated by mutations in the protease-coding domain (Stewart *et al.*, 1990) or the use of proteinase inhibitors (Sommerfelt *et al.*, 1992), otherwise non-infectious virus containing unprocessed Gag and Gag-Pro-Pol is produced. After cleavage, the viruses take on a mature form in which the core is detached from the membrane, as opposed to the immature morphology in which Gag precursor proteins are anchored by the MA protein to the inner face of the viral envelope.

#### **1.4 Retroviral Vectors**

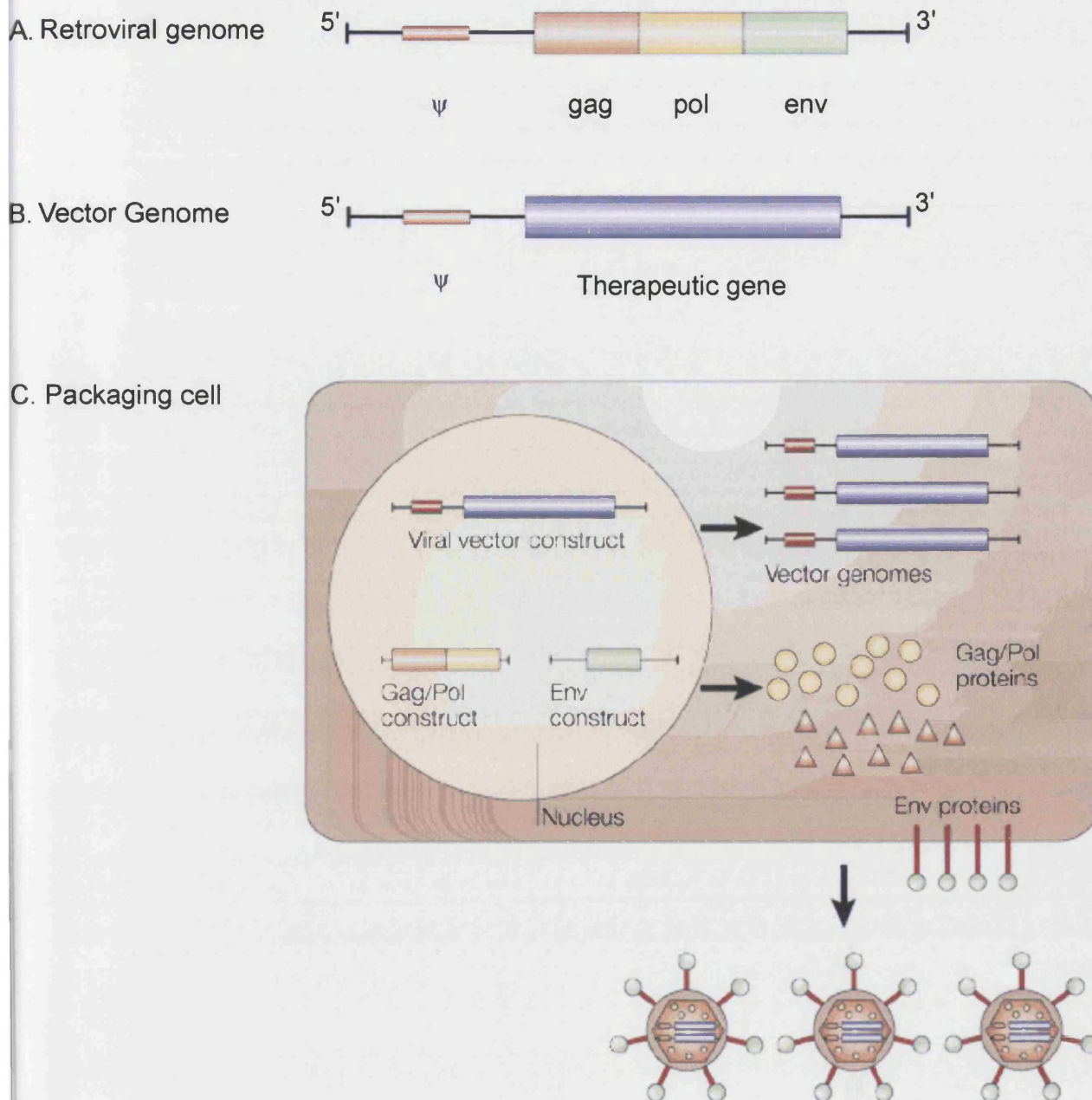
Vectors based on members of the retrovirus family are the most widely used today (Table 1.1). The majority of the retroviral vectors currently used in gene therapy models are derived from the murine leukaemia virus (MLV) and were among the first viral vectors to be used in human gene therapy trials (Blaese *et al.*, 1995). MLV has a small, simple and well-characterised genome, which allows extensive vector



manipulation. To produce retroviral vectors all of the protein-encoding sequences are removed from the virus and replaced by the transgene of interest. The essential *cis*-acting sequences such as the packaging sequence ( $\psi$ ) have to be included in the vector construct. The viral sequences necessary for reverse transcription of the vector RNA and integration of the proviral DNA, the LTRs, the transfer RNA-primer binding site, and the polypurine tract (PPT) also have to be present for efficient gene transduction and integration (Vogt, 1997). Thus the retroviral vector is replication-defective as it is incapable of making the proteins required for additional rounds of replication.

Viral proteins needed for infection are provided in *trans* in the packaging cell line. Retroviral packaging constructs are either transiently transfected into the packaging cells or a cell line is established that stably expresses the viral proteins. The packaging constructs are modified to reduce the chances of generating replication-competent virus (RCV) through recombination in the packaging cells. To further decrease the possibility of developing helper virus in the packaging cells a split genome packaging strategy was developed. Here two packaging constructs, one containing gag and pol and the other containing env are used (Danos and Mulligan, 1988; Markowitz *et al.*, 1988) (Figure 1.5). This not only increases the safety of retroviral vectors but also facilitates the pseudotyping of retroviral vectors with different envelope constructs.

Retroviral vectors possess several features that make them suitable for gene therapy. They are able to integrate into the target cell chromatin and thus have the potential for long-term gene expression. Although integration does not guarantee stable expression of the transduced gene, it is an effective way for the genetic material to be maintained in a self-renewing tissue and in the clonal outgrowth of a stem cell. The design of the retroviral vector means that target cells do not express viral proteins, which are responsible for most of the pathological and immunological consequences of viral infection. Thus gene transduction is usually well tolerated. Vector design allows up to 8 kb of exogenous DNA to be inserted and expressed in place of the viral genes (Kay *et al.*, 2001). Recent packaging cell lines produce titres above  $10^7$  transducing particles/ml and are suitable to be scaled-up for manufacturing large amounts of vector free from replication competent viruses (RCV) for clinical use.



**Figure 1.5 Retrovirus-based vectors**

A. The retroviral genome contains *gag*, *pol* and *env* genes. The  $\psi$  sequence is the packaging sequence that differentiates viral RNA from all other RNA in the cell and is recognised by the viral proteins for packaging.

B. The vector genome. *gag*, *pol* and *env* genes are replaced by the therapeutic gene.

C. The packaging cell. The *gag* and *pol* genes are separated from the *env* gene making regeneration of a replication competent virus unlikely. The vector genomes, by virtue of the  $\psi$  sequence are encapsulated along with the Pol and Gag proteins. The virus, buds from the packaging cell, resulting in the retroviral vector.

Adapted from (Somia and Verma, 2000).

A critical limitation to the use of C-type retroviral vectors is their inability to infect non-dividing cells (Vogt, 1997). As discussed previously, nuclear entry of simple retroviruses requires disruption of the nuclear membrane to allow the pre-integration complex to gain access to the chromatin (Roe *et al.*, 1993) and is thus dependent on target cell mitosis shortly after entry (Miller *et al.*, 1990). Recently, a nuclear localisation signal was engineered in the matrix protein of an avian C-type retrovirus, spleen necrosis virus (SNV), to enable an SNV vector to transduce non-proliferating cells (growth-arrested human T lymphocytes and quiescent primary monocyte-derived macrophages) (Parveen *et al.*, 2000). However, most work has concentrated on lentiviruses which are able to penetrate an intact nuclear membrane and transduce non-dividing cells. This characteristic means that lentiviral vectors will probably become important vector systems in the future treatment of a wide range of diseases. They are able to transduce haematopoietic stem cells *ex vivo* without first inducing them to proliferate with cytokine stimulation. They have been shown to be effective tools for gene delivery to the central nervous system (CNS), generating long-term expression without detectable pathology (Naldini *et al.*, 1996a). Therapeutic efficacy has also been demonstrated in a primate model for Parkinson's disease (Kordower *et al.*, 2000), which is the first successful gene therapy in such a model. Lentiviral transduction of muscle and liver has also been shown in animals, but studies in the liver have shown that not all non-dividing cells are equally susceptible to transduction by lentiviral vectors (Park *et al.*, 2000). Some cell types, such as the hepatocyte, might require cell cycling for efficient gene transfer

## **1.5 Safety of Retroviral vectors**

### **1.5.1 Insertional mutagenesis**

Integration is a mutagenic event with the potential to activate or inactivate cellular genes, including oncogenes or tumour-suppressor genes. A classical example of this is the integration of ALV upstream of a gene encoding a cellular transcription factor (c-myc), which causes lymphoma in chickens (Hayward *et al.*, 1981). One of the key advantages of retrovirus derived vectors, insertion of the transgene into the host chromosome with sustained gene expression, was until recently a theoretical

disadvantage with the potential for insertional mutagenesis. The potential for transformation by a gene therapy vector was first described in a murine model using a retroviral vector (Li *et al.*, 2002). This issue has come to prominence recently with the recent report of two cases of T-cell leukaemia in patients treated with retroviral gene therapy for X-linked severe combined immune deficiency (X-SCID) (2003), which will be discussed in greater detail below.

### **1.5.2 Gene therapy of X-linked SCID.**

X-SCID is an inherited disorder characterised by an early block in T and natural killer (NK) lymphocyte differentiation. This results in the absence of both T and B cells leading to severe and recurrent infections that are usually fatal in the first years of life. The block is caused by mutations of the gene encoding the  $\gamma$ c cytokine receptor subunit of interleukin 2, 4, 7, 9 and 15 receptors, which participate in the delivery of growth, survival and differentiation signals to early lymphoid precursors. Bone marrow transplantation (BMT) can be used to successfully treat X-SCID, but it works best when there is a fully compatible donor. Unfortunately this is the case in under one third of X-SCID children. In unmatched recipients, BMT carries the risk of graft failure, graft-versus-host disease, lymphoma and other medical problems.

The lack of therapeutic options in X-SCID led to the development of a gene therapy trial by Fischer and colleagues (Cavazzana-Calvo *et al.*, 2000). Bone marrow stem cells were obtained from the affected children, cultured with growth factors and transfected on three successive days with a Moloney derived retroviral vector carrying the  $\gamma$ c gene. 10 out of the first 11 patients achieved effective and life-saving immune reconstitution (2003). The patients recovered well and were able to lead a normal life for periods of up to 3 years from the first group of patients. Expression of the  $\gamma$ c gene was detected in T and NK cells with T, B and NK cell counts and function comparable to age matched controls (Cavazzana-Calvo *et al.*, 2000). Thus gene therapy was able to correct the disease phenotype and from a clinical perspective may have been considered cured by this pioneering treatment.

However, 30 months after treatment one of the patient developed a monoclonal gamma-delta T-cell lymphoproliferative disorder (leukaemia like disorder) (2003). Subsequently a second child has developed a T-cell leukaemia. Both leukaemias appear to be caused by insertional mutagenesis i.e. retroviral activation of a cellular oncogene at the site of insertion. The gene LIM domain only 2 (LMO2), located on chromosome 11, is normally involved in the control of blood cell proliferation and differentiation and is known to be activated in certain types of T-cell leukaemias. Its expression has shown to be elevated in both cases. It is thought that the cancerous T cells in both patients are derived from single transduced cells in which the retrovirus genome has inserted near, or in, the LMO2 oncogene activating LMO2 expression (Kohn *et al.*, 2003). A similar insertion into the LMO2 region has recently been identified in a third child in this study, although this child has not developed leukaemia (Thomas *et al.*, 2003).

The complication of leukaemia has not occurred in any other clinical trial nor in any large animal model that used retroviral vectors to modify haematopoietic stem cells. Leukaemia has been linked to vector integration in only one mouse study using this approach (Li *et al.*, 2002). Multiple factors may have contributed to the development of leukaemia in the patients involved in the X-SCID trial. These include the high level of engraftment and expansion of genetically modified cells, unique properties of the haematopoietic stem and progenitor cells in the bone marrow of X-SCID patients, the immune deficiency of X-SCID patients and/or the transferred gene itself. The gene itself is one that lends itself perfectly to gene replacement in that it provides a stimulus to growth and survival but this may have contributed to the malignant transformation. Further use of current gene-transfer methods for the treatment of X-SCID poses a complex dilemma in the consideration of potential risks and benefits. New recommendations from the United States Food and Drug Administration (FDA) Biological Response Modifiers Advisory Committee (BRMAC) state that this form of therapy should not be the first line of treatment for X-SCID, but it can be considered in the absence of other options such as matched bone-marrow transplantation (Check, 2003).

Insertional mutagenesis had always been considered a potential risk associated with the use of retroviral vectors. It is important to recognise that the risks could be

different for each disease, each therapeutic gene and each class of patient and every subsequent gene therapy trial involving the use of retroviral (or other integrating vectors) should be preceded by a careful assessment of the risk-benefit ratio.

Understanding the risk of oncogenesis by vector integration requires further investigation into the mechanisms that underlie transformation. This will lead to the development of approaches to minimise the likelihood of leukaemia or tumour formation. One of the first priorities is to analyse the site-selection patterns of integration for different vectors. For oncoretroviruses it is established that integration is biased towards DNase I hypersensitive chromatin (Vijaya *et al.*, 1986; Rohdewohld *et al.*, 1987). This also appears to be the case for HIV-1 (Schroder *et al.*, 2002). This bias results in preferential integration in gene-rich regions, particularly in or near actively transcribed genes. This preference is likely to be conserved in the replication defective vectors that are derived from these viruses. The potential sites and frequency of integration will probably differ between vector types as well as between target cells and further research is needed to determine likely sites that will aid decisions about future treatments.

## **1.6 Cancer Gene Therapy**

The direct targeting of cancer cells with gene therapy offers tremendous promise for the future of cancer treatment with new molecular technology offering several ways of targeting tumour cells. Cancer has become the most common disease treated in current gene therapy protocols with 68.5% of all patients treated (Table 1.1). The field of cancer gene therapy embraces a range of ideas and technologies from direct attack on tumour cells to harnessing the immune response to tumour antigens.

### **1.6.1 Immuno-gene therapy**

The immune system has several features that are ideally suited to gene therapy most notably an amplification of the therapeutic effect following relatively low level gene delivery and high level specificity of body-wide target cell killing once correctly activated (Vile *et al.*, 2000). The potential of harnessing these two features to fight

metastatic disease is why the majority of cancer gene therapy protocols are aimed at immune stimulation (54% of open trials listed by the US recombinant advisory committee; data from /www.nih.gov/science/).

The timing of treatment in immuno-gene therapy, like most, if not all, treatment modalities for cancer is crucial. The smaller the tumour the more likely that appropriately activated immune cells can control and eradicate it. Animal models show that with tumour vaccines directed at the immune system, gene therapy was more successful in smaller tumours (Forni *et al.*, 1995). Early in the disease, immunomodulatory gene therapy can eradicate disseminated metastases that are antigenically similar to the primary tumour and that do not yet exert an inhibitory immunosuppressive effect. In the clinical setting tumours may evolve over long periods when the immune system is intact. Variants that are poorly immunogenic are probably selected and these escape immune surveillance. Thus, the smaller the volume of disease, the shorter the time in which tumour cells can acquire mutations allowing them to evade the immune system. In addition, immune dysfunction has been shown to correlate with disease extent in cancer patients, making effective activation of the immune system less likely in advanced disease (Kavanaugh and Carbone, 1996). Finally, the more advanced the tumour the more likely that the patient will have received chemotherapy (and/or radiotherapy) regimens that may suppress the immune system and decrease further the efficacy of gene therapy.

The first clinical protocols for cancer gene therapy involved the *ex vivo* modification of freshly isolated tumour cells with cytokines (Pardoll, 1995). It became apparent that in many cases cytokine modification was little better than conventional adjuvant-based cancer cell therapies with no gene transfer component (Hock *et al.*, 1993). Additionally, the recovery and establishment in culture of patient tumour cells is time consuming and expensive and may also significantly alter the phenotype of the cells.

However, animal models showed clear efficacy for this treatment modality. For example, a vaccine of irradiated tumour cells (B16 melanoma model) engineered to secrete murine granulocyte-macrophage colony-stimulating factor (GM-CSF) showed potent, specific, and long-lasting anti-tumour immunity in mice (Dranoff *et al.*, 1993). It was subsequently shown that cytokine modified vaccines can generate significant

immune responses in patients with minimal toxicity. Irradiated autologous melanoma cells melanoma cells engineered to secrete human GM-CSF using either retroviral or adenoviral vectors have shown promising activity in patients with metastatic melanoma with evidence of potent antitumour immunity. (Soiffer *et al.*, 1998; Soiffer *et al.*, 2003). However, given the preparation and subsequent modification of autologous tumour cells needed to produce vaccines it is unlikely that this will ever become a universal approach.

The two areas in which immuno-gene therapy may make most progress are likely to be the use of tumour-associated antigens and the exploitation of the central significance of the dendritic cell in generating anti-tumour immune responses. The identification of tumour-associated antigens (TAA) from human tumour cells (commonly melanoma), which are recognised by either CD8<sup>+</sup> (Boon and van der Bruggen, 1996) or CD4<sup>+</sup> T cells (Wang *et al.*, 1999a; Wang *et al.*, 1999b; Walker *et al.*, 2002), has provided evidence that tumours can express antigens against which T cell-mediated responses can be raised. The identification of these antigens means that tumour vaccination can move from the relatively crude level of whole cell vaccines to more defined targets. It is important to note that tumours are highly heterogeneous and unlikely to express one antigen on all of the cells. Thus, vaccination with a 'cocktail' of antigens is likely to be necessary.

The most potent antigen presenting cell in the immune system is the dendritic cell (DC) so called because of its extensive cellular processes with which it interacts with T cells in lymphatic tissue (Banchereau and Steinman, 1998). The presence of anti-tumour immune responses against defined tumour antigens has led to investigations in the use of modified DCs to break tolerance and induce anti-tumour immune responses. Vaccination of patients with advanced melanoma with dendritic cells pulsed with tumour lysate or a cocktail of peptides has shown impressive anti-tumour activity (Nestle *et al.*, 1998; Nestle *et al.*, 2001). Several clinical trials using patient DCs pulsed with peptide antigen or tumour lysates have shown similar results raising the possibility that DC based vaccines may allow the development of effective therapies against melanoma and other cancers (Timmerman and Levy, 1999).



However, the disadvantages of DCs pulsed with synthetic peptides from TAA include the uncertainty regarding the longevity of antigen presentation, the restriction by the patient's haplotype and the relatively low number of known MHC class I and in particular of MHC class II helper cell-related epitopes (Humrich and Jenne, 2003). In addition whole tumour cell preparations are difficult to standardise, and depend on the availability of tumour cells. Thus the utilisation of viral vectors genetically modified to express TAA for the *ex vivo* transduction of DCs is an attractive alternative to achieve a MHC I- and MHC II-restricted presentation of tumoural antigens. To induce protective anti-tumoural immune responses an increasing number of modified viral vectors have been used to transduce DCs.

Initial murine studies using DCs transduced *ex vivo* by retroviruses encoding a model tumour antigen ( $\beta$ -galactosidase) were able to generate antigen-specific cytotoxic T lymphocytes (CTLs), with significant anti-tumour activity, against tumours expressing the antigen (Specht *et al.*, 1997). Delivery of genes by adenoviral vectors has also been demonstrated (Song *et al.*, 1997; Song *et al.*, 2000), but the high antigenicity of these vectors seems likely to preclude their use in this situation. DCs are non-dividing and this has led to great interest in the use of lentiviral vectors to modify them, which has successfully been achieved (Chinnasamy *et al.*, 2000; Schroers *et al.*, 2000). Transduced cells have been shown to maintain T-cell stimulatory activity and antigen specific responses have been demonstrated (Dyall *et al.*, 2001).

It is important to recognise the possibility of inducing autoimmune damage by modification of DCs with tumour associated antigens that are displayed on normal tissues. The first goal in the modification of DCs must be the establishment of potent anti-tumour immune responses; the second will be to learn how to restrict such responses solely to the tumour.

### **1.6.2 Tumour suppressor genes and oncogenes**

A more direct approach for gene therapy is the altering of a DNA sequence that is specifically responsible for a malignant transformation or its maintenance, such as the

ablation of an oncogene or the replacement of a functional tumour suppressor gene. The replacement of tumour suppressor genes has proved a particularly attractive target for cancer gene therapy. This concept is based on two assumptions. Transformation of a normal cell to a malignant cell is causally related to the acquisition of a series of genetic lesions. The first assumption is therefore that restoration of a single genetic defect will be effective in inhibiting tumour cells that have multiple additional defects. Despite the multiplicity of these lesions within a single cancer cell studies have shown that correction of a single critical genetic lesion is sufficient to abrogate tumorigenicity in human cancer cells (McCormick, 2001). This has been demonstrated for the principal tumour suppressor genes, for example: adenomatous polyposis coli (APC) (Morin *et al.*, 1996), retinoblastoma (RB) (Nikitin *et al.*, 1999) and p53 (Roth *et al.*, 1996). Expression of each of these genes in tumour cells *in vitro* causes an acute change in cell physiology and gene expression, resulting in cell-cycle arrest or death. As well as validating the concept of this form of gene therapy, these experiments clearly demonstrate the selective advantage of losing tumour-suppressor gene expression in tumour development.

The second assumption regarding the use of tumour suppressor genes is that collateral delivery of these genes to normal tissue will have little effect because these genes are already expressed in normal cells where they are appropriately regulated. This issue has not been addressed rigorously in model systems. The normal tissue counterpart of the tumour would seem a logical choice for experimental investigation, but the toxic effects of the gene are more likely to be seen in cells exposed to the highest levels of the vector, such as the liver or vascular endothelial cells. In one study delivery of p53 to normal bronchial epithelial cells showed no effects on cell growth, with a 2-3 log therapeutic window relative to the tumour cells (Zhang *et al.*, 1994).

These results supported the rationale of delivering tumour suppressor genes, but the effects were thought to be cell autonomous with negligible effects on surrounding uninfected cells. This means that virtually every tumour cell would need to be infected, an enormous technical hurdle especially for disseminated disease. However, it appears that a bystander effect whereby adjacent cells to those that have been transfected are also killed is seen with gene delivery of p53 (McCormick, 2001). This may be because p53 is anti-angiogenic: it downregulates expression of vascular

endothelial growth factor (VEGF) and upregulates expression of thrombospondin – a potent inhibitor of angiogenesis (Nishizaki *et al.*, 1999).

The first clinical effects of delivering a tumour suppressor gene, p53, used a retroviral vector containing the wild-type p53 gene to mediate transfer of wild-type p53 into human non-small cell lung cancers (NSCLC) by direct injection (Roth *et al.*, 1996). The treatment was well tolerated with clear signs of apoptosis in injected tumours, and three out of the nine treated patients showed regression of their tumours as well as evidence of a bystander effect. More recent studies have used adenoviral vectors to deliver p53 (Ad-p53). Repeated intratumoural injections of Ad-p53 appear to be well tolerated, result in transgene expression of wild-type p53, and seem to mediate antitumour activity in a subset of patients with advanced NSCLC (Swisher *et al.*, 1999) and squamous cell carcinoma of the head and neck (Clayman *et al.*, 1999). Ad-p53 is now being tested in phase II and III clinical trials as well in combination with chemotherapeutic agents (Nemunaitis *et al.*, 2000) or radiotherapy. Replacement of p53 may have particular relevance to cytotoxic chemotherapy as cells that contain mutant p53 appear to be more resistant to chemotherapy. Thus correction of p53 function could in theory allow apoptosis in response to chemotherapy-induced DNA damage.

The opposite approach to introducing tumour suppressor genes is the blocking of overactive oncogenes. Again viral vectors have been used to deliver antisense oligonucleotides or ribozymes to block oncogene expression (McCormick, 2001). The applicability of this approach is reliant on similar assumptions to those of tumour suppressor gene therapy and again these have been fulfilled. Tumour cells with many genetic defects still depend on oncogene expression for growth and survival. For example, tumours that are driven by HRAS are destroyed when it is eliminated by either genetic or pharmacological approaches (Chin *et al.*, 1999). Furthermore, ribozymes that target mutant RAS selectively should have no effect on normal cells.

The effectiveness of this approach may be hindered by a lack of a bystander effect and also by the increasing number of alternative approaches to oncogene-directed therapy. Recent successes with small molecules, particularly inhibitors of the RAS pathway (Sebolt-Leopold *et al.*, 1999) and p210 BCR-ABL (Druker *et al.*, 2001), show that

more attention is likely to be directed to this area of oncogene-based research and therapy. In contrast, it is more difficult to develop small molecules that reactivate mutated tumour suppressors.

### 1.6.3 Suicide genes

The next broad approach to direct targeting of cancer cells with gene therapy is the delivery of suicide genes. These are enzyme encoding genes which, once expressed, allow the cancer cell to metabolise a harmless prodrug into a toxic metabolite. Examples include cytosine deaminase which converts 5-fluorocytosine into the toxic agent 5-fluorouracil and herpes simplex thymidine kinase (HSV-tk), which initiates the phosphorylation of the non-toxic drugs acyclovir and ganciclovir to their toxic triphosphate forms. A bystander effect is commonly seen with suicide gene therapy and may be mediated by either a local or immune effect or a combination of the two (Vile *et al.*, 2000). The prototypical bystander effect involves the transfer of toxic metabolites between cells (Moolten, 1994) but there may be other effects such as suppression of angiogenesis.

The first clinical study of suicide gene therapy used direct injection of murine retroviral producer cells to treat brain tumours (Ram *et al.*, 1997). The aim was to release retrovirus encoding the herpes simplex virus thymidine kinase (HSVtk) suicide gene, which would infect surrounding tumour cells (the only cells that should be replicating at that site and thus be susceptible to C-type retroviral infection) and render them susceptible to the prodrug ganciclovir (GCV) (Culver *et al.*, 1992). This would kill the retroviral producer cells, the infected cells and bystander tumour cells. This approach proved successful in the rat model (Culver *et al.*, 1992), however there was only limited success when used to treat human brain tumours (Ram *et al.*, 1997). There was some antitumour activity in five smaller tumours (1.4 +/- 0.5 ml), but there appeared to be limited gene transfer to tumours suggesting that this effect was due to 'bystander' mechanisms from the vector-producing cells. The injected producer cells remained stuck in close proximity to the injecting needle and tk cDNA transfer by the retroviral vector was limited to a few cells away. The response of only very small

tumours showed the feasibility of this approach, but highlighted the need to improve delivery of the therapeutic gene.

Several enzyme-prodrug combinations are being evaluated but HSV-tk has been most widely evaluated and has progressed farthest into the clinic with many phase I and II trials currently ongoing. The first phase III trial has recently reported its results (Rainov, 2000). This multicentre trial randomised 248 patients with newly diagnosed, previously untreated glioblastoma multiforme (GBM) to either standard therapy (surgical resection and radiotherapy) or standard therapy plus adjuvant gene therapy (using retroviral producer cells) during surgery. Progression-free median survival in the gene therapy group was 180 days compared with 183 days in control subjects. Median survival was 365 versus 354 days, and 12-month survival rates were 50 versus 55% in the gene therapy and control groups, respectively. These differences were not significant. Therefore, the adjuvant treatment improved neither time to tumour progression nor overall survival time, although the feasibility and good biosafety profile of this gene therapy strategy were further supported. The failure of this specific protocol may be due mainly to the presumably poor rate of delivery of the HSV-tk gene to tumour cells. In addition, the current mode of manual injection of vector-producing cells with a non-migratory fibroblast phenotype limits the distribution of these cells and the released replication-deficient RV vectors to the immediate vicinity of the needle track. Further evaluation of the RV-mediated gene therapy strategy must incorporate refinements such as improved delivery of vectors and transgenes to the tumour cells, non-invasive in vivo assessment of transduction rates, and improved delivery of the prodrug across the blood-brain and blood-tumour barrier to the transduced tumour cells.

A more promising approach appears to be the local injection of adenoviral vectors that express HSV-tk into brain tumours, which has shown a survival benefit in a small number of patients when compared to retroviral producer cells or lacZ control. Mean survival times for retrovirus, adenovirus, and control groups were 7.4, 15.0, and 8.3 months, respectively. The difference in the survival times between the adenovirus and retrovirus groups was significant ( $p < 0.012$ ) (Sandmair *et al.*, 2000). Despite this promising result there remains a need to improve the efficacy of suicide gene therapy. Efforts are underway to improve to achieve this using different vectors and suicide

genes that might produce stronger bystander effects and investigating the potential of combining suicide genes that act synergistically (McCormick, 2001). Another potential method of potentiating the bystander effect is by using lovastatin and other compounds that upregulate the number of gap junctions (Touraine *et al.*, 1998). In a murine tumour model only two or three injections of lovastatin during ganciclovir treatment doubled the antitumour response rate, with 60-70% of the mice achieving complete remission (Touraine *et al.*, 1998). This supports the hypothesis that the transfer of phosphorylated ganciclovir from HSV-tk gene-expressing cells to neighbouring tumour cells is a major component of the bystander effect and that pharmacological manipulation of gap junction function with lovastatin can result in striking improvement in the antitumour response in mice with tumours modified to contain as few as 10% HSV-tk cells.

#### **1.6.4 Anti-angiogenic genes**

Bystander effects can also be achieved in other ways with one of the most promising being suppression of angiogenesis. Several anti-angiogenic agents have been developed that show promising activity in animal tumour models. Gene therapy has the potential to produce these therapeutic agents in high concentrations in a local area for a sustained period, thereby avoiding the problems encountered with long-term administration of recombinant proteins, monoclonal antibodies, or anti-angiogenic drugs. For example, adenoviral expression of a soluble form of VEGF receptor was shown to suppress tumour growth in mouse models (Takayama *et al.*, 2000). And angiostatin and endostatin expressed from plasmid DNA complexed with liposomes are able to inhibit the growth of breast cancer in mice (Chen *et al.*, 1999).

#### **1.7 Targeting gene delivery**

One of the major goals of all anti-cancer treatments is to destroy tumour cells without affecting normal tissues. Gene therapy protocols would be improved by the availability of targetable vectors that could deliver genes to specific target cells or disease sites. Non-target cells should not be infected as gene delivery could be potentially harmful e.g. suicide genes, and would deplete the pool of vector particles.

There are two contrasting ways of achieving targeted gene therapy. On the one hand a non-targeted, non-specific vector can be delivered to achieve a selective effect e.g. directly injecting a tumour. This is called extrinsic targeting. Alternatively the capacity for selective delivery may reside within the agent itself: intrinsic targeting. This is achieved by designing elements into the gene therapy vector that enable it to selectively transduce the target cells of choice.

Intrinsic targeting may be achieved by transcriptional or transductional targeting. Transcriptional targeting is achieved by altering the promoter sequence within the vector so that the gene is only expressed in certain tissues. Several 'tissue specific promoters' have been identified e.g. the tyrosinase gene is only expressed in melanocytes and control of its expression is dependent on a promoter which is only active within these cells. Thus if this promoter is inserted upstream of a particular gene that gene will only be expressed in cells of melanocyte lineage including melanoma cells (Vile and Hart, 1993). Several potentially useful tissue specific promoters for cancer gene therapy have been identified that enable selective gene delivery *in vitro* and in animal models (McCormick, 2001). Examples of these include the androgen receptor promoter for prostate cancer, thyroglobulin for thyroid cancer and surfactant protein B for bronchial cancers.

Another approach to targeting has been transductional targeting i.e. delivering DNA to specific cells. There have been numerous attempts to develop reliable approaches for targeting gene delivery using both viral and non-viral gene delivery systems. One approach has been to use adaptor molecules to cross-link gene delivery vehicles to receptors on the target cell surface. An alternative approach has been to introduce receptor binding polypeptides into the surface of viral vectors (Russell and Cosset, 1999). Depending upon the vector system used and the targeting strategy employed, the gene delivery vehicle may or may not be competent to deliver its gene after it has bound to the targeted receptor. A targeting element may therefore be used to direct delivery through a cell surface receptor, to inhibit gene delivery to a specific target cell or to modulate the trafficking and localisation of a gene delivery vehicle in the body.

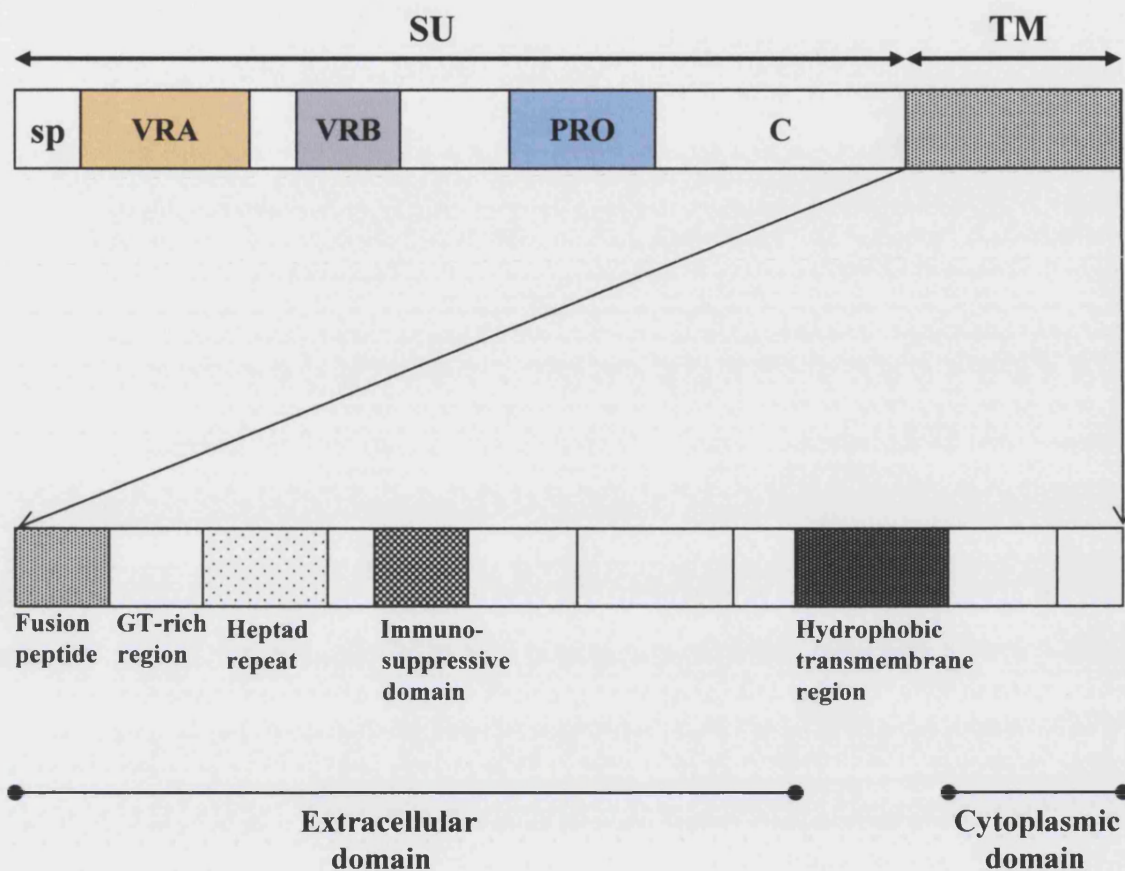
As previously stated retroviral vectors are the ones most commonly used in current clinical trials (Table 1.1). At the present time most retroviral gene therapy protocols require that target cells be removed from the body, transduced *ex vivo* and then reimplanted. This method is inconvenient, inefficient and expensive and this provides a strong impetus for continued efforts to develop targetable, injectable retroviral vectors. The most common method used to achieve such targeting is via alterations in the retroviral envelope. Thus in order to see how retargeting may be achieved it is necessary to understand the structure and function of the retroviral envelope glycoprotein complex.

### 1.7.1 Retroviral envelope glycoprotein

The first steps of retroviral entry are attachment to the target cell surface and subsequent fusion of the viral and cellular membranes (Hunter, 1997). These steps of attachment and fusion are mediated by the viral envelope glycoproteins. These homotrimeric proteins have an extraviral surface (SU) subunit that is linked to a membrane-anchored transmembrane (TM) subunit, which spans the lipid membrane of the virus (Figure 1.6). The viral envelope glycoprotein is synthesized as a polyprotein precursor that is directed by its N-terminal signal peptide in to the lumen of the endoplasmic reticulum (Hunter and Swanstrom, 1990). In the endoplasmic reticulum the signal peptide is cleaved from the protein and cellular chaperone proteins guide the folding of the monomer subunits. These are assembled into trimers and exported from the endoplasmic reticulum to the Golgi compartment. The precursor polypeptide is cleaved by a Golgi protease that recognises a cleavage signal at the SU-TM junction. After cleavage the SU and TM remain associated primarily through non-covalent bonds (Gliniak *et al.*, 1991), but are also linked by a labile disulphide bond that may play an important part in the fusion triggering mechanism (Pinter *et al.*, 1997). Cleavage activation at the SU-TM junction is required for correct functional maturation of the protein to a fusion-competent state.

From the Golgi compartment, the mature SU-TM complex is transported to the cell surface and incorporated into budding retroviral particles. The precise mechanism by which envelope glycoproteins are preferentially concentrated in budding virions is not fully understood (Hunter, 1997). Several cell surface and viral heterologous





**Figure 1.6 Functional domains of the retroviral envelope proteins**

Schematic representation of the functional domains of MLV envelope proteins.

SU: surface subunit, SP: signal peptide, VRA/VRB: variable regions A+B, PRO: proline rich region, C: C-terminal domain and TM: transmembrane region

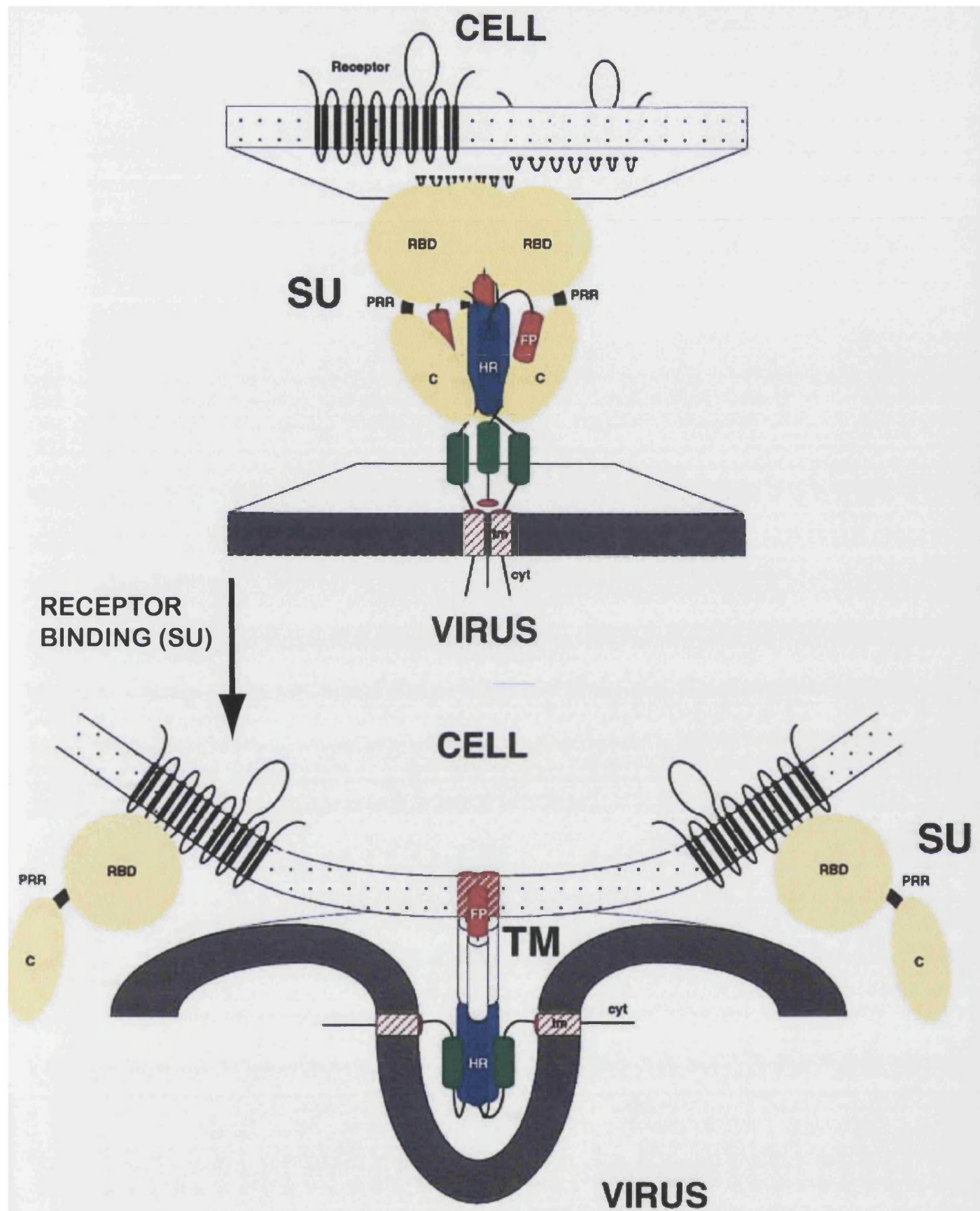
This figure does not reflect the size difference between SU and TM regions; MLV SU is approximately 480 amino acid and TM 200 amino acids.

Adapted from (Battini *et al*,1995, Zhao *et al*, 1998)

glycoproteins can also be incorporated into retroviral particles (Suomalainen and Garoff, 1994).

Final maturation of the envelope glycoprotein complex to the fully fusion-competent state only occurs after the protein has been incorporated into a retroviral particle. At the time of, or shortly after budding, the viral protease becomes active and cleaves a C-terminal peptide (the R-peptide) from the cytoplasmic tail of the TM protein (Ragheb and Anderson, 1994; Rein *et al.*, 1994). Before this final cleavage step occurs, the envelope glycoprotein is competent to bind to its receptor on the target cell but is unable to trigger the post-binding events that lead to membrane fusion. Exactly how the R-peptide inhibits this fusion triggering is unknown, but its importance is shown by the fact that truncated envelope glycoproteins lacking the R-peptide are potent inducers of cell-cell fusion and are thus strongly cytotoxic to the cells from which they are expressed (Ragheb and Anderson, 1994; Rein *et al.*, 1994).

When a virus decorated with mature envelope glycoprotein encounters a permissive target cell, the SU glycoprotein binds with high affinity to its receptor and this triggers a series of post-binding events that lead to membrane fusion (Figure 1.7). The SU glycoprotein comprises an N-terminal receptor-binding domain connected through a proline-rich hinge rich region to a C-terminal domain believed to mediate interactions between the SU and TM that lead to fusion. Comparing the receptor-binding domains of SU glycoproteins of type C-mammalian retroviruses reveal the presence of a conserved framework containing two hypervariable regions (VRA and VRB) that are thought to be the main determinants of receptor-binding specificity (Battini *et al.*, 1992; Baer *et al.*, 1993; Battini *et al.*, 1995). Mutagenesis of critical residues in these hypervariable regions can abolish receptor-binding activity (Bae *et al.*, 1997). Close to N-terminus of SU is a conserved peptide motif centering on a histidine residue that has been shown to be critical for post-binding events that lead to membrane fusion (Bae *et al.*, 1997; Lavillette *et al.*, 2000). Deletion or mutation of this histidine residue has no effect on receptor recognition and virus binding, but abrogates fusion triggering. By exposing cells to a soluble receptor-binding fragment of the SU that contains the histidine motif it is possible to restore their susceptibility to infection by viruses that carry the histidine mutated envelope (Lavillette *et al.*, 2000). This infers



**Figure 1.7 Molecular rearrangements of the TM structure following binding.** RBD, receptor binding domain; PRR, proline-rich region; C, SU carboxyl terminal domain; TM, transmembrane subunit; FP, fusion peptide; HR, heptad repeat.

Adapted from (Russell and Cosset, 1999).

that the N-terminal histidine-containing motif transmits a post-binding signal to the target cell and/or envelope complex that triggers membrane fusion.

The proline-rich region provides a flexible linker between the N- and C- terminal domains of SU. However, recent reports suggest that it also acts as a functional domain critical to the structure and function of the SU. It has been shown to influence receptor recognition (Battini *et al.*, 1995), to be important for stabilisation of the SU-TM interaction (Gray and Roth, 1993) and also to alter viral fusogenicity (Andersen, 1994).

The TM subunit is divided into an extraviral domain, a membrane-spanning domain that anchors the envelope glycoprotein complex in the viral membrane and a short C-terminal peptide that remains, projecting from the interior surface of the viral membrane after the R-peptide has been cleaved. A hydrophobic fusion peptide is present close to the N-terminus of TM, which is critical for fusion triggering (Jones and Risser, 1993; Zhu *et al.*, 1998). The exposed fusion peptide is believed to penetrate the target cell membrane and directly catalyse the process of fusion between the viral and target cell membranes (Figure 1.7).

### **1.7.2 Targeting with adaptor molecules**

Targeting can be achieved using adaptor molecules to cross-link retroviruses to receptors on target cells. One binding site of the adaptor interacts with the retrovirus and the other interacts with the target cell. This was first demonstrated using a streptavidin bridging approach in which viruses were coated with biotinylated antibodies against the retroviral envelope, cells were coated with retrovirus-specific biotinylated antibodies and the streptavidin was used to cross-link the virus and cell-associated antibodies (Goud *et al.*, 1988; Roux *et al.*, 1989). This bridging strategy established proof of principle that ecotropic retroviruses could be retargeted to human cells although transduction of target cells was very inefficient (Roux *et al.*, 1989).

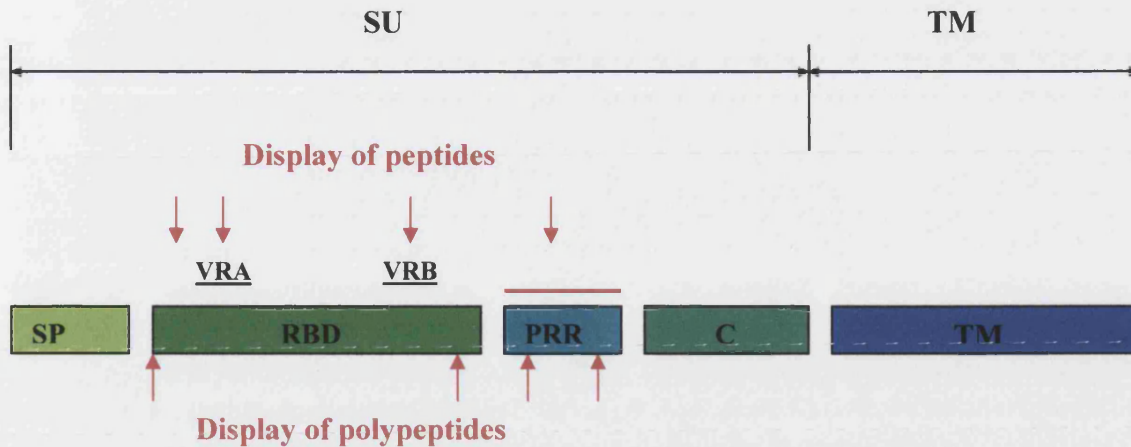
Despite the relative inefficiency of these early results, interest in this strategy has recently been revived with the development of adaptor molecules that can simultaneously retarget virion binding and trigger conformational changes in the

glycoprotein of retroviruses that belong to the group of avian leukosis viruses (ALVs) (Snitkovsky and Young, 1998). These adaptors consist of recombinant polypeptides in which ligands are fused to soluble forms of the receptors for ALVs. They allow the virions to bind to specific cell-surface molecules and the retroviral receptor moiety activates viral entry into target cells. This elegant targeting strategy has been demonstrated *in vitro* for adaptor molecules that carry EGF-receptor binding determinants such as EGF itself (Snitkovsky *et al.*, 2000). The bridge proteins can either be preloaded on target cells or, more interestingly, preloaded on ALV vector particles (Boerger *et al.*, 1999). This latter approach is more likely to have greater interest for gene therapy although the adaptor may dissociate from the virus particle or induce dissociation of the ALV SU before targeting has been achieved.

### 1.7.3 Targeting by envelope modification

The viral envelope glycoprotein determines which cells the retrovirus can infect and it has thus become the primary focus of retroviral targeting studies that aim to alter viral tropism. Retroviruses recognise a relatively limited number of cell-surface proteins as entry receptors (Sommerfelt, 1999) and several retroviral glycoproteins (e.g. those of ecotropic MLV) do not recognise a receptor on human cells. Thus initial attempts were made to extend their host-range so that they could bind to human cell surface molecules (Russell and Cosset, 1999). These attempts to retarget retroviral tropism consisted of the insertion of ligands (i.e. growth factors, hormones, peptides or single-chain antibodies) into various locations on the viral surface glycoproteins. Figure 1.8 shows examples of insertion sites into the MLV glycoprotein that have been characterised and found to be functional (Lavillette *et al.*, 2001b). They include modifications of the glycoprotein such as domain replacement (Kasahara *et al.*, 1994), peptide insertion into prefolded domains (Battini *et al.*, 1998; Wu *et al.*, 2000), and the display of polypeptides as additional folded domains (Russell *et al.*, 1993; Cosset *et al.*, 1995a; Zhao *et al.*, 1999).

Many of these chimeric glycoproteins fold correctly and are stably incorporated on virions achieving efficient retargeted virion binding to the new cell-surface molecules. Unfortunately, upon binding to the new target receptors most, if not all, of the chimeras are unable to induce membrane fusion and subsequent penetration of the



**Figure 1.8. Insertion sites into the MLV glycoprotein.**

Schematic representation of chimeric retroviral envelopes. SU: surface subunit, SP: signal peptide, RBD: receptor binding domain, VRA/VRB: variable regions A+B, PRR: proline-rich region, C: C-terminal domain and TM: transmembrane subunit.

The red arrows show the positions of the MLV glycoprotein that accommodate insertion/substitutions of peptides and/or polypeptides.

Adapted from (Lavillette *et al*, 2001b).



viral core into the cytosol. In many cases, this failure is not caused by inactivation of the fusion machinery of the chimeric glycoproteins themselves as the chimeras are able to achieve membrane fusion once they are allowed to bind cells expressing the natural retroviral receptors. Instead the poor fusion activity of chimeric glycoproteins is thought to be due to a loss of coupling between retargeted binding and fusion activation (Zhao *et al.*, 1999).

It therefore appears that the interaction of the envelope chimera with the targeted cell surface molecule is not able to activate the fusion function of the chimeras and the infectivity of the recombinant retrovirus is inhibited at a post-binding block. It is critically important when designing retargeted retroviruses to understand the molecular mechanism that converts receptor binding into a signal that can activate the retroviral fusion machinery. Closer examination of the molecular mechanisms that couple binding via the natural retroviral receptors to fusion activation show that it involves complex inter-relations between the different subdomains of the glycoprotein complex (Lavillette *et al.*, 1998; Barnett and Cunningham, 2001; Barnett *et al.*, 2001; Lavillette *et al.*, 2001a).

#### **1.7.4 Targeting by host range restriction**

Several strategies have been developed to overcome this loss of binding-to-fusion coupling. Many of which are based on the display of polypeptides on retroviral glycoproteins that naturally recognise a target receptor and thus infect these cells (e.g. amphotropic MLV). The aim here is to restrict the range of host cells, which may be infected. An important advantage of host-range restriction is that the natural virus entry pathway is utilised and thus fusion activation of the chimeric envelope is more readily achieved (Russell and Cosset, 1999; Lavillette *et al.*, 2001b). This approach also has a potential safety advantage in that the host-range is not expanded and so there is not the risk of generating a more pathogenic strain of the virus from which the vector is derived.

### 1.7.5 Retroviral display of cell-specific blocking domains to restrict tropism

Several ligands displayed at the N-terminus of C-type retroviral envelope glycoproteins have been shown to block infectivity on cells expressing the targeted receptor (Russell and Cosset, 1999). This was first seen when epidermal growth factor (EGF) was displayed on the amphotropic MLV glycoprotein (Cosset *et al.*, 1995a). These vectors could efficiently bind to EGF receptor-positive target cells but could not infect them. This sequestration can be competitively abrogated by adding the displayed ligand (EGF) as soluble polypeptide. The blocking effect occurs even if the amphotropic receptor is co-expressed on the target cells and despite full infectivity on EGF receptor-negative cells. This is due to the fact that some ligand-receptor pairs have the capacity to sequester cell-bound retargeted virions. This phenomenon of receptor-mediated sequestration has mainly been seen, although not exclusively, for molecules belonging to the tyrosine kinase family of receptors (Lavillette *et al.*, 2001b)

Inverse targeting exploits receptor-mediated virus neutralisation and allows targeted gene delivery in mixed cell populations to cells that express only the viral receptor. For example, EGF displaying retroviral vectors could efficiently infect EGF receptor-negative haematopoietic cells, but were non-infective for EGF-receptor positive epithelial carcinoma cells (Fielding *et al.*, 1998). This approach can thus be used to selectively transduce haematopoietic cells with an advantageous gene (e.g. multi-drug resistance gene), whilst minimising the risk of inadvertent transduction of contaminating cancer cells which express EGF receptors. An advantage of inverse targeting is that infection occurs via the natural virus entry pathway and so fusion activation of the chimeric envelope is efficient. However, there is a slight reduction (2-10 fold depending on the ligand) in the efficiency with which these targeted envelopes mediate entry into receptor-negative cells. This is thought to be due to a steric effect of the displayed ligand.

Inverse targeting has also been demonstrated for lentiviral vectors (Peng *et al.*, 2001). Targeted HIV-1-based lentiviral vectors were generated by pseudotyping them with chimeric murine leukaemia virus (MLV) envelope glycoproteins displaying EGF at their N-terminus. Intravenous inoculation of mice with non-targeted lentiviral vectors,



carrying wild-type glycoprotein leads to maximal gene delivery to the liver and spleen with minimal delivery to heart, muscle, lung, brain, kidney, ovary and bone marrow. In contrast, EGF-displaying vectors administered intravenously are expressed maximally in the spleen with very low expression in EGF receptor-rich liver cells. Transduction of liver cells can be restored by pre-treating the animals with soluble EGF, suggesting that these vectors are inversely targeted to spleen cells (Peng *et al.*, 2001). This important study shows that it is possible to generate lentiviral vectors carrying retargeted glycoproteins and establishes the feasibility of retargeting them *in vivo*.

A method of utilising this sequestration to allow specific gene delivery to the targeted cell itself has also been explored; protease targeting. This relies on enabling the virus to escape from the sequestering receptor, and has been achieved by inserting, between the displayed ligand and the envelope glycoprotein, peptide sequences that are cleavable by cell-surface-specific proteases. This has been shown in EGF-displaying amphotropic vectors with a matrix metalloprotease (MMP) cleavage site, which were able to preferentially infect EGF-receptor positive MMP-rich target cells *in vitro* (Peng *et al.*, 1997) and also to discriminate between MMP-rich and MMP-poor tumour xenografts in nude mice (Peng *et al.*, 1999). The retroviral envelope may also be masked in a more general manner by homotrimeric polypeptides that efficiently block infection on all cell types. For example, the N-terminal expression of the trimerising extracellular domain of the CD40 ligand on the amphotropic MLV glycoprotein is able to block infection (Morling *et al.*, 1997). It either hinders receptor binding and/or fusion activation and thus prevents infection. This block in infection can be overcome by inserting, between such blocking domains and the viral glycoprotein, a protease cleavage site that is recognised by a cell surface protease expressed on target cells (Morling *et al.*, 1997).

These results have therefore opened the possibility that proteases on the cell surface, rather than receptors, could be used to target gene delivery. Retroviral vectors that can be activated by cell-surface proteases involved in carcinogenesis can be generated and may also be used to identify novel proteases, by using random peptide display libraries inserted between the infection-blocking domain and the viral glycoprotein (Buchholz *et al.*, 1998).

### 1.7.6 Escorting viral entry

Another way of expanding viral tropism is the co-expression of a 'targeted' glycoprotein with the wild-type glycoprotein. The targeted glycoprotein, which is usually defective for fusion, carries a specific binding domain that enables binding to tissues that express the target molecules (Hall *et al.*, 1997; Jiang *et al.*, 1998). Recent strategies using this approach have concentrated on incorporating matrix-targeting motifs (i.e. collagen-binding peptides) on amphotropic MLV vector particles. It has been shown to improve retrovirus binding and transduction of human endothelial cells *in vitro* (Gordon *et al.*, 2000; Gordon *et al.*, 2001). Importantly these vectors were able to target gene delivery to sites of vascular injury *in vivo* in rats (Hall *et al.*, 2000) and to the tumour vasculature in human xenografts in nude mice (Gordon *et al.*, 2001). This 'preferential' targeting cannot be highly specific in principle and closer examination of vector biodistribution is needed to determine the extent of the 'leak' to non-target cells.

More specific targeting may be achieved through a similar approach using vectors derived from avian spleen necrosis virus (SNV) (Jiang *et al.*, 1998). SNV and simian D-type retroviruses belong to the same receptor interference group and appear to use the same receptor; the human allele of this receptor cannot mediate SNV entry into human cells. It is believed that this is because of its low affinity for the SNV glycoprotein (Jiang *et al.*, 1998). However, viruses that display both the wild type glycoprotein and a chimeric glycoprotein with an engineered high-affinity binding motif improve the SNV glycoprotein interaction with its receptor and promote membrane fusion in a restricted manner (Jiang *et al.*, 1998). This vector-mediated, cell type-specific gene delivery is maintained *in vivo* in a murine tumour model (Jiang and Dornburg, 1999).

## 1.8 Tumour antigens

The identification of tumour-associated antigens (TAA) provides an attractive target for various therapeutic approaches including retroviral gene therapy. Tumour associated antigens are antigens which are expressed at an increased level on cancer cells. They may also be expressed in very low quantities on normal tissues, some

being strongly restricted to certain stages/sites in tissue development. My project concerns the targeting of specific tumour antigens: primarily carcinoembryonic antigen (CEA) and also high-molecular-weight melanoma associated antigen (HMW-MAA).

HMW-MAA is an integral membrane proteoglycans that is expressed in greater than 90% of melanomas but not in normal adult tissues (Natali *et al.*, 1981). Its expression in melanoma is associated with a poor prognosis (Kageshita *et al.*, 1993). HMW-MAA has been used successfully *in vivo* as a target for both radioimaging and for immunotherapy (Mittelman *et al.*, 1994).

### 1.8.1 Carcinoembryonic antigen (CEA)

CEA was first described by Gold and Freedman in 1965, when they identified an antigen that was present in both foetal colon and adenocarcinoma of the colon but appeared to be absent from adult tissues (Gold and Freedman, 1965). It was suggested that CEA was an oncofoetal antigen; expressed during foetal life, absent in healthy adults and re-expressed in cancer. Subsequent work demonstrated that CEA was also present in healthy adult tissues although at much lower levels than in malignant tissues (Boucher *et al.*, 1989).

Later it was discovered that CEA could be measured in serum from patients with colorectal and other carcinomas using a sensitive radioimmunoassay (Thomson *et al.*, 1969). These findings prompted the widespread use of CEA as marker for colorectal cancer. The main reasons why CEA is useful as a serum tumour marker are probably because it is a stable molecule shed from the cell surface, with a restricted expression in normal adult tissue and high level of expression in positive tumours (Hammarstrom, 1999).

The main clinical use of serum CEA is in the post-surgical surveillance of colon cancer. Two large studies showed that an increase in CEA was the first indicator of recurrent disease in over 80% of patients (Minton *et al.*, 1985; Wanebo *et al.*, 1989). Monitoring of CEA levels post-operatively allows for second-look surgery or other treatment modalities to be instigated at an earlier time. Recently it has been shown that serum CEA measurement is the most cost-effective test in detecting potentially

curable recurrent disease (Graham *et al.*, 1998). Serum CEA levels may also be used as a prognostic indicator as high pre-operative level is associated with poor 5-year survival (Hammarstrom, 1999).

The gene encoding CEA is a member of the immunoglobulin supergene family (Thompson *et al.*, 1991). The human CEA gene family is clustered on chromosome 19q and comprises 29 genes. Of these, 18 are expressed, with 7 belonging to the CEA subgroup and 11 to the pregnancy-specific glycoprotein (PSG) subgroup. The domain structure of the 7 expressed CEA subgroup members is shown in. Two types of immunoglobulin domains are seen in the CEA family: an N-terminal domain of 108 amino acids homologous to the Ig variable domain (IgV-like) and between zero and six domains homologous to the Ig constant domain of the C2 set (Williams and Barclay, 1988). The IgC2 domains may either be of type A containing 93 amino acids or of type B containing 85 amino acids. A signal peptide of 34 amino acids precedes the N-domain, which is cleaved from the mature protein following transport to the cell surface. Molecules from the CEA and PSG subgroups differ at their C-terminal end. The CEA subgroup members are attached to the cell surface while the PSGs are secreted molecules. CEA is attached to the cell surface by a glycosyl phosphatidyl inositol moiety and is probably released as a soluble form by a phospholipase C or D (Hammarstrom and Baranov, 2001).

Two features appear to be characteristic for all expressed members of the CEA family: (i) they contain a single IgV-like N-domain, which lacks the intrachain disulfide linkage- the latter being replaced by a salt bridge. However, this type of IgV-domain is also seen in a few other immunoglobulin superfamily members notably CD2 and CD8; and (ii) the molecules are extensively glycosylated on asparagine residues, mainly (if not exclusively) by complex carbohydrate chains (Yamashita *et al.*, 1987).

When isolated from liver metastases CEA is a glycoprotein consisting of 60% carbohydrate and a molecular mass from 180-200 kD. CEA has been shown to exhibit considerable heterogeneity, which appears to be attributable to variations in its carbohydrate side chains. CEA itself shows a more limited tissue expression in normal adult tissue than other members of the CEA family. It is expressed in columnar

epithelial cells and goblet cells in the colon, in mucous neck cells and pyloric mucous cells in the stomach, in squamous epithelial cells of the tongue, oesophagus and cervix, in secretory epithelia and duct cells of sweat glands and in epithelial cells of the prostate (Nap *et al.*, 1992; Hammarstrom and Baranov, 2001). CEA expression in the tissues listed above generally commences early in foetal life (9-14 weeks) and persists throughout life.

In the colon CEA has been demonstrated by immunoelectron microscopy to localise specifically to the apical surface of mature enterocytes with no staining seen at the basolateral surface of the enterocytes (Hammarstrom, 1999). It appears to be specifically located at the apical glycocalyx/microvillus region of the mature enterocytes. This area is made up of microvesicles, which are formed by the blebbing of the microvillus membrane and subsequent pinching off. This vesiculation is a response to the conditions affecting the microvillus membrane and may serve as a means to rapidly remove membrane active agents from the gut luminal surface. Thus, CEA is released via CEA-coated vesicles in normal colon. The production and release of CEA in the normal adult colon is substantial. Over 24 hours a healthy adult is believed to excrete approximately 50-70mg of CEA in faeces, which may even underestimate the amount secreted.

The role of CEA in healthy adults remains unknown. The structural similarity of CEA to immunoglobulin-related proteins, such as ICAM-1 and ICAM-2, suggested that it might act as an adhesion molecule. In vitro experiments have shown that CEA was capable of both homophilic (CEA binding to CEA) and heterophilic (CEA binding to non-CEA molecules) interactions (Thompson *et al.*, 1991). The fact that cancer invasion and metastasis may be caused by alterations in cell adhesion led to the hypothesis that CEA may be involved in these processes. Evidence for this role was provided by Hostetter, who showed that after transplantation of colorectal tumours into nude mice, the number of liver metastases increased from 2% to 48% following injection of the mice with CEA (Hostetter *et al.*, 1990). However, to date there is no direct evidence that CEA is causally involved in cancer invasion and metastasis.

In normal physiology it seems unlikely that CEA is involved in intercellular adhesions because of its apical localization on polarised cells. It has been suggested

that CEA may play a role in innate immunity protecting the colon and potentially other areas such as the upper alimentary tract, urinary bladder and the skin (sweat glands) from microbial attack. CEA has been found to bind certain strains of *Escherichia Coli* and may protect the colon by binding and trapping infectious microorganisms, which are subsequently excreted (Hammarstrom and Baranov, 2001).

CEA is expressed on and secreted by a number of tumours of epithelial origin such as: colorectal carcinoma, lung adenocarcinoma, gastric carcinoma, breast carcinoma and pancreatic carcinoma. It is considered to be up regulated in certain carcinomas e.g. colorectal and gastric carcinomas. However, whether CEA in breast or pancreatic carcinomas, for example, represents ectopic expression cannot be resolved at present, as it is possible that CEA may be produced by a few normal cells in these tissues but at a level below the current threshold of detection.

Unlike the strict apical localisation of CEA in normal colonic epithelium, in colonic carcinoma CEA is expressed over the entire cell surface, in intraglandular lumina and even in intracellular lumina. This results in 'shed' CEA having access to the blood and lymphatics through the intercellular spaces as reflected in increased serum levels. A key issue with regards to tumour targeting is whether there is a difference between tumour CEA and normal CEA. At the genetic level no difference is apparent. In an early study it was shown that normal colon CEA was indistinguishable from tumour CEA by several immunological, physiochemical and chemical criteria (Fritsche and Mach, 1977). Despite this it seems possible that subtle post-translational changes such as trimming of the C-terminus after release from the membrane or modifications in the carbohydrate side chains, might create differences between tumour and normal CEA. A further source of heterogeneity may be that CEA from different organs may display different post-translational changes.

### **1.8.2 Targeting carcinoembryonic antigen (CEA)**

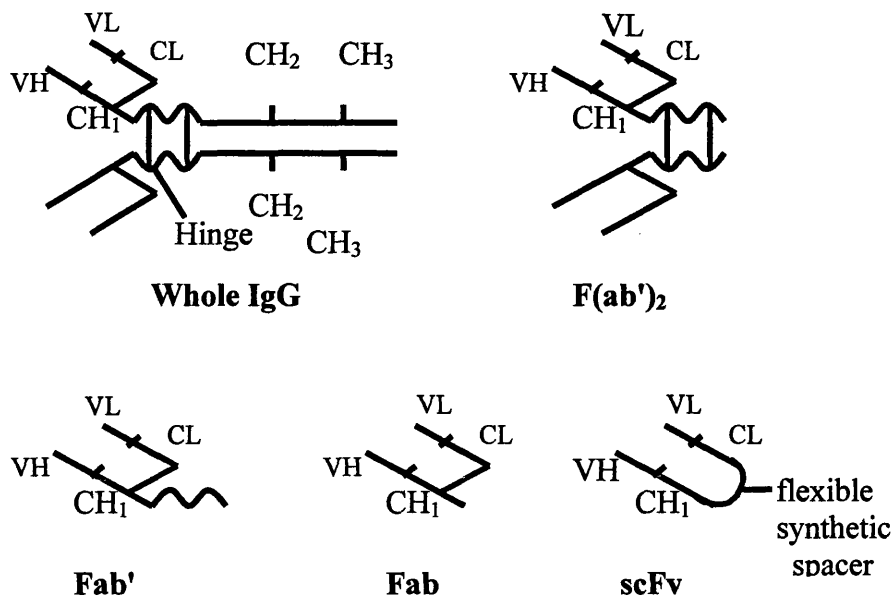
CEA was one of the first target antigens for radioimmunolocalisation of colorectal and other tumours of epithelial origin. Targeted therapy against CEA on cancer cells initially used monoclonal antibodies raised against it. This approach has shown some

success for radioimaging and recently for radioimmunotherapy (Behr *et al.*, 1996; Behr *et al.*, 2002). However, there are limitations to their use due to their large size and cross-reactivity with various normal tissues (Nap *et al.*, 1992; Yokota *et al.*, 1992). These limitations result in part from the presence of effector or linking regions that are not required for antigen recognition.

This has led to the development of single chain (scFv) antibodies. scFvs consist of the antibody variable heavy (VH) and light (VL) regions linked by a short synthetic peptide to form a single molecule of 27 kDa (Huston *et al.*, 1988) (Figure 1.9). scFvs have better tumour penetration because they are less than one-fifth of the molecular weight of an IgG antibody, whilst retaining full specificity for the antigen. They have the further advantage that immunogenicity may be reduced, as protein that is not required for antigen binding is not included.

There has been particular interest in the development of scFvs against CEA. MFE-23, an scFv against CEA was selected using a combinatorial phage library of  $10^7$  scFvs generated from the cDNA from the spleen of mice immunized with CEA (Chester *et al.*, 1994a). MFE-23 has shown a higher affinity to CEA ( $K_d$  of 2.5nM) than a high-affinity monoclonal antibody ( $K_d$  of 25nM). The specificity of MFE-23 was examined against a range of human tissues and the only reactivity seen was weak and with normal colon. This is in contrast to many anti-CEA monoclonal antibodies, which react against various normal tissues. The ability of MFE-23 to target CEA was initially shown by localization of radiolabelled scFv to human colorectal tumour xenografts in nude mice (Chester *et al.*, 1994a). A subsequent human clinical study, performed in 10 patients with CEA positive tumours, showed that MFE-23 had a more efficient tumour targeting capacity than the high-affinity monoclonal antibody (Begent *et al.*, 1996). In this study MFE-23 located all known tumour deposits and demonstrated advantages over current imaging technology (computerised tomography).

One potential clinical use for scFvs is antibody targeted therapy. Antibody directed enzyme prodrug therapy (ADEPT) is a two-step system with the potential to treat solid tumour deposits. The targeted antibody (or scFv) delivers an enzyme selectively to a tumour deposit. When there is a high tumour to plasma ratio of the enzyme a non-



**Figure 1.9. The structure of IgG and its fragments.**

The blue lines indicate disulphide bridges.

Adapted from (Huston *et al.*, 1988).



toxic prodrug is administered. The enzyme (localised to its tumour target) catalyses the prodrug to a cytotoxic agent at the tumour sites. MFE-23 has been successfully linked to the enzyme carboxypeptidase G2 (CPG2). This enzyme can cleave glutamic acid from a variety of prodrugs to release potent nitrogen mustards. It has been shown that the MFE-23:CPG2 fusion protein successfully localises tumour deposits giving high tumour; normal tissue ratios of CPG2 in human colon carcinoma xenografts in nude mice (Bhatia *et al.*, 2000).

### **1.8.3 Retroviral targeting to tumour antigens by envelope modification**

Targeting to tumour antigens using scFvs provides an attractive option for altering retroviral tropism by modification of the retroviral envelope glycoprotein. Insertion of an scFv against CEA into the Moloney MLV envelope has been attempted as a way of achieving host range extension (Konishi *et al.*, 1998; Khare *et al.*, 2001). The chimeric envelopes were successfully incorporated into viral particles with binding to CEA expressing cells. MFE23 was fused to ecotropic MMLV and coexpressed with wild-type ecotropic envelope in one study achieving a titre of  $10^3$  (Konishi *et al.*, 1998). In another study a different scFv that recognises CEA was developed and fused to ecotropic MLV envelope and coexpressed with wild-type ecotropic envelope (Khare *et al.*, 2001). The titre was  $10^4$ , but co-centrifugation of virus and cells (2000 rpm for 2 hours) was used in addition to Polybrene to enhance infection.

This level of infection is disappointing when compared to viruses displaying the wild type envelope ( $10^{3-4}$  versus  $10^7$ ). However, the second retroviral vector showed promising efficacy when used to deliver a suicide gene to CEA-positive tumour xenografts in nude mice (Khare *et al.*, 2002). The subcutaneous administration of the retroviral vector directly to the xenografts produced tumour suppression with a 70% reduction in tumour weight for the treated group as compared to the control group.

As stated earlier these chimeras are probably unable to efficiently induce membrane fusion and subsequent penetration of the viral core into the cytosol. This is likely to reflect the fact that after binding the scFv is unable to signal the trigger for fusion and also that the human target cells do not express the natural viral receptor of the

backbone envelope. In order to overcome both of these problems we have developed envelope chimeras with an amphotropic backbone and also used a protease cleavage site to unmask the natural viral receptor after binding. These chimeras will be discussed in detail in subsequent chapters. The protease cleavage site chosen is one recognised by matrix metalloprotease 2 (MMP-2). The rationale for this is discussed below.

A combination of tumour antigen and protease targeting has been used to target amphotropic MLV to a melanoma antigen (high-molecular-weight-melanoma-associated antigen, HMWMAA) (Martin *et al.*, 1999). It used an scFv against HMWMAA, LMH2 (Kupsch *et al.*, 1995), linked to the amphotropic MLV envelope. A proline-rich hinge and MMP-2 cleavage site linked the two proteins. This targeted vector (LMH2/ProMMP previously named scLPMA) showed a preferential infection for HMWMAA-positive cells with a 1000-fold increase in titre over HMWMAA-negative cells. It was also able to selectively differentiate between a cell mixture of antigen positive and negative cells that were both shown to be protease positive. Experiments using human tumour xenografts in nude mice showed that targeting could be maintained *in vivo* using this model (Martin *et al.*, 2002). This targeting approach has several features that make it attractive for clinical gene therapy. The target cell surface must express both a specific antigen and a specific protease and this extra requirement provides an added degree of safety

## **1.9 Matrix metalloproteases**

The matrix metalloproteases (MMPs) constitute a multigene family of which 22 human homologues have been identified to date (Sternlicht and Werb, 2001). The enzymes are structurally related and take their name from a zinc atom in their active site. The enzymes range from the well-characterised interstitial collagenase (MMP-1), which degrades fibrillar collagens, to the more obscure membrane-type (MT-) MMPs whose functions and substrates are yet to be fully elucidated.

Extracellular proteinases are required for numerous developmental and disease-related processes. An ability to degrade extracellular proteins is essential for any individual cell to interact with its immediate surroundings. MMPs also regulate cell behaviour by

cleaving cell surface molecules and other non-matrix proteins. They play a central role in tissue remodelling associated with growth, development and repair. In these physiological processes MMP activity is tightly regulated, but aberrant expression can contribute to the pathogenesis of several diseases e.g. rheumatoid arthritis and tumour invasion and metastasis. The enzymes are secreted in an inactive proform with an amino-terminal domain blocking the active site (Kleiner and Stetler-Stevenson, 1993). In nearly all cases the removal of this domain and consequent activation occurs extracellularly. Once activated MMPs are tightly controlled by a group of inhibitors, 'tissue inhibitors of metalloproteinases' (TIMPS), of which four have been identified to date (Gomez *et al.*, 1997).

MMPs are generally present in greater amounts and activated more often in and around carcinomas than in normal, benign tissues, with the highest levels of expression seen in areas of active invasion at the tumour-stroma interface. Significant positive correlations have been found between MMP expression and various indicators of poor prognosis in many types of cancer including colorectal and gastric cancers (Liabakk *et al.*, 1996; Sier *et al.*, 1996). In some instances increased MMP levels have been shown to be an independent poor prognostic feature of both disease-free and overall survival. Several MMPs have been implicated in tumour invasion, metastasis and neoangiogenesis including MMPs 1, 2, 3, 9 and 14 (Sternlicht and Werb, 2001).

Thus, MMPs are a valid target for anti-cancer therapies. It has been shown that overexpression of TIMPs in tumours of various origins leads to reduced tumour growth and formation of metastases (McCormick, 2001). More recently, antitumour efficacy by *in vivo* gene transfer of TIMPs has been reported in several clinically relevant animal models. For example, in a nude mouse model of colorectal liver metastasis, overexpression of TIMP-2 in the liver using an adenoviral vector prior to, or following, tumour challenge resulted in 95% reduction in metastasis compared with controls (Brand *et al.*, 2000). As stated previously MMP can be used as a target for protease-activatable vectors, such as EGF-displaying amphotropic vectors with an (MMP) cleavage site, with selective infection both *in vitro* and *in vivo*.

## **1.10 Thesis aims**

The aims of this thesis were to explore the use of retroviral vectors based on amphotropic murine leukaemia virus to target tumour cells. Targeting was attempted by modification of the retroviral envelope. The aim of the work described in chapter 3 was to produce vectors that targeted tumour antigens by receptor cooperation. Work described in chapter 4 examined protease targeting of a specific tumour antigen (carcinoembryonic antigen) *in vitro* and *in vivo*. The ultimate aim of this work is to produce targeted retroviral vectors that would be suitable for use in clinical cancer gene therapy.

## **Chapter 2**

### **Materials and Methods**

#### **2.1 Plasmid preparation and manipulation**

##### **2.1.1 Preparation of heat shock competent *E. coli***

1 ml of an overnight culture of *E. coli* HB101 in Luria Broth (LB) (GibCoBRL) was subcultured into 100ml of fresh LB and cultured for 2 hours on a shaker at 37°C. The culture was then cooled on ice for 10 minutes and pelleted at 4°C and the supernatant then discarded. The pellet was resuspended in 30 ml ice-cold transformation buffer (250 mM PIPES, 2.5 mM CaCl<sub>2</sub>·2 H<sub>2</sub>O, 60mM KCl dissolved in 250 ml H<sub>2</sub>O, pH adjusted to 6.7 using KOH, then 55mM MnCl<sub>2</sub> is added to this solution). The bacteria were pelleted and resuspended in 10 ml cold transformation buffer containing 10% DMSO and frozen at -80°C in 100-200 µl aliquots.

##### **2.1.2 Introduction of plasmid DNA into *E. coli***

10-50 ng of plasmid were mixed with 50 µl of heat shock competent *E. coli* HB101 and incubated on ice for 10 minutes. The cells were then heat shocked for 90 seconds at 42°C, cooled on ice and pelleted. The pellets were resuspended in 20 µl of LB and plated on LB-agar + 50 µg/ml ampicillin (Sigma) and incubated at 37°C overnight.

##### **2.1.3 Plasmid DNA mini-preps**

Single colonies of *E. coli* HB101 were picked into 5 ml of LB supplemented with 50 µg/ml ampicillin and agitated at 37°C overnight. Mini-preps of plasmid DNA were produced from 4-5 ml overnight cultures of transformed bacteria using a Concert<sup>TM</sup> Rapid plasma DNA Mini-prep kit (GibCoBRL) as per the manufacturers instruction.

#### **2.1.4 Restriction enzyme digests**

All restriction enzymes were purchased from Promega. 0.5-2 µg of plasmid DNA was mixed with 1 µl of enzyme. Digests were performed at 37°C for 2 hours and 10 µl of the digest was mixed 6:1 with Orange G loading buffer then loaded on a 1 % TAE:agarose (SeaKem) gel containing 0.4 µg/ml ethidium bromide. Gels were run at 100V and observed under UV using a Syngene gel documentation system. 0.5 µg of a commercial DNA ladder (1 kb ladder; Life Technologies) was run as a marker.

#### **2.1.5 Isolation of DNA restriction fragments**

DNA fragments were isolated from bands cut from TAE/agarose gels using a Concert<sup>TM</sup> Rapid DNA fragment gel purification kit (GibCoBRL) as per the manufacturer's instructions.

#### **2.1.6 Sub-cloning of DNA fragments**

Isolated fragments were mixed at a ratio of 3:1 with vectors linearised by restriction enzyme digest. Vector and insert were mixed 1:1 with Takara DNA ligase solution and incubated at 15°C for 30 minutes. Ligation solutions were transformed by heat shock into *E. coli* HB101 and subsequently plated onto LB-agar + 50µg/ml ampicillin. Resultant colonies were mini-prepped and screened for insert and orientation by restriction digest.

#### **2.1.7 Plasmid DNA maxi-preps**

Plasmid DNA for transfection was prepared by polyethylene glycol extraction. Single colonies of *E. coli* HB101 carrying the desired plasmid were picked into 5 ml of LB supplemented with 50 µg/ml ampicillin (Sigma) and agitated at 37°C for 8 to 10 hours. 500 µl of bacteria was subcultured into 500 ml of LB + 50 µg/ml ampicillin and shaken at 37°C for 16 hours. Bacteria was then harvested by centrifugation (15 minutes at 4000g), washed in STE and resuspended in 10 ml of ice cold Solution I (50 mM glucose, 1mM EDTA, 10 mM Tris-HCL (pH 8.0)).

20 ml of Solution II (0.2 M NaOH, 1% SDS) was added and the cells inverted and left to lyse for 5 minutes at room temperature. The mixture was neutralised by adding 15 ml of Solution III (3M Sodium Acetate buffer (pH 6.0)) and left on ice for 15 minutes. Protein/SDS precipitates were removed by centrifugation at 10,000g for 15 minutes at 4°C. Pellets were washed once in 70% ethanol and re-suspended in 0.5ml of TE (pH 8.0).

An equal volume of cold 5M LiCl was added to precipitate RNA, the mixture was centrifuged as above and the supernatant transferred to an equal volume of isopropanol and the resulting precipitate harvested and washed as previously. The pellet was resuspended in 0.5 ml of TE pH 8.0 + 20 µg/ml RNase (Sigma) and incubated at room temperature for 30 minutes. Supercoiled plasmid DNA was precipitated by the addition of an equal volume of 1.6M NaCl, 20% PEG 8000 (Sigma) solution and harvested by microfuge centrifugation. PEG pellets were resuspended in 500 µl TE (pH 8.0), phenol:chloroform extracted twice, ethanol precipitated and resuspended in TE. DNA purity was assessed by the ratio of optical densities at 260 and 280 nm and was typically >1.75. Plasmids were checked by restriction digest, and aliquots diluted to 1 mg/ml and stored at -20°C.

#### **2.1.8 Plasmid DNA transfer into eukaryotic cells**

Plasmid transfections were carried out using the liposomal reagent LipoFectamine (Invitrogen) as per manufacturers instructions. Cells were seeded in 6-well plates such that on the day of transfection they were 60% confluent. On the day of transfection cells were washed gently with OptiMEM (GibCoBRL) and 800 µl of OptiMEM left in each well. A transfection mixture of 16 µl of plasmid DNA at a concentration of 0.1 µg/µl, 10 µl of OptiMEM, and 6 µl of LipoFectamine was incubated for 25 minutes at room temperature before being mixed with 170 µl of OptiMEM and added drop by drop to the cells. Cells were incubated at 37°C for 6 hours, after which time the cell supernatants were removed by aspiration and were replaced by normal medium. 48 hours later, transfected cells were analysed for gene expression or placed in selective medium.

## 2.2 Polymerase Chain Reaction

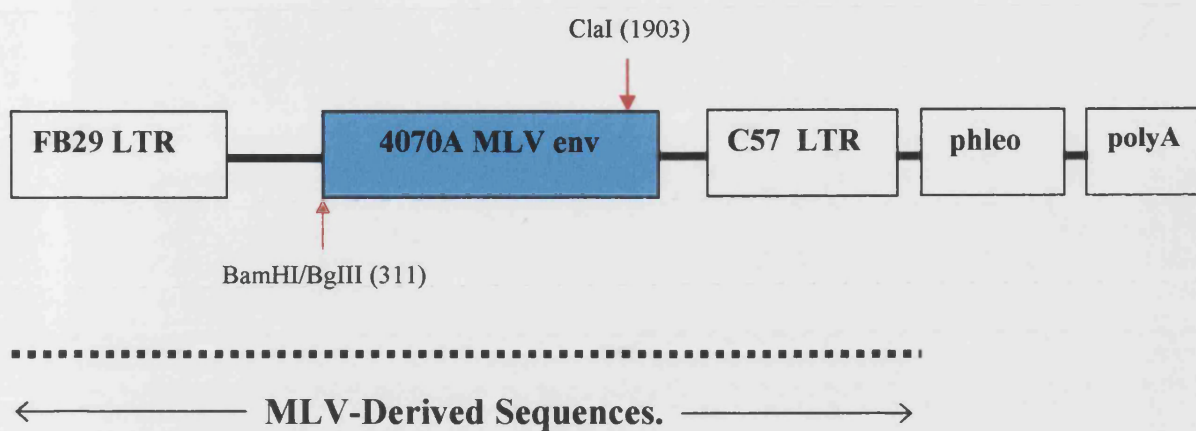
Polymerase chain reactions (PCRs) were set up in 50 µl volumes consisting of 10 µl of 10 x Ex Taq™ buffer (Mg<sup>2+</sup> concentration 20 mM (Takara Bio Inc)), 8 µl of dNTP mixture (2.5 mM each (Takara)), 0.2 µM of each primer (Sigma), template DNA, Takara Ex Taq™ (5units/µl) and sterilised distilled water up to 50 µl. PCRs were performed using a Hybaid multiblock system. Typical reaction conditions used an initial denaturation stage of 94°C for 60 seconds, followed by a 30 cycle stage of 94°C for 30 seconds, an annealing stage at the appropriate annealing temperature for 30 seconds with a polymerisation stage of 72°C for 30 seconds before a final incubation at 72°C for 5 minutes. Precautions were taken to avoid false positives including the use of appropriate controls for each PCR reaction, water controls, stringent preparation of all solutions and careful physical precautions.

## 2.3 Plasmids

The ALF plasmid (Figure 2.1), which had previously been constructed in the Collins lab (Cosset *et al.*, 1995b) is an envelope expression plasmid generated by linking the MLV-A *env* gene (Ott *et al.*, 1990) with the FB29 Friend MLV promoter (Perryman *et al.*, 1991). To generate ALF a *Bgl*II-*Cla*I fragment containing the *env* gene was cloned into the *Bam*HI and *Cla*I sites of plasmid FB3LPh (Cosset *et al.*, 1995b), which also contained the C57 Friend MLV LTR driving the expression of a phleomycin resistance gene (Gatignol *et al.*, 1988).

The chimeric envelope expressing plasmids were derived from plasmids designed initially to target the epidermal growth factor receptor (EGFR), which had previously been constructed (Cosset *et al.*, 1995a). Briefly, the EA plasmid contained a DNA fragment encoding EGF introduced at the N terminus of the 4070A surface domain (SU) (Cosset *et al.*, 1995a). EGFP4070A and EGFP24070A were constructed by inserting differing proline-rich spacers between EGF and the 4070A SU in the EA chimera. These Pro linkers were derived from the proline-rich region (Pro) of 4070A SU. This region separates the N-terminal receptor binding domain (RBD) and the C-terminal domain. Pro is the full proline-rich region, while Pro2 is a truncated version





**Figure 2.1 ALF envelope expression plasmid.**

Schematic diagram of ALF expression plasmid. The thick dotted line show MLV-derived sequences. The red arrows show the restriction sites of the respective enzymes.

FB29LTR, FB29 Friend MLV long terminal repeat; C57 LTR, C57 Friend MLV long terminal repeat; phleo, phleomycin resistance gene.

consisting of 9 amino acid that encompasses the first 2 turns of the predicted  $\beta$ -helix of full-length Pro (Valsesia-Wittmann *et al.*, 1997). Constructs expressing LMH2 (Kupsch *et al.*, 1995), an scFv which recognises high-molecular-weight melanoma associated antigen (HMWMAA), had been constructed in the Collins lab from the EGFPPro chimeras (Martin *et al.*, 1999). LMH2/Pro and LMH2/Pro2 were made by inserting the scFv coding sequence after EGF was removed by digestion with *Sfi*I and *Not*I. LMH2/ProMMP was constructed by insertion of the peptide Pro-Leu-Gly-Leu-Trp-Ala as an MMP cleavage site between the Pro linker and the env protein (Martin *et al.*, 1999).

To construct the MFE23 expressing plasmids the scFv that recognises CEA, MFE23, was removed from full length MFE23 (which includes a pelB leader peptide at its N-terminus and a C-terminal 11-residue Myc-tag: both removed) (Chester *et al.*, 1994a) by digestion with *Sfi*I and *Not*I. MFE23/Pro, MFE23/Pro2 and MFE23/ProMMP were made by inserting the scFv coding sequence into the respective plasmids LMH2/Pro LMH2/Pro2 and LMH2/ProMMP. This was after the removal of the coding sequence for LMH2 by digestion with *Sfi*I and *Not*I.

To construct CEA expressing plasmids the CEA coding sequence of 2,108 nucleotides was amplified by PCR from an expressed sequence tag (IMAGE 587714, from the UK human genome mapping project resource centre) homologous to the human carcinoembryonic antigen.

Primers used, forward: GCGCTGATCACCAGCCATGGAGTCTCCCCTCGCCCC.

Reverse: GCGCCTCGAGCTATATCAGAGCAACCCCAACC.

This fragment was subcloned into the *Bam*HI-*Xho*I sites of pcDNA 3.1(+) (Invitrogen) to give the plasmid pcDNA3.1-CEA. It was also subcloned into the *Bam*HI-*Xho*I sites of vector plasmid pHR'-CMVLacZ-IRESGFP (Naldini *et al.*, 1996b; Low *et al.*, 2001) to give the plasmid pHR'-CMVCEA-IRESGFP. A GFP expressing plasmid pHR'-CMVv-FLIP-IRESGFP was used as a control.

## 2.4 Cell culture

### 2.4.1 Cell lines

TELCeB6 cells are derived from the TE671 cell line (American Tissue Culture Collection, ATCC, CRL-8805) and harbour the MFGnslacZ genome and an MLV-Gag-Pol expression plasmid, CeB (Cosset *et al.*, 1995b). The A375 cell line is a human melanoma cell line (ATCC; CRL-1619). The HT1080 cell line is a human fibrosarcoma cell line (ATCC; CCL-121). The HT29 cell line is a human colonic adenocarcinoma cell line (ATCC; HTB-38). The Mawi cell line is a human colonic adenocarcinoma cell line (Baer *et al.*, 1993) (obtained from C. Porter, Chester Beatty Laboratories, Institute of Cancer Research, London). The 293T cell line is a human embryonic cell line that expresses the SV40 large T antigen (obtained from M. Calos, Stanford University, USA).

All cell lines used were maintained in Dulbecco's Modified Eagle Medium (DMEM) (GibcoBRL) supplemented with 10% Foetal Calf Serum (FCS), Penicillin (100 units/ml) and Streptomycin (100 µg/ml). Cells were grown at 37°C in a humidified incubator at 10% CO<sub>2</sub>. Upon confluence cells were washed in Hank's Balanced salt (HBBS) (Gibco™), detached in a minimal volume of Trypsin-EDTA (GibcoBRL) and replated at a concentration of 1/5 to 1/10 or at the desired density for transfection of infection. All tissue culture ware was purchased from Nunc.

Transient expression of CEA by 293T cells was achieved by LipoFectamine transfection of pHR'-CMVCEA-IRESGFP or pHR'-CMVv-FLIP-IRESGFP as described previously. 48 hours post-transfection, efficiency was checked by analysing the percentage of green cells, as both plasmids harbour green fluorescent protein (GFP). Cells were subsequently lysed for Western blotting to detect CEA or used as target cells for infection.

## **2.5 Construction of retroviral producer cell lines and infection with recombinant viruses**

### **2.5.1 Generation of stable producer cell lines**

Envelope expression plasmids MFE23/Pro2, MFE23/Pro, MFE23/ProMMP and ALF were transfected into TELCeB6 cells using LipoFectamine. 48 hours later transfected cells were selected using normal medium containing phleomycin (Cayla) (50 µg/ml) and pools of phleomycin-resistant clones were used for virus production. The TELCeB6-MFE23/Pro2 and TELCeB6-MFE23/ProMMP bulk populations were also cloned by serial dilution and the clone that produced the highest viral titer was identified.

### **2.5.2 Virus harvesting and concentration**

To harvest viruses, producer cells were grown at 37°C until they became confluent and then cultured at 32°C for 48 hours with feeding of fresh DMEM supplemented with 10% FCS every 24 hours. The medium was then replaced with serum-free OptiMEM and supernatant was collected 14 to 16 hours later. The harvested virus was filtered through 0.45 µm-pore size filters, and in some cases, concentrated by centrifugation at 2,500 x g and 4°C for 12 hours. Concentrated virus was kept frozen at -70°C.

### **2.5.3 Viral titration**

Target cells were seeded in 24-well plates at a density of  $5 \times 10^4$  cells/well 18 hours before infection. Viral supernatants were serially diluted in OptiMEM. The viral dilutions were either added neat or after incubation with Polybrene (4 µg/ml) or LipoFectamine (10 µg/ml). Cells were incubated in the presence of the viruses for 4 hours at 37°C. The cells were then washed once in OptiMEM and then cultured in DMEM-10% FCS for 48 hours.

Infected target cells were fixed by the addition of 0.5 ml of 2% glutaraldehyde, made from a 25% stock (Sigma) in phosphate buffered saline (PBS: 170 mM NaCl, 3.4 mM KCl, 10 mM monopotassium phosphate, 1.8 mM disodium phosphate), and incubated for 15 minutes at room temperature. Cells were washed in PBS before adding 0.5 ml of X-gal buffer (0.01% sodium deoxycholate, 0.02% Nonidet P-40, 2 mM MgCl<sub>2</sub>, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide made up in PBS) containing X-gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) at a concentration of 0.64 mg/ml and 20 mM Tris-HCl, pH 8.3 (to inhibit endogenous lacZ activity). A 40 mg/ml stock solution of X-gal was made in dimethylformamide (DMF) and stored at -20°C. Cells were incubated at 37°C for 2 hours for the substrate to be metabolised. Titers of the lacZ pseudotype were determined by counting the number of clusters of blue cells. Clusters of blue cells were attributed to a single infection and expressed as lacZ infectious units per millilitre (iu/ml) of virus supernatant.

Initial titres were determined using 1, 10 or 100  $\mu$ l of viruses. Subsequently 0.1, 1, 10, or 100  $\mu$ l of viruses were used depending on initial viral titer with the titer being in the linear range for the volume of viruses used in these experiments.

#### **2.5.4 Inhibition of infection by single chain antibody.**

To demonstrate that scFv targeted infection required initial binding to the tumour antigen blocking experiments were performed using either the relevant scFv or an irrelevant scFv control. The two scFvs used for the blocking experiments were LMH2, an scFv that recognises high-molecular-weight-melanoma-associated antigen (HMWMAA) and MFE23, an scFv that recognises carcinoembryonic antigen (CEA). Target cells expressing either HMWMAA or CEA were preincubated with 50  $\mu$ g/ml of LMH2 or MFE23 for 2 hours at 37°C. Viral infections were then performed in the presence of 50  $\mu$ g/ml of LMH2 or MFE23 and Polybrene (4  $\mu$ g/ml) for 4 hours at 37°C. After infection cells were washed once in OptiMEM and then cultured in DMEM-10% FCS for 48 hours and then X-Gal stained.

### **2.5.5 Inhibition of infection by receptor interference**

To show that Pit-2 was required for infection receptor interference experiments were performed on target cells. A375 and HT29 cells were infected with the replication competent amphotropic 1504 MLV strain derived from 293T-MLV-A cells (Hartley and Rowe, 1976). These cell lines were deemed to be 'chronically infected' after two weeks of passage from the addition of the above virus (Jobbagy *et al.*, 2000). Viral infections were then performed in the presence of Polybrene (4 µg/ml) for 4 hours at 37°C. In addition to the 4070A-enveloped and chimeric-enveloped viruses, MLV pseudotyped with the gibbon ape leukaemia virus (GALV) envelope glycoprotein was used. This virus was harvested from TELCeB6 cells that stably express the GALV envelope (Tailor *et al.*, 1993). After infection cells were washed once in OptiMEM, cultured in DMEM-10% FCS for 48 hours and then X-Gal stained.

### **2.5.6 Protease inhibition**

To demonstrate that protease cleavage was required for gene delivery by viruses expressing the MFE23/ProMMP envelope, infections were performed in the presence of tissue inhibitor of metalloproteinase (TIMP)-2 (Sternlicht and Werb, 2001). Viral infections were performed with TIMP-2 (at a final concentration of 5µg/ml) and Polybrene (4 µg/ml) for 4 hours at 37°C. After infection cells were washed once in OptiMEM, cultured in DMEM-10% FCS for 48 hours and then X-Gal stained.

### **2.5.7 Infection of mixed cell populations**

293T cells were transfected with pHR'-CMVCEA-IRESGFP or pHR'-CMVv-FLIP-IRESGFP (vector control). The efficiency of transfection was determined by analysing the percentage of green cells, as both plasmids harbour green fluorescent protein (GFP). The aim of transfection was to achieve an efficiency of 50% and thus a mixed cell population. This was achieved by titrating the amount of plasmid transfected and determined by counting four separate microscopy fields. 48 hours post-transfection, viral infections were performed in the presence of Polybrene (4 µg/ml) for 4 hours at 37°C. After infection cells were washed once in OptiMEM,

cultured in DMEM-10% FCS. On the same day as the infection the cell mixture was separated by fluorescent-activated cell sorting (FACS) using an Epics Elite (Coulter) flow cytometer into GFP-positive and –negative populations. The cells were replated, cultured in DMEM-10% FCS for 48 hours and then X-Gal stained.

## **2.6 Protein analysis**

### **2.6.1 Preparation of cell lysates**

CEA expression by target cells was determined from cell lysates. Target cells were incubated in NP-40 lysis buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris pH 8.0) at 4°C for 30 minutes with rocking every 5 minutes. The lysates were then removed and centrifuged for 10 minutes at 10,000 x g at 4°C to pellet the nuclei. 6x loading buffer (375 mM Tris-HCL (pH 6.8) containing 6% SDS, 30% 2-mercaptoethanol, 10% glycerol and 0.06% bromophenol blue) was diluted to 1x with the supernatant. Samples were heated to 95°C for 5 minutes and either processed immediately or stored at -80°C.

### **2.6.2 Preparation of viral supernatants**

Viral supernatant (8ml) was pelleted for analysis by ultracentrifugation in an SW41 Beckman Rotor (30,000 rpm for 1 hour at 4°C). Pellets were resuspended in 30 µl of 6x loading buffer diluted in OptiMEM. Samples were heated to 95°C for 5 minutes and either processed immediately or stored at -80°C.

### **2.6.3 Immunoblotting**

Supernatant and lysate samples (10-20 µl) were then run on 10-14% polyacrylamide (SDS) gels. After protein transfer using semi-dry transfer apparatus and transfer buffer (39 mM glycine, 48 mM Tris base, 20% methanol) onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech) total protein was stained with Ponceau S to ensure equal loading. Nitrocellulose membranes with blotted proteins were blocked for 1-16 hours by gentle agitation in blocking solution (PBS

containing 0.1% v/v Tween-20 and 5% w/v non-fat milk powder). Membranes were then incubated with the appropriate primary antibody (for details of antibodies used see table 2.1) diluted in blocking solution with gentle agitation at room temperature for 1 hour. The membranes were washed three times for 10 minutes in large volumes of PBS-T (PBS containing 0.1% v/v Tween-20). Bound first antibody was detected by addition of 10 ml of blocking solution containing a horseradish peroxidase (HRPO) conjugated antibody (Table 2.1) and gentle agitation at room temperature for 1 hour. The membranes were extensively washed as before and developed using enhanced chemiluminescence (ECL: Amersham Pharmacia) as per the manufacturers protocol.

**Table 2.1 Antibodies used**

<i>Antibody</i>	<i>Species</i>	<i>Concentration</i>	<i>Source</i>
Anti-human CEA	Mouse monoclonal	1/100	Dako ltd, United Kingdom.
Anti-Rauscher Leukaemia virus (RLV) gp70	Goat polyclonal	1/1000	Quality Biotech inc., Camden, N.J.
Anti-Rauscher Leukaemia virus (RLV) p30	Goat polyclonal	1/10000	Quality Biotech inc., Camden, N.J.
HRPO-conjugated Anti-mouse IgG	Rabbit polyclonal	1/1000	Dako ltd, United Kingdom.
HRPO-conjugated Anti-goat IgG	Rabbit polyclonal	1/1000	Dako ltd, United Kingdom.



#### **2.6.4 Analysis of envelope cleavage by gelatinase A**

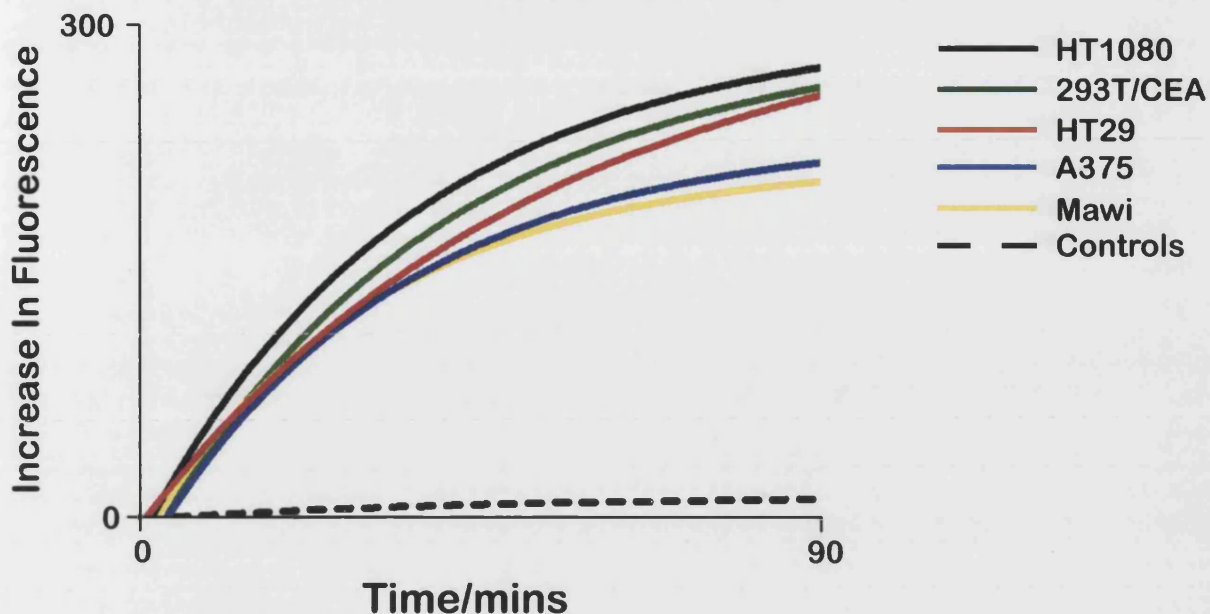
Accessibility of the MMP cleavage site, in the MFE23/ProMMP chimera, to protease cleavage was assessed by treatment of pelleted viruses with activated gelatinase A (Boehringer, Mannheim). Viruses were centrifuged at 100,000 x g for 1 hour at 4°C, resuspended in 50 µl of 100 mM Tris (pH 7.5), 200 mM NaCl, 1U activated gelatinase and then incubated for 6 hours at 37°C. The samples were then analysed by immunoblotting as described previously.

#### **2.7 Protease activity**

The protease activity of target cells and controls was determined using the fluorogenic peptide 2,4 dinitrophenol (DNP)-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH<sub>2</sub> (Bachem, Switzerland). This peptide contains an MMP cleavage site, which separates the DNP group (which acts as a quencher) from the tryptophan, leading to an increase in fluorescence with excitation at 280 nm. Target cells were grown to semi-confluency and then washed twice in buffer A (50mM Tris [pH 7.5], 10mM CaCl<sub>2</sub>, 0.2M NaCl). The DNP-peptide was diluted to 20 µM in buffer A, added to the different target cells and incubated at 37°C for 90 minutes. Samples were collected at 15-minute intervals and substrate hydrolysis was determined by monitoring the increase in fluorescence emission at 346nm using an excitation wavelength of 280nm. Controls were: peptide incubated at 37°C in the absence of target cells and peptide incubated with target cells at 4°C. Figure 2.2 shows the time course of peptide cleavage and hence increase in fluorescence for each cell line.

#### **2.8 Detection of cell surface CEA on target cells.**

Target cells were grown to confluence on 10 cm tissue culture plates. Cells were washed twice in PBS and detached after incubating at 37°C for 5 minutes in 5 ml of versene (GibCoBRL). The mixture was resuspended in PBS and then 10 µl of the concentrated cell suspension was added to each slide. The supernatant was removed and the slide was allowed to air dry at room temperature. PBS containing 1% w/v bovine serum albumin (BSA) was added to the slide and incubated for 15 minutes at



**Figure 2.2 Time course of protease expression by target cells.**

Target cells were incubated with dansylated peptide DNP-PLGLWADR-NH<sub>2</sub>. The cleavage of the peptide at its MMP site separates the DNP group (which acts as a quencher) from the tryptophan, leading to an increase in fluorescence with excitation at 280nm. Samples were collected at 15-minute intervals and substrate hydrolysis was determined by monitoring the increase in fluorescence emission at 346nm using an excitation wavelength of 280nm.

Controls were: peptide incubated at 37°C in the absence of target cells and peptide incubated with target cells at 4°C.

room temperature. Slides were washed once with PBS before incubation with the primary antibody A5B7 (mouse monoclonal antibody to carcinoembryonic antigen (CEA)) (Chester *et al.*, 1994b) diluted to 1/100 with PBS for 2 hours at 4°C. Slides were then washed twice in PBS and incubated with the secondary antibody (FITC-conjugated rat anti-mouse kappa light chain (Serotec)), diluted to 1/50 with PBS, for 2 hours at 4°C. After three washes with PBS coverslips were mounted on the cell samples with a drop of immunofluorescence mounting medium (Dako). Cells were analysed by confocal microscopy (MRC 1024, Bio-Rad equipped with krypton-argon laser). Pictures were acquired using Kalman filtration and analysed with lasersharp software (Bio-Rad) and Confocal Assistant/CAS software.

## **2.9 *In Vivo* Experiments**

### **2.9.1 Establishment of tumours**

Tumour xenografts from A375, HT1080, Mawi and HT29 cell lines were established in athymic female Balb/c nu/nu mice. Target cells were expanded, washed in HBBS and detached in a minimal volume of Trypsin-EDTA. Cells were washed twice with HBBS, counted, pelleted and then resuspended in the correct volume of HBBS to give a concentration of  $5 \times 10^6$  cells/0.2 ml HBBS. 0.2 mls of this cell mixture was injected subcutaneously into the left side of the abdomen.

### **2.9.2 Intratumoural injections**

When the tumours had reached 5-7 mm in diameter they were injected with lethally irradiated (40 Gy) producer cells (producing 4070A-enveloped, MFE23/ProMMP-enveloped or unenveloped vectors). The producer cells were expanded, washed in HBBS and detached in a minimal volume of Trypsin-EDTA. Cells were washed twice with HBBS, counted, pelleted and then resuspended in 50 mls HBBS prior to irradiation. Lethal irradiation was achieved by irradiating the cells with 40Gy at 4°C. After irradiation the cells were pelleted and then resuspended in the correct volume of HBBS to give a concentration of  $1 \times 10^6$  cells/0.1 ml HBBS. 0.1 ml of this cell mixture was injected directly into the tumours on 2 successive days. One week after the last

producer cell injection the tumours were excised, trimmed and disaggregated before incubation with 2 volumes of 5 mg/ml collagenase 1a (Sigma) for 2 hours at 37°C with occasional agitation. The separated tumour cells were pelleted, washed once in HBBS and resuspended in DMEM supplemented with 10% FCS. These suspensions were plated in 10cm tissue culture dishes. After 2 hours of incubation, nonadherent cells were discarded and adherent tumour cells were incubated overnight before staining for  $\beta$ -galactosidase expression. An average of  $10^6$  tumour cells were analysed for blue nuclear staining as an indication of tumour transduction.

### 2.9.3 Analysis of vector spreading

Nude mice were injected intraperitoneally with either  $10^7$  irradiated (40Gy) MFE23/ProMMP or unenveloped producer cells or  $10^6$  irradiated (40Gy) amphotropic producer cells (all three sets of irradiated producer cells were suspended in 0.2 ml of HBBS). The reason for the higher number of MFE23/ProMMP and unenveloped producer cells injected was to compensate for the higher titer seen with amphotropic vectors. Two weeks after injection the mice were sacrificed and the spleen, liver and kidneys were removed and snap frozen at  $-80^{\circ}\text{C}$ . DNA was extracted from 5-10 mg of tissue using a DNAeasy tissue kit (Qiagen) as per manufacturer's instructions. Proviral analysis was performed by nested PCR using primers that amplify a 480-bp DNA fragment located between the 3' end of the  $\beta$ -galactosidase gene and the 3' LTR of the integrated vector.

External primers were:

LacZ1 5'-GCACATGGCTGATATCGAACGG-3'

LTR1 5'-GCTTCAGCTGGTGATATTGTTGAG-3'

Internal primers were:

LacZ2 5'-ATTGGTGGCGACGACTCCTG-3'

LTR2 5'-AGCCTGGACCACTGATATCCTG-3'

PCR conditions were 35 cycles of 30 seconds at 94°C, 1 minute at 60°C and 1 minute at 72°C. For qualitative analysis, DNA was quantified by the ratio of optical densities at 260 and 280 nm in a spectrophotometer (Hitachi, U-1500). 10 µg of DNA (approximately 10<sup>6</sup> mouse cell equivalents) were used for the first PCR reaction (LTR1/LacZ1) and 1/10 of this reaction was used as template for the second round of the nested PCR (LTR2/LacZ2). Semi-quantitative PCR was carried out by serial dilutions of the DNA samples. Plasmid DNA harbouring one copy of the MFGnslacZ vector genome was used to determine the sensitivity of the system (1 plasmid = 0.1 fg).

## **Chapter 3**

### **Targeted retroviral infection by receptor cooperation**

#### **3.1 Introduction**

##### **3.1.1 Overview**

This chapter describes the construction and characterisation of retroviral vectors designed to target the tumour antigens high-molecular-weight melanoma associated antigen (HMWMAA) and carcinoembryonic antigen (CEA) by receptor cooperation. Targeted vectors were produced by insertion of single-chain antibodies (scFvs) in the normal retroviral surface protein (SU) of amphotropic murine leukaemia virus (MLVA). This introduction describes the initial studies examining receptor cooperation and the likely mechanisms that underlie it.

##### **3.1.2 Targeting by host-range restriction**

Gene therapy protocols would be greatly improved by the availability of targetable vectors that could deliver genes to specific target cells or diseases sites. Non-target cells should not be infected as gene delivery could be potentially harmful and would deplete the pool of vector particles. As unmodified retroviral vectors transduce a range of host tissues *in vivo* (Peng *et al.*, 2001; Martin *et al.*, 2002), cell surface targeting is an attractive way of achieving targeted transduction.

The host range of a retrovirus is partly determined by an amino-terminal domain of the envelope protein, responsible for receptor binding (Heard and Danos, 1991; Battini *et al.*, 1995). Amphotropic MLV can infect cells of many species because the envelope recognises an epitope present in a phosphate ion transporter (Pit-2) that is widely distributed (Miller *et al.*, 1994). This gives it the ability to infect a wide range of human target cells. The basis for host-range restriction is the introduction of modifications into the retroviral envelope glycoprotein that allow selective infection of target cells (Russell and Cosset, 1999). A particular advantage of this strategy of

targeting is that the natural virus entry pathway is exploited and thus fusion activation of the chimeric envelope is more readily achieved. There is also a potential safety advantage as the host-range properties of the virus are not expanded and thus the risk of developing a more pathogenic strain of the virus from which the vector is derived is decreased.

### 3.1.3 Receptor cooperation

The initial experiments concerning receptor cooperation were described by Cosset and colleagues (Valsesia-Wittmann *et al.*, 1996; Valsesia-Wittmann *et al.*, 1997). This group constructed envelope chimeras where the amphotropic 4070A Pit-2 binding domain was linked to the N terminus of the Moloney murine leukaemia virus (MMLV) SU using different proline-rich spacers. The resulting envelope chimeras have two receptor binding domains: the 4070A Pit-2 binding domain and the MMLV receptor for infection mCAT-1 (an amino acid transporter). They examined the role that a variety of different amino acid spacers, placed between the two binding domains, played in receptor binding and infection.

The linkers used were derived from the proline-rich region (Pro) of 4070A SU. This region separates the N-terminal receptor binding domain (RBD) and the C-terminal domain. Pro was initially thought solely to act as a flexible linker but it now appears to be a functional domain that influences receptor recognition, stabilises the SU-transmembrane (TM) protein interaction and affects virus fusogenicity (Andersen, 1994; Battini *et al.*, 1995; Lavillette *et al.*, 1998; Lavillette *et al.*, 2002). The full length proline-rich region is 59 amino acids long and predictive structural analysis suggests it is organised as a  $\beta$ -turn polyproline consisting of 11  $\beta$ -turns (Valsesia-Wittmann *et al.*, 1997). Truncated proline-rich spacers were constructed to encompass the first 2-4 predicted  $\beta$ -turns respectively (Valsesia-Wittmann *et al.*, 1997).

The chimeric envelope with no spacer between the two binding domains was able to infect cells that expressed either receptor alone or both receptors together (Valsesia-Wittmann *et al.*, 1997). The level of infection was considerably lower (approximately 10,000-fold) than that seen with the wild-type envelopes on cells that expressed both

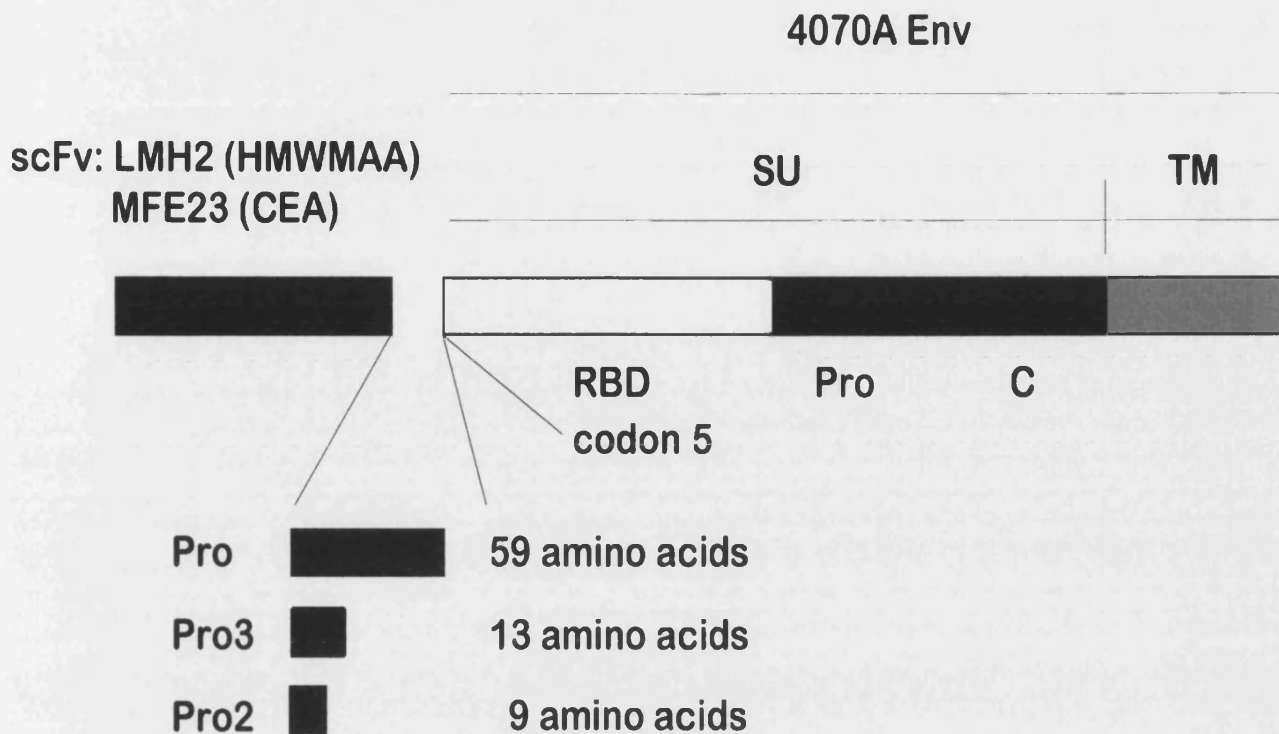
receptors (Cearl 13). The insertion of the differing proline-rich linkers was shown to affect viral tropism and efficiency of infection. As the length of the proline-rich linkers increased the MMLV mCAT-1 binding domain becomes increasingly masked with at least three to four  $\beta$ -turns required to mask the MMLV backbone. With the full-length 59 amino acid proline-rich linker this masking becomes complete and only cells expressing both receptors are permissive to infection. The fact that both receptors are needed to allow infection implies that after binding to Pit-2 the envelope undergoes a conformational change that reveals the mCAT-1 binding domain and permits infection. The level of infection remains lower than seen with wild-type envelope (approximately 500-fold) implying that the conformational change that occurs following binding to Pit-2 is not 100% efficient. If the arrangement of the binding domains is reversed i.e. MMLV mCAT-1 binding domain is fused to the 4070A envelope backbone a similar situation of receptor cooperation is seen although it appears to be less stringent (Valsesia-Wittmann, 2001).

These experiments showed that the composition, length and flexibility of the interdomain spacer played a crucial role in determining two-step receptor cooperation. This mechanism of entry that utilises the natural virus entry pathway is attractive for the design of retroviral vectors. Initial attempts to target 4070A amphotropic vectors to tumour antigens fused an scFv directly to the 4070A MLV-SU with no connecting linker (Martin *et al.*, 1998). Although this vector showed preferential infection of cells that displayed both the tumour antigen and Pit-2 masking of the Pit-2 RBD was not achieved. The lack of stringency seen with this model has resulted in it not being developed further. The use of proline-rich linkers to link the scFv to the 4070A RBD may allow true receptor cooperation, i.e. the requirement of both cell surface receptors to allow infection. This chapter describes the construction and characterisation of such vectors targeted to tumour antigens by receptor cooperation.

### **3.2 Construction of targeted envelopes**

MFE23 (Chester *et al.*, 1994a), a single-chain antibody (scFv) that recognises carcinoembryonic antigen (CEA) was fused to codon 5 of the mature amphotropic 4070A surface domain (SU) of the envelope glycoprotein by proline-rich linkers





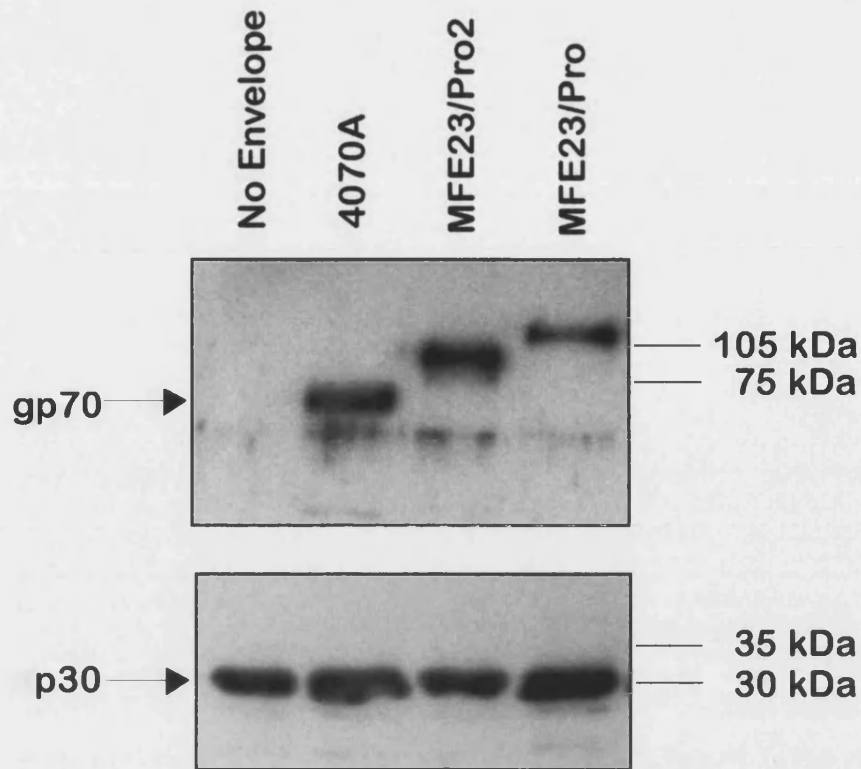
**Figure 3.1. Construction of targeted envelopes.**

LMH2 and MFE23 scFvs recognising HMWMAA and CEA were fused to the N terminus of amphotropic 4070A MLV-SU by using three spacers derived from the Pro of 4070A SU. The Pro spacer contains all 59 amino acids of Pro, Pro2 and Pro3 are truncated versions that encode the first two or three predicted B-turns of Pro. The spacers were introduced in the +5 position of the 4070A envelope.

RBD, receptor binding domain; TM transmembrane; C Carboxy terminal

(Figure 3.1). LMH2 (Kupsch *et al.*, 1995), an scFv which recognises high-molecular-weight melanoma associated antigen (HMWMAA) had previously been fused to codon 5 of 4070A SU using proline-linkers (Martin *et al.*, 2003) (Figure 3.1). These linkers were derived from the proline-rich region (Pro) of 4070A SU. This region separates the N-terminal receptor binding domain (RBD) and the C-terminal domain. Pro is the full proline-rich region, while Pro2 and Pro3 are truncated versions consisting of 9 and 13 amino acids respectively that encompass the first 2 or 3 predicted  $\beta$ -turns (Valsesia-Wittmann *et al.*, 1997).

Plasmids expressing the different envelopes or a 4070A envelope expression plasmid (ALF) (Cosset *et al.*, 1995b) were transfected into TELCeB6 cells that harbour the MFGnslacZ vector genome and a murine leukaemia virus (MLV) Gag-Pol expression plasmid, CeB (Cosset *et al.*, 1995b). Transfected cells were then selected with phleomycin and supernatant from pools of phleomycin-resistant clones were analysed. In order to demonstrate the incorporation of the chimeric envelope glycoproteins into retroviral particles, supernatants of the various TELCeB6-transfected cell lines were ultracentrifuged to pellet viral particles. Pellets were then analysed by Western blotting for their Gag (p30 CA) and envelope (gp70) protein contents (Figure 3.2). The LMH2 chimeras had been previously analysed and been shown to have significantly less envelope found in the viral pellet when compared to that of 4070A (Martin *et al.*, 2003). As expected analysis of supernatant from non-transfected TELCeB6 cells showed no detectable envelope (labelled No Envelope in figure 3.2). The MFE23 chimeras all showed high levels of envelope expression in the viral pellets with env-to-capsid ratios at comparable levels to that of 4070A. This demonstrates that the insertion of MFE23 coupled to proline linkers at the N terminus of the MLV SU did not impair expression, processing and viral incorporation of the mutant envelopes. Envelope incorporation of the CEA-targeted chimeras appears to be better than that seen with other polypeptides inserted at the +5 position of MLV-A such as epidermal growth factor (EGF) (Cosset *et al.*, 1995a) or other scFvs such as LMH2 (Martin *et al.*, 1999; Martin *et al.*, 2003). This may reflect the fact that the MFE23 chimeras, unlike other N-terminally substituted envelopes, allow correct folding and assembly into trimers with successful incorporation into retroviral particles.



**Figure 3.2. Targeted envelope incorporation in retroviral particles.**

MFE23-4070A chimera. Concentrated supernatants from TELCeB6 cells (No envelope) and TELCeB6 transfected with 4070A, MFE23/Pro or MFE23/Pro2 envelopes were separated on a 10% sodium dodecyl sulphate polyacrylamide gel, electroblotted, incubated with goat anti-Rauscher leukaemia virus SU (gp70) and anti-Rauscher leukaemia virus CA protein (p30) antisera followed by anti-goat horseradish peroxidase, and then developed with ECL (Amersham).

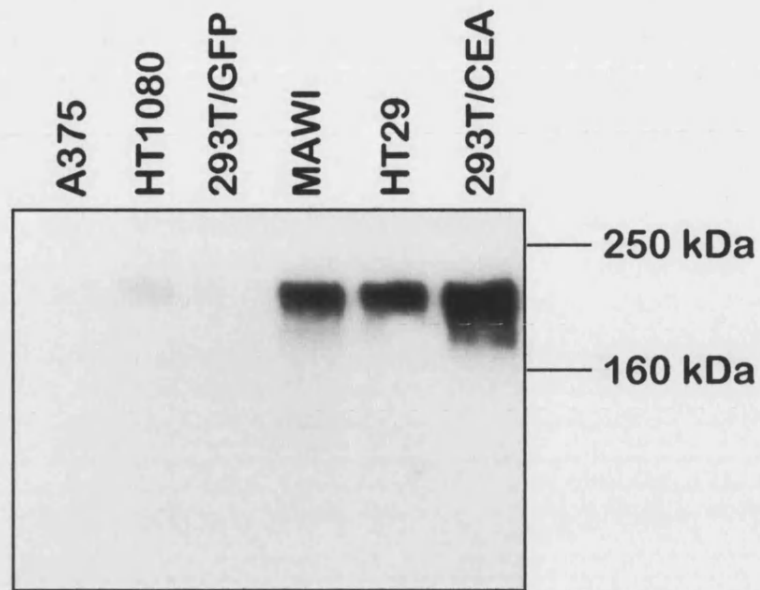
### 3.3 Carcinoembryonic antigen (CEA) expression

Target cells used to determine infection by MFE23 chimeras were two human colorectal cell lines (HT29 and Mawi), a human melanoma cell line (A375), a human fibrosarcoma cell line (HT1080) and a human embryonal kidney cell line (293T) transfected with pHR'-CMVCEA-IRESGFP or an irrelevant vector control (pHR'-CMVv-FLIP-IRESGFP). CEA expression of these target cells was characterised by Western blotting of cell lysates using a monoclonal mouse anti-human CEA antibody (Figure 3.3).

CEA is a glycoprotein with a molecular mass ranging from 180-200 kDa (Hammarstrom, 1999). The cell lysates from both colorectal carcinoma cell lines show CEA is highly expressed by these cells. The 293T cells transfected with the CEA expressing plasmid pHR'-CMVCEA-IRESGFP also highly express CEA at a similar if not higher level than that of the colorectal carcinoma cell lines. HT1080, A375 and 293T transfected with the vector control all showed no evidence of CEA expression.

Analysis of CEA by Western blotting of cell lysates determines total expression of CEA by the cells concerned. Tumour antigen targeting by altering retroviral envelope tropism relies on interactions at the cell surface between the chimeric envelope and the new target, the tumour antigen. This system is thus dependent on differential expression of tumour antigens at the cell surface. CEA expression at the cell surface was determined by immunostaining with an anti-CEA antibody A5B7 (Figure 3.4). The differences seen in CEA expression by Western blotting were confirmed by immunostaining. HT29 and A375 are shown as representative positive and negative cell lines respectively.

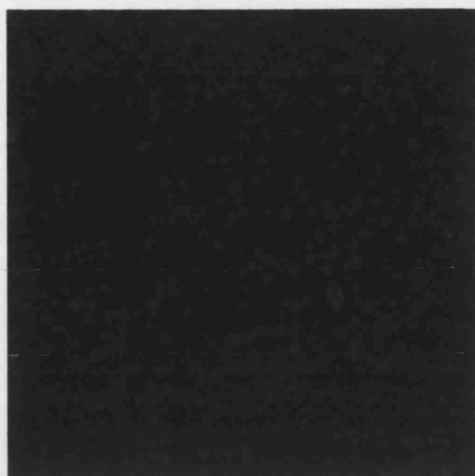
Expression of HMWMAA on the target cells had previously been determined using LMH2 antibody and fluorescence-activated cell sorting (Kupsch *et al.*, 1995). This showed that A375 (human melanoma cell line) and TE671 (human rhabdomyosarcoma cell line) were HMWMAA positive where as B1 (human melanoma cell line) and ECV (human endothelial cell line) were HMWMAA negative (Martin *et al.*, 1999).



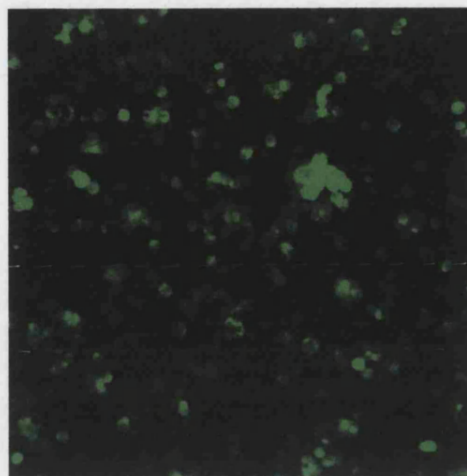
**Figure 3.3. Detection of carcinoembryonic antigen (CEA).**

Target cells were lysed and then run on a 10% sodium dodecyl sulphate-polyacrylamide gel, electroblotted, incubated with a monoclonal mouse anti-human CEA antibody (Dako) followed by anti-mouse horseradish peroxidase (Dako) and then developed with ECL.

**A.**

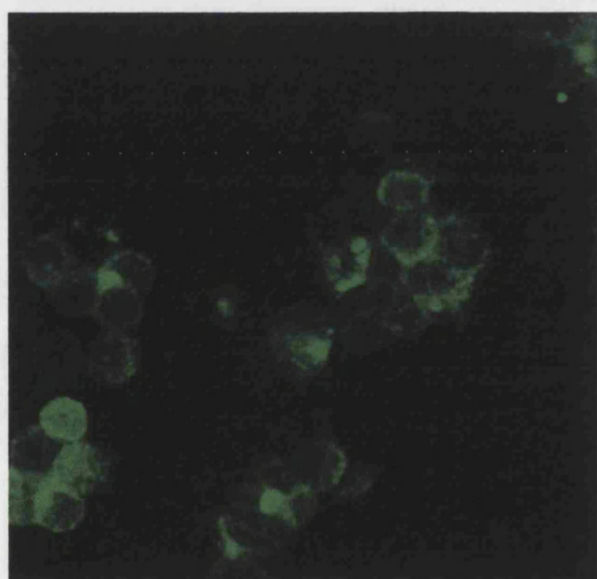


**A375**



**HT29**

**B.**



**HT29**

**Figure 3.4 Surface expression of CEA.**

A. Immunostaining of A375 and HT29 using anti-CEA antibody A5B7 (magnification x 10).

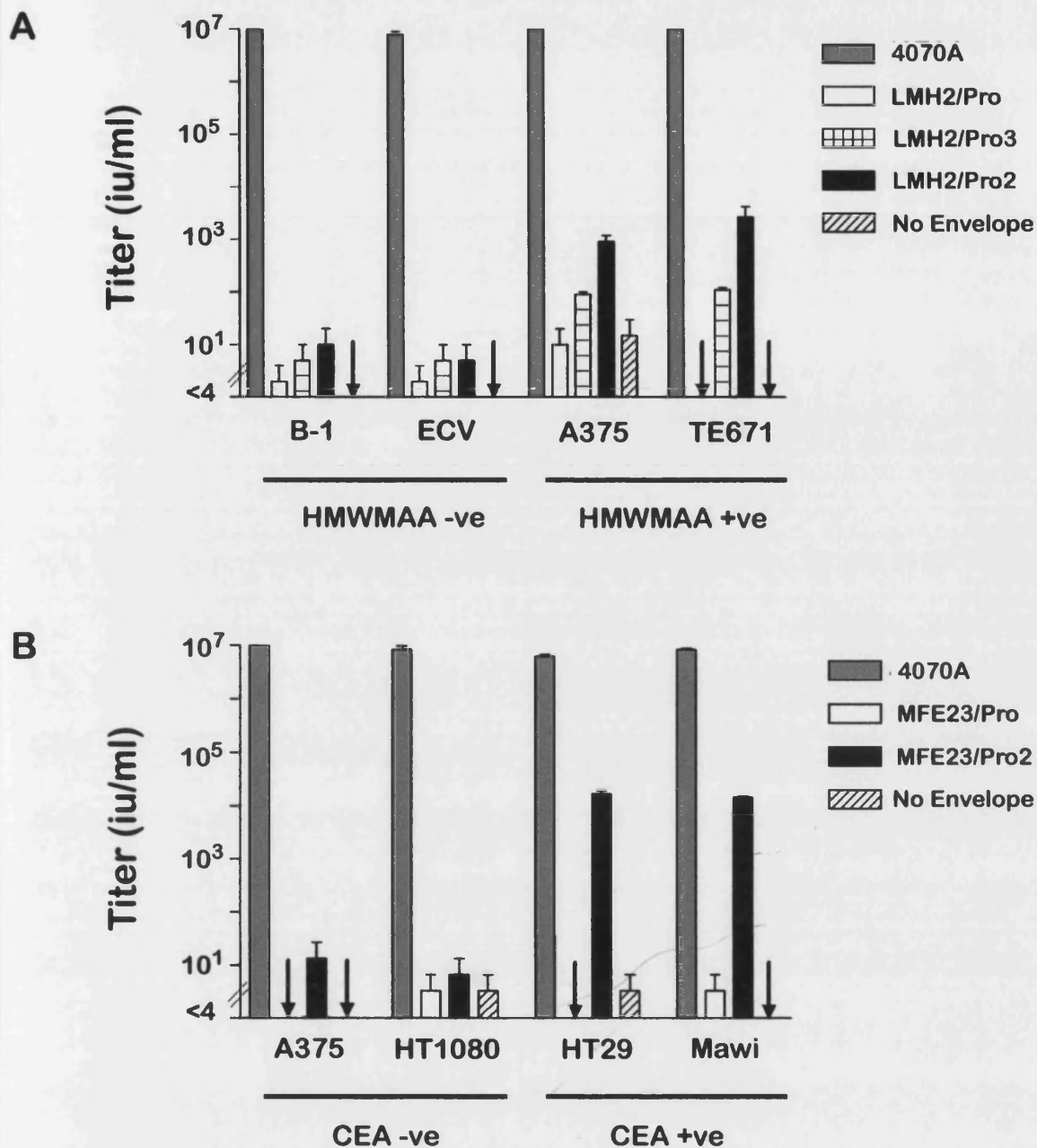
B. Magnified image of HT29 (x 40).

### 3.4 Targeted infection

Viruses were harvested from the selected producer cells, in some cases concentrated at 2,500 x g at 4°C for 12 hours and titered on the respective cell lines either immediately or having been frozen at -70°C. Figure 3.5 shows infection by viruses incubated with target cells for 4 hours at 37°C in the presence of Polybrene and then washed and analysed by X-gal (5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside) staining after 48 hours (Takeuchi *et al.*, 1994). Figure 3.5A shows that viruses with LMH2 chimeric envelopes and the shorter proline-linkers, Pro2 or Pro3, were able to selectively infect HMWMAA-positive cells. The higher titer was seen with LMH2/Pro2, has the shorter linker, 9 amino acids encompassing 2 predicted B-turns, and was at least 10-fold more infectious on HMWMAA-positive cells than LMH2/Pro3. LMH2 with the full-length proline linker, LMH/Pro, was not infectious. Its titer was less than 10 on all 4 cell lines and was even lower than virus with no envelope (supernatant from TELCeB6 cells). LMH2/Pro2 gave the best titer, up to 1000 IU/ml unconcentrated and was approximately 100-fold more infectious on HMWMAA-positive cells. Viruses with the wild-type 4070A envelope were able to infect all 4 cell lines non-selectively at high-titer (ranging from 10<sup>6</sup> IU/ml to 10<sup>7</sup> IU/ml depending on cell line and infection conditions).

The highest titer with MFE23 chimeric envelopes was also seen in the envelope with the shortest linker, MFE23/Pro2 (Figure 3.5B). MFE23/Pro2-enveloped vectors were able to selectively infect CEA-expressing cells, with over 10,000 IU/ml on CEA-positive cells and approximately 10 IU/ml on CEA-negative cells. Again the chimeric envelope with the full length-proline linker, MFE23/Pro, is not infectious with a titer of less than 10 on all 4 cell lines. The higher titer seen with MFE23/Pro2 than LMH2/Pro2 probably reflects a higher level of envelope incorporation into virions as reflected by Western blotting (Figure 3.2). Viruses with the wild-type 4070A envelope were able to infect all 4 cell lines non-selectively at high titer (ranging from 10<sup>6</sup> IU/ml to 10<sup>7</sup> IU/ml depending on cell line and infection conditions).

It appears from these experiments that the length of proline-linker is crucial in determining level of selective infection when using single-chain antibodies to alter the



**Figure 3.5 Titers of targeted viruses.**

A. Viruses with HMWMAA-targeted chimeras, 4070A or no envelope were added (with 4ug of Polybrene/ml) to HMWMAA -ve HMWMAA +ve cell lines as indicated.

B. Viruses with MFE23-targeted chimeras, 4070A or no envelope were added (with 4ug of Polybrene/ml) to CEA -ve or +ve cell lines as indicated.

Target cells were infected with serial dilutions of virus. Titer was calculated from values in the range where the number of infectious events was directly proportional to the volume of virus added (as discussed in Materials and Methods).



tropism of the MLVA envelope. We propose that the incorporation of the single chain antibody and proline-linker into the retroviral envelope masks the Pit-2 receptor binding domain of the wild-type 4070A allowing selective infection of target cells expressing the relevant tumour antigen. In this model the binding of the single-chain antibodies to their cell surface induces a conformational change within the shorter proline-rich linkers, most efficiently with Pro2, that reveals the 4070A Pit-2 receptor binding domain and allows subsequent fusion and infection. Thus, the shorter linkers are able to transiently mask the Pit-2 RBD, a phenomenon not seen with the full length Pro that appears unable to undergo such changes that would reveal the Pit-2 RBD after binding to the tumour antigen. It is clear that even the MFE23/Pro2 chimeric envelope is 100 fold less infectious than the wild-type 4070A envelope, which implies that the conformational change that follows scFv binding is not 100% efficient.

Table 3.1 shows that lipofectamine could enhance the efficiency of transduction of CEA-positive cells by MFE23/Pro2-enveloped virus sevenfold without affecting its specificity, as has been described for other scFv-targeted viruses (Martin *et al.*, 1999).

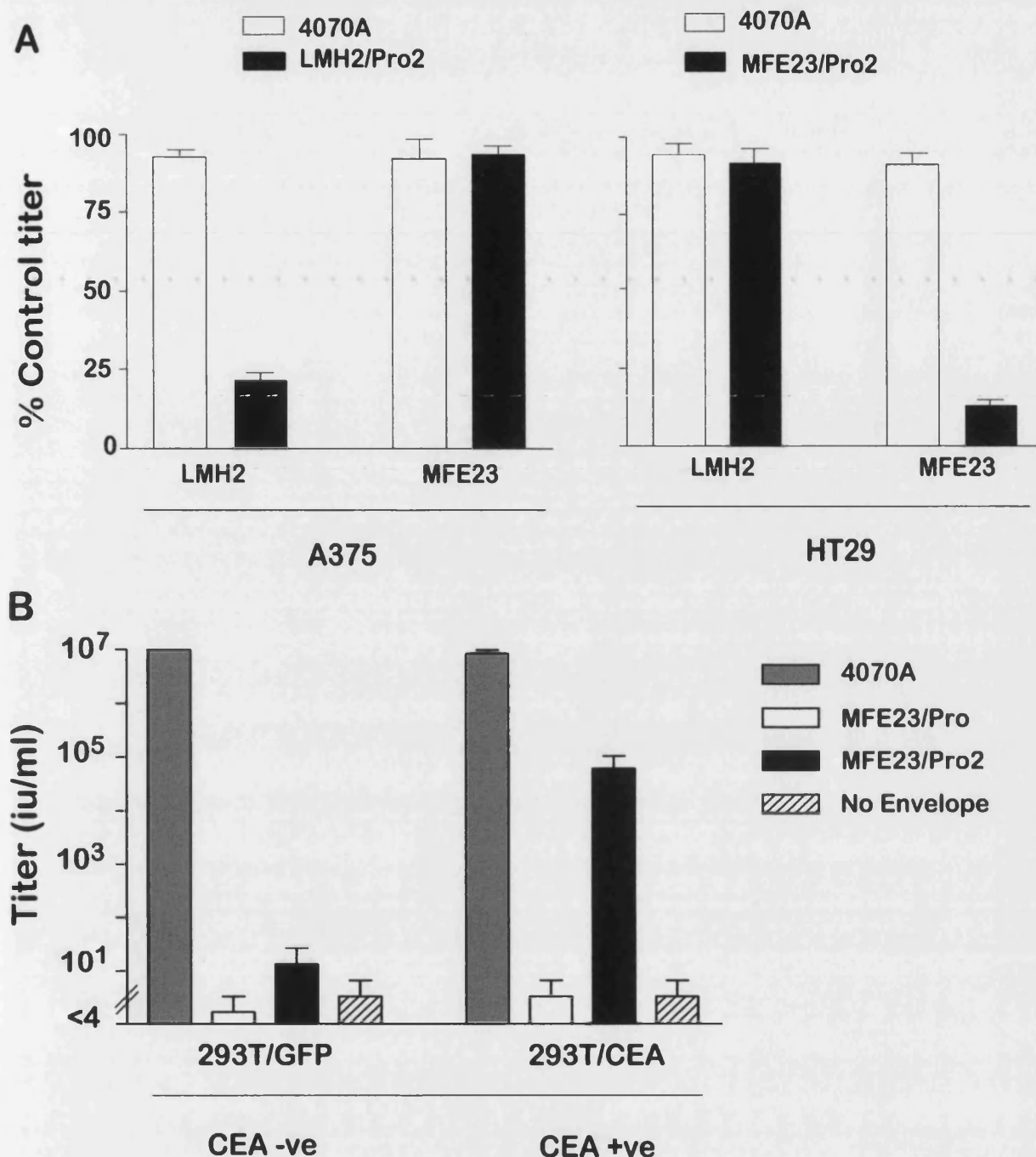
### **3.5 Requirement of tumour antigen for infection**

The mechanism of infection proposed above is reliant on the chimeric envelope binding to the relevant tumour antigen before undergoing conformational changes unmasking the Pit-2 RBD and allowing subsequent fusion and infection. To demonstrate that scFv targeted infection required initial binding to the tumour antigen blocking experiments were performed using either the relevant scFv or an irrelevant scFv control. Figure 3.6A shows that addition of the appropriate, but not the irrelevant, scFv could inhibit targeted infection. Inhibition of infection of LMH2/Pro2 by LMH2 to 20% of control titer was achieved. A slightly greater inhibition of MFE23/Pro2, 15% of control titer, was achieved using MFE23. Blocking of infection with the monovalent scFv is incomplete, presumably because of the higher avidity of viral binding. No significant effect on the titer of 4070A-enveloped viruses was seen showing that neither scFv affects the interaction of the 4070A RBD with its Pit-2 receptor.

	A375			HT29		
	Nil	PB	LIP	Nil	PB	LIP
No Env	<4	<4	$1.1 \times 10^2$	<4	$5.0 \times 10^1$	$1.2 \times 10^2$
MFE23/Pro	<4	<4	$9.0 \times 10^1$	<4	<4	$6.0 \times 10^1$
MFE23/Pro2	$1.1 \times 10^2$	$2.0 \times 10^1$	$1.3 \times 10^2$	$1.2 \times 10^4$	$1.7 \times 10^4$	$8.1 \times 10^4$
4070A	$6.0 \times 10^6$	$1.0 \times 10^7$	$1.4 \times 10^7$	$1.4 \times 10^6$	$6.3 \times 10^6$	$9.0 \times 10^6$

**Table 3.1 Enhancement of receptor cooperation.**

MFE23/Pro2 vectors were incubated with 4ug of Polybrene (PB)/ml or 10ug of lipofectamine (LIP)/ml or with no addition (Nil) for 10 min at room temperature before addition to the target cells. Results are indicated in IU/ml.



**Figure 3.6 Targeted infection requires tumour antigen.**

A. Antigen blocking. HMWMAA+ve (A375) and CEA+ve (HT29) were treated with LMH2 or MFE23 scFv prior to infection. Infection by virus with 4070A envelope and LMH2/Pro2 (left panel) or MFE23/Pro2 (right panel) was then measured. Titer is expressed as a percentage of that of untreated cells.

B. Transfection of CEA. 293T cells were transfected with a GFP or CEA expressing plasmid. 48 hours after transfection viruses with MFE23-targeted chimeras, 4070A or no envelope were added (with 4ug of Polybrene/ml) to each target cell.

Further evidence to support the requirement of CEA expression by target cells for infection by MFE23/Pro2 was provided by the infection of 293T cells transfected with pHR'-CMVCEA-IRESGFP or pHR'-CMVv-FLIP-IRESGFP (vector control). The efficiency of transfection was determined by analysing the percentage of green cells, as both plasmids harbour green fluorescent protein (GFP). Infection was carried out 48 hours post-transfection when transfection efficiency was greater than 90% as determined by counting four separate microscopy fields. Expression of CEA was confirmed by Western blotting of lysates of cells transfected with the relevant plasmids. Lysates were performed 48 hours post-transfection and confirmed that the 293T cells transfected with the CEA expressing plasmid pHR'-CMVCEA-IRESGFP expressed a high level of CEA comparable to that of CEA-positive colorectal carcinoma cell lines (Figure 3.3). 293T cells transfected with the vector control showed no evidence of CEA expression.

Figure 3.6B shows that the titer of MFE23/Pro2 was significantly increased (greater than 1000 fold) on 293T cells expressing CEA compared to 293T cells not expressing CEA. The titer of MFE23/Pro2 on the CEA-expressing 293T cells was similar if not greater than that seen on cells that endogenously express CEA (HT29 and Mawi). The titer of non-enveloped, 4070A- and MFE23/Pro-enveloped viruses was unaffected by transfection with either of the plasmids (Figure 3.6B). This shows that the differential infection seen with MFE23/Pro2 is dependent on CEA expression rather than any other feature of the colorectal cells or the transfection procedure itself.

### **3.6 Requirement of Pit-2 expression for infection**

The mechanism of infection proposed is also reliant on the RBD of the 4070A backbone of the envelope chimeras mediating infection via its Pit-2 receptor as explained previously. To show that Pit-2 was required for infection receptor interference experiments were performed. Receptor interference occurs in cells 'chronically infected' with one retrovirus. This masks or down-regulates the availability of receptors to subsequent infection by another virus that utilises the same receptors (Weiss, 1993). Although viral interference is believed to mainly occur at the plasma membrane due to competition for receptors, other sites such as the

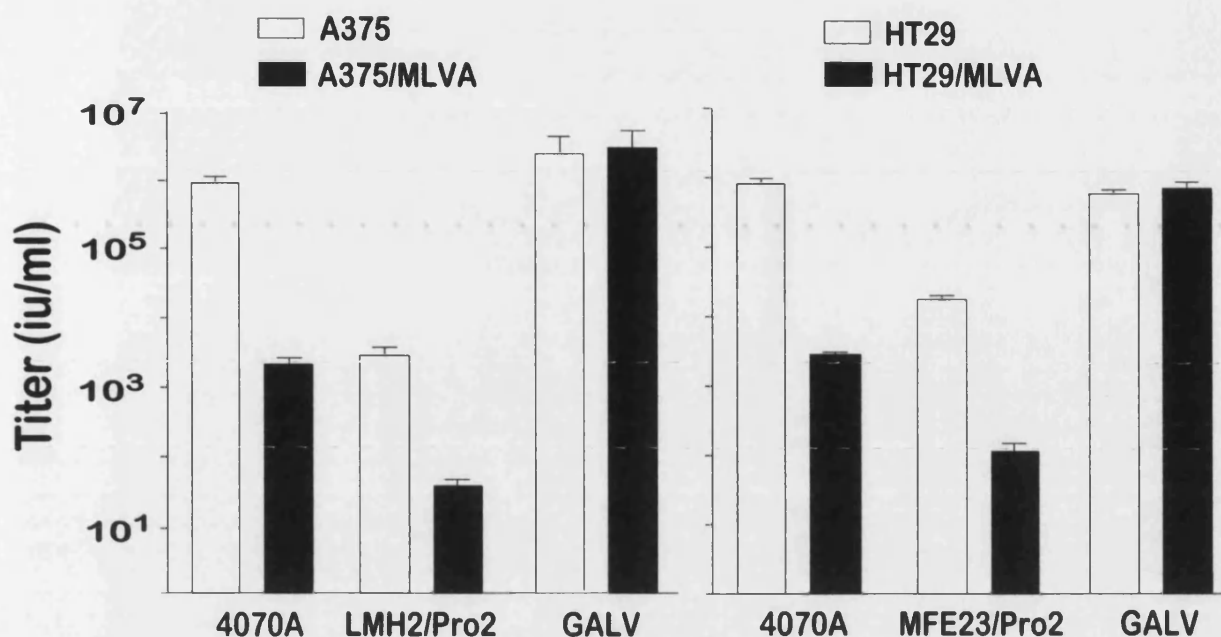
endoplasmic reticulum exist where disruption of translocation processes may decrease the number and availability of receptors on the host cell surface (Weiss, 1993).

Receptor interference assays were performed using A375 (HMWMAA-positive) and HT29 (CEA-positive) cells 'chronically infected' (two weeks of passage) with replication-competent 4070A MLV (Jobbagy *et al.*, 2000). Figure 3.7 shows that transduction of 4070A-infected A375 cells by LMH2/Pro2 and of 4070A-infected HT29 cells by MFE23/Pro2 was reduced by approximately 500-fold, similar to the reduction of infection by viruses carrying the unmodified 4070A envelope. The titer of amphotropic MLV pseudotyped with the GALV envelope, which recognises a different surface receptor Pit-1 (Lam *et al.*, 1996), was unaffected indicating that it is the effect on Pit-2 that affects the titer of the targeted viruses and viruses carrying the unmodified 4070A envelope.

### 3.7 Discussion

This series of experiments have shown that it is possible to achieve selective targeting to the tumour antigens HMWMAA and CEA by tropism restriction of amphotropic MLV. Infection is dependent on cells expressing both tumour antigen and Pit-2 on their cell surface, implying that receptor cooperation occurs. The titers achieved with the different envelope chimeras shows that the length of the interdomain proline-rich spacer has a crucial effect on infectivity. As stated earlier with no linker the Pit-2 RBD is not masked and the vector is able to infect both tumour antigen positive and negative target cells (Martin *et al.*, 1998). With the full-length Pro linker the level of infection is negligible and optimal infection for the chimeras is seen with the 9 amino acid Pro2. It appears that Pro2 is able to transiently mask the Pit-2 RBD, which becomes accessible after binding to the relevant tumour antigen via the scFv. Thus, an optimised interdomain spacer may diminish steric hindrance and favour conformational changes that allow infection after initial binding to the tumour antigen.

The titer achieved here for MFE23/Pro2 of  $10^4$  IU/ml is approximately 1 log higher than those reported in a previous study where MFE23 was fused to ecotropic MMLV and coexpressed with wild-type ecotropic envelope (Konishi *et al.*, 1998). Khare and



**Figure 3.7 Targeted infection requires Pit-2.**

Pit-2 blocking. HMWMAA +ve (A375) and CEA +ve (HT29) cells were infected with wild type amphotropic MLV. After 2 weeks A375, HT29, infected A375/MLVA and HT29/MLVA cells were infected with viruses carrying targeted (LMH/Pro2 or MFE23/Pro2) envelopes or 4070A or GALV envelopes and their titers were determined.

colleagues have also developed a chimera in which an scFv that recognises CEA is fused to ecotropic MLV envelope and coexpressed with wild-type ecotropic envelope (Khare *et al.*, 2001). The titer that they reported was similar to that of MFE23/Pro2, but co-centrifugation of virus and cells (2000 rpm for 2 hours) was used in addition to Polybrene to enhance infection.

The titer of MFE23/Pro2 is similar to that which was previously reported for virus targeted to HMWMAA by an MMP-cleavable scFv and Pro linker (Martin *et al.*, 1999). This virus gave reasonable infection (about 5%) in HMWMAA-positive tumour xenografts (Martin *et al.*, 2002), which suggests that MFE23/Pro2-targeted virus will be suitable for experiments in CEA-positive tumour xenografts. This is further supported by *in vivo* experiments from Khare and colleagues. They used the retroviral vector described above to deliver a suicide gene to CEA-positive tumour xenografts in nude mice (Khare *et al.*, 2002). The subcutaneous administration of the retroviral vector directly to the xenografts produced tumour suppression with a 70% reduction in tumour weight for the treated group as compared to the control group.

The level of targeted infection seen with MFE23/Pro2 is characteristic of approaches that use retroviral envelope interaction with its natural receptor to trigger efficient fusion. Previous strategies have included inverse targeting, where the cells that express the target molecule are not infectible (Cosset *et al.*, 1995a; Fielding *et al.*, 1998; Chadwick *et al.*, 1999), and protease targeting, where infection of the target cell requires cleavage of an incorporated domain by a protease (Nilson *et al.*, 1996; Martin *et al.*, 1999; Peng *et al.*, 1999). Both of these strategies are limited, either by the type of molecules that can be targeted (inverse targeting) or by the requirement for an active protease for infection (protease targeting). Targeting by receptor cooperation that relies only on the expression of a target molecule and a retroviral receptor may provide a more general approach.

Further modifications to improve efficiency could involve engineering scFvs to allow efficient envelope incorporation. The level of incorporation of MFE23 was acceptable but the level of LMH2 was suboptimal and almost certainly had a detrimental effect on infection. It has been demonstrated that receptor cooperation can be achieved using different non-viral  $\beta$ -spiral peptides to act as the interdomain linker (Valsesia-

Wittmann, 2001). Thus, modifications in composition and length of the  $\beta$ -turn helix may optimise conformational changes after initial binding. It is possible that point mutations introduced into 4070A envelope may improve its stability and thus improve transduction efficiency as has been demonstrated for the ecotropic MLV envelope (Zavorotinskaya and Albritton, 2001).



## **Chapter 4**

### **Retroviral infection by protease targeting**

#### **4.1 Introduction**

##### **4.1.1 Overview**

This chapter describes the construction and characterisation of retroviral vectors designed to target the tumour antigen carcinoembryonic antigen (CEA) by protease targeting. Targeted vectors were produced by insertion of a single-chain antibody (scFv) in the retroviral surface protein (SU) of amphotropic murine leukaemia virus (MLVA). The scFv was joined to the 4070A SU by a full-length proline linker and a matrix metalloprotease (MMP) cleavage site. This introduction includes an overview of protease targeting of retroviral vectors explaining the development of this approach as well as the rationale that underlies it.

##### **4.1.2 Protease targeting**

Protease targeting has become an important way of restricting the host-range of retroviral vectors. It is achieved by grafting a blocking domain onto the viral envelope protein via a linker containing a protease cleavage site. The block in infection is overcome by cleavage at this site, which removes the blocking domain and allows infection via the normal receptor pathway. By inserting protease cleavage sites for specific cell-surface proteases targeting may be directed to specific proteases on target cells rather than cell-surface receptors.

The choice of blocking domain used is important and two broad categories have been determined (Russell and Cosset, 1999). The first category consists of high affinity ligands that lead to viral sequestration. This was initially shown for vectors displaying epidermal growth factor (EGF) (Nilson *et al.*, 1996). Sequestration has subsequently been demonstrated for vectors displaying several other ligands such as stem cell factor (Fielding *et al.*, 1998), insulin-like growth factor (Chadwick *et al.*, 1999) and for antibodies against EGF receptor and the lymphocyte surface antigen CDw-52 (Russell

and Cosset, 1999). This category of blocking domain is not ideal for gene delivery as blocking only occurs on receptor-positive target cells. There is a high level of residual infection on non-target cells because the blocking domain does not completely stop the ability of the SU trimer to attach to its natural Pit-2 receptor. This can be confirmed for the EGF-displaying viruses, whose infectivity on EGF-receptor positive cells is restored by adding soluble EGF to block the EGF receptor, indicating that the displayed EGF domain does not sterically hinder attachment to Pit-2 (Cosset *et al.*, 1995a).

It has been shown that trimeric leucine zipper peptides and globular domains that are capable of forming homotrimeric interactions, such as tumour necrosis factor or the c-terminal domain of CD40 ligand, can efficiently block infection on both target and non-target cells (Morling *et al.*, 1997). These homotrimeric polypeptides form the second category of blocking domains. Binding assays show that these homotrimers are able to block interaction with the natural viral receptor and it is believed that they form a homotrimeric cap at the tip of the envelope glycoprotein to which they are grafted thus sterically blocking virus attachment (Morling *et al.*, 1997; Russell and Cosset, 1999). This mechanism does not depend upon competitive sequestration as blocking is seen on target cells that do not express the cognate receptors for the displayed domains. Thus, the block to retroviral infection is more generalised with these trimeric polypeptides and they would appear to be more useful for clinically relevant protease targeting.

Initial experiments to show proof of principle for protease targeting used vectors displaying a factor Xa cleavable EGF domain (Nilson *et al.*, 1996). These vectors showed low levels of infectivity on EGF receptor-positive target cells, but became fully infectious for the same target cells after they had been treated with exogenous factor Xa. Similar experiments showed that full infectivity could be restored following factor Xa treatment of vectors containing a factor Xa cleavable homotrimeric blocking domain, such as the trimeric C-terminal domain of the CD40 ligand (Morling *et al.*, 1997).

In principle, any protease that does not degrade the envelope glycoprotein can be targeted using this strategy and retroviral vectors, which could be activated by

plasmin and by furin have been demonstrated (Buchholz *et al.*, 1998; Russell and Cosset, 1999). This approach was expanded by developing vectors that displayed blocking domains that could be cleaved and activated by what was felt to be a more clinically relevant protease: endogenous membrane-associated matrix metalloproteases (MMP) (Peng *et al.*, 1997). The initial vectors linked EGF to the N-terminus of the 4070A SU via an MMP cleavable linker. In cell lines that expressed both the EGF receptor (EGFR) and MMPs on their cell surface, the vector was able to give a 100-fold higher titer than on EGFR-positive, MMP-negative cells. Subsequent vectors were developed using the C-terminal domain of the CD40 ligand again linked to the N-terminus of the 4070A SU via an MMP cleavable linker (Peng *et al.*, 1999). This vector was also able to preferentially infect MMP-positive cells. In mixed cell cultures both vectors were able to discriminate between the two different cell types with preferential infection of MMP-positive cells (Peng *et al.*, 1997; Peng *et al.*, 1999). This is important as it suggests that MMPs secreted by the target cell do not significantly activate the vector and that activation is localised to the surface of the target cell and mediated by membrane-bound MMPs. It also appears that a cell-bound vector particle will not dissociate from its target cell when proteolytically activated because of the multivalent nature of the virus-cell interaction. It seems unlikely that a cell-bound vector would be cleaved simultaneously at all its points of attachment and as envelope chimeras are sequentially cleaved they will interact with Pit-2 receptors on the cell to which the vector is already attached thus ensuring gene delivery to the targeted cell.

The EGF targeted vectors were subsequently shown to be able to discriminate *in vivo* between protease-rich and protease-poor tumour xenografts in nude mice after direct intratumoural injection (Peng *et al.*, 1999). These experiments are extremely important as they address the concern that protease activation in a tissue culture environment might have little relevance to the *in vivo* situation. It had been postulated that increased levels of protease expression *in vivo* might lead to non-specific cleavage or that protease activity might be negated by the local accumulation of natural protease inhibitors.

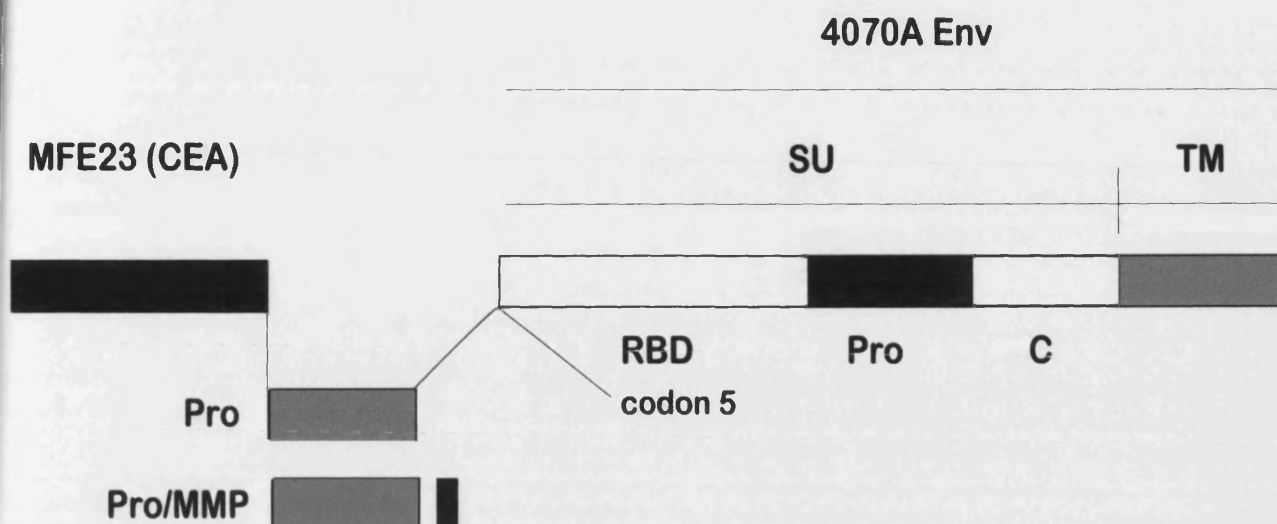
Protease targeting has been used to target specific tumour types by inserting different ligands into the retroviral envelope. Successful targeting of melanoma cells was

achieved by linking an scFv (LMH2 (Kupsch *et al.*, 1995)) that recognises high-molecular-weight melanoma associated antigen (HMWMAA) to the extreme N-terminus of 4070A. The full-length proline-rich spacer and a cleavage site for matrix metalloproteases was used to link the scFv to the SU (Martin *et al.*, 1999). The blocking domain (scFv, full-length proline-rich linker and MMP cleavage site) seen with this model acts similarly to the homotrimeric polypeptides described previously that block infection of non-target cells via the Pit-2 receptor binding domain. This targeted vector (LMH2/ProMMP previously named scLPMA) showed a preferential infection for HMWMAA-positive cells with a 1000-fold increase in titer over HMWMAA-negative cells. It was also able to selectively differentiate between a cell mixture of antigen positive and negative cells that were both shown to be protease positive. This targeting approach has several features that make it attractive for clinical gene therapy. The target cell surface must express both a specific antigen and a specific protease and this extra requirement provides an added degree of safety. This chapter describes the construction and characterisation of vectors designed to target carcinoembryonic antigen (CEA) using the protease targeting model of the vector described above (LMH2/ProMMP). This chapter will also describe *in vivo* experiments using this vector.

## 4.2 Construction of targeted envelope

To construct the envelope chimera targeted to CEA, MFE23 (Chester *et al.*, 1994a) an scFv that recognises CEA was fused to codon 5 of the mature amphotropic 4070A surface domain (SU) by the full-length (59 amino acid) proline-rich linker (Pro). The matrix metalloproteases (MMP) cleavage site PLGLWA (Ye *et al.*, 1995) was introduced between the Pro linker and the envelope protein (Figure 4.1). The envelope that this plasmid expresses is called MFE23/ProMMP.

Plasmids expressing the different envelopes or a 4070A envelope expression plasmid (ALF) (Cosset *et al.*, 1995b) were transfected into TELCeB6 cells that harbour the MFGnslacZ vector genome and a murine leukaemia virus (MLV) Gag-Pol expression plasmid, CeB (Cosset *et al.*, 1995b). Transfected cells were then selected with phleomycin and supernatant from pools of phleomycin-resistant clones were analysed. In order to demonstrate the incorporation of the chimeric envelope glycoproteins into



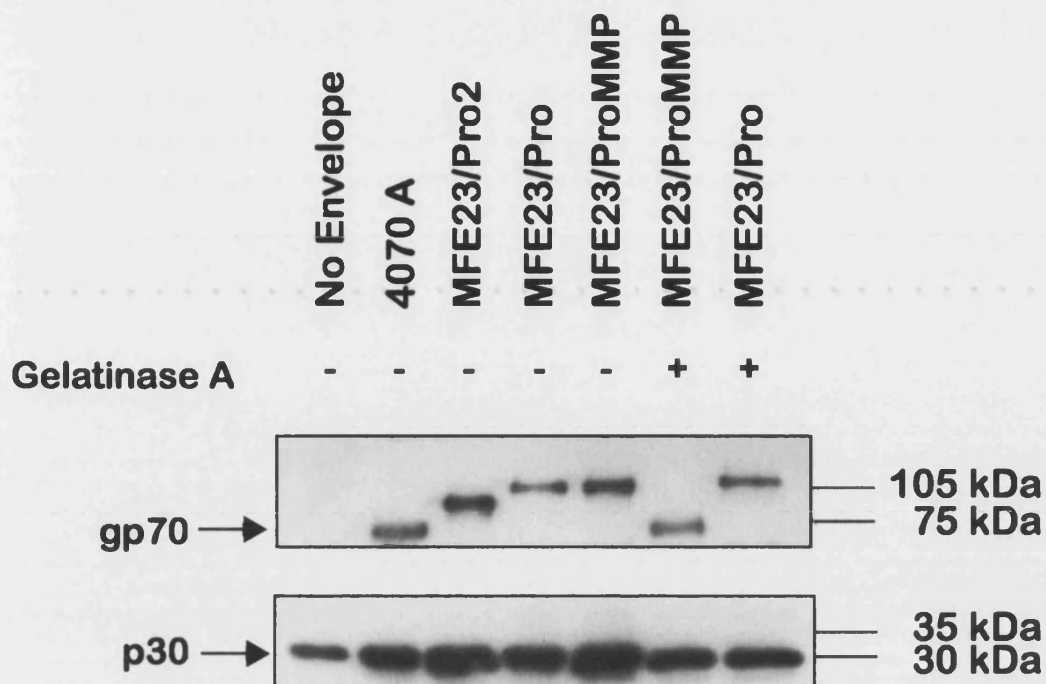
**Figure 4.1. Construction of targeted envelope MFE23/ProMMP.**

MFE23, an scFv recognising CEA, was fused to the N terminus of amphotropic 4070A MLV-SU by using the proline-rich spacer (Pro) derived from MLV-A 4070A SU. An MMP cleavage site was introduced between Pro and the SU of 4070A.

RBD, receptor binding domain; TM, transmembrane; C, carboxy terminal domain .

retroviral particles, supernatants of the various TELCeB6-transfected cell lines were ultracentrifuged to pellet viral particles. Pellets were then analysed by Western blotting for their Gag (p30 CA) and envelope (gp70) protein contents (Figure 4.2). As expected analysis of supernatant from non-transfected TELCeB6 cells showed no detectable envelope (labelled No Envelope in figure 4.2). The MFE23/ProMMP chimera showed high levels of envelope expression in the viral pellet with env-to-capsid ratios at comparable levels to that of 4070A. This demonstrates that the insertion of MFE23 coupled to full-length proline-linker and MMP cleavage site at the N terminus of the MLV SU did not impair expression, processing and viral incorporation of the mutant envelope. Envelope incorporation of this CEA-targeted chimera appears to be better than that seen with other polypeptides, that incorporate protease cleavage sites, inserted in the N-terminus of MLV-A. Examples of which include: epidermal growth factor (EGF) and C-terminal domain of the CD40 ligand both linked via an MMP-cleavable linker (Peng *et al.*, 1997; Peng *et al.*, 1999), where expression is lower than 4070A. The LMH2/ProMMP chimera had been previously analysed and shown to have significantly less envelope found in the viral pellet when compared to that of 4070A (Martin *et al.*, 1999). The higher level of envelope expression seen with MFE23/ProMMP may reflect the fact that the MFE23 chimera, unlike other N-terminally substituted envelopes, allows correct folding and assembly into trimers with successful incorporation into retroviral particles.

Protease targeting of MFE23/ProMMP is dependent on cleavage of the chimeric envelope to the 4070A backbone and subsequent infection via Pit-2. To assess whether the cleavage site was accessible to MMPs, MFE23/ProMMP viruses were incubated with activated gelatinase A. This treatment reduced the size of the chimeric envelope to the size of the unmodified SU (Figure 4.2), demonstrating that the MMP cleavage site was cleaved. Cleavage appears to be efficient with all of the chimeric envelope reduced to the size of the backbone SU. Incubation of MFE23/Pro, which lacks the MMP cleavage site, was unaffected by incubation with gelatinase A showing that this treatment doesn't degrade the envelope glycoprotein. LMH2/ProMMP treated with gelatinase A showed a similar result with the chimeric envelope successfully cleaved to the size of the unmodified SU (Martin *et al.*, 1999). However, some cleavage of LMH2/ProMMP was observed without gelatinase A, which was attributed to production of MMPs by the producer TELCeB6 cells. This is an



**Figure 4.2. Targeted envelope incorporation in retroviral particles.**

MFE23/4070A chimera. Concentrated supernatants from TELCeB6 cells (No envelope) and TELCeB6 transfected with 4070A, MFE23/Pro2, MFE23/Pro or MFE23/ProMMP envelopes were separated on a 10% sodium dodecyl sulphate polyacrylamide gel, electroblotted, incubated with goat anti-Rauscher leukaemia virus SU (gp70) and anti-Rauscher leukaemia virus CA protein (p30) antisera followed by anti-goat horseradish peroxidase, and then developed with ECL (Amersham).

The last 2 lanes show the effect of treatment of viral supernatants MFE23/ProMMP and MFE23/Pro with gelatinase A (as described in Materials and Methods).

important point as cleavage by the producer cells to the wild-type envelope will result in loss of targeting and infection of non-target cells. Thus, the absence of cleavage of MFE23/ProMMP without gelatinase A is potentially advantageous in maintaining the titer and specificity of the targeted virus.

### **4.3 Protease expression by target cells**

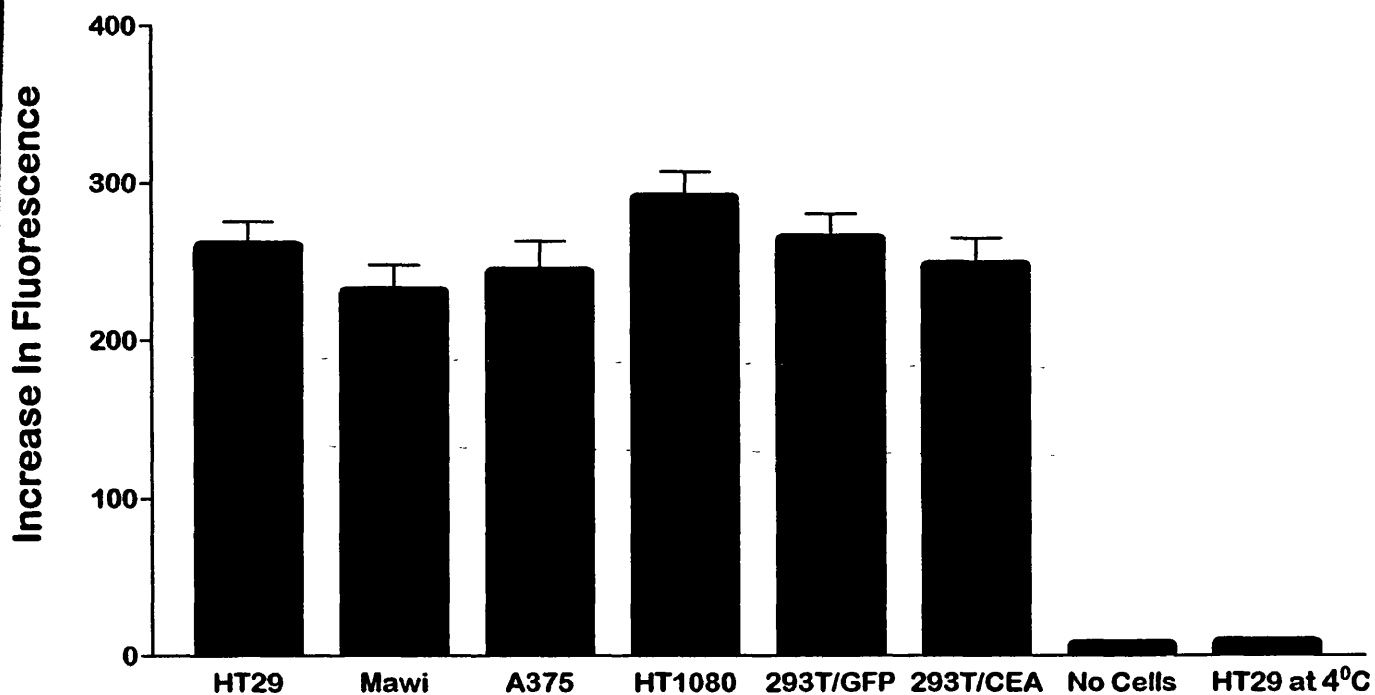
Target cells used to determine infection by MFE23/ProMMP were two human colorectal cell lines (HT29 and Mawi), a human melanoma cell line (A375), a human fibrosarcoma cell line (HT1080), a human embryonal kidney cell line (293T) transfected with pHR'-CMVCEA-IRESGFP or an irrelevant vector control (pHR'-CMVv-FLIP-IRESGFP). CEA expression by these target cells was characterised by Western blotting of cell lysates using a monoclonal mouse anti-human CEA antibody and by immunostaining with an anti-CEA antibody A5B7 (as described in chapter 3; Figures 3.3 and 3.4).

The level of MMPs capable of cleaving MFE23/ProMMP present at the surface of each target cell was determined using the dansylated peptide DNP-PLGLWADR-NH<sub>2</sub> (Stack and Gray, 1989). The cleavage of the peptide at its MMP site separates the DNP group (that acts as a quencher) from the tryptophan, leading to an increase in fluorescence with excitation at 280nm. A DNP-Peptide buffer was incubated with each of the target cells for 90 minutes at 37<sup>0</sup>C and the change in fluorescence measured. All of the cell lines showed similar levels of DNP-Peptide cleavage and hence MMP activity (Figure 4.3). Thus, any difference of infectivity on target cells was not caused by differences in protease expression. No change in fluorescence was seen in the absence of cells or without incubation at 37<sup>0</sup>C.

### **4.4 Targeted infection**

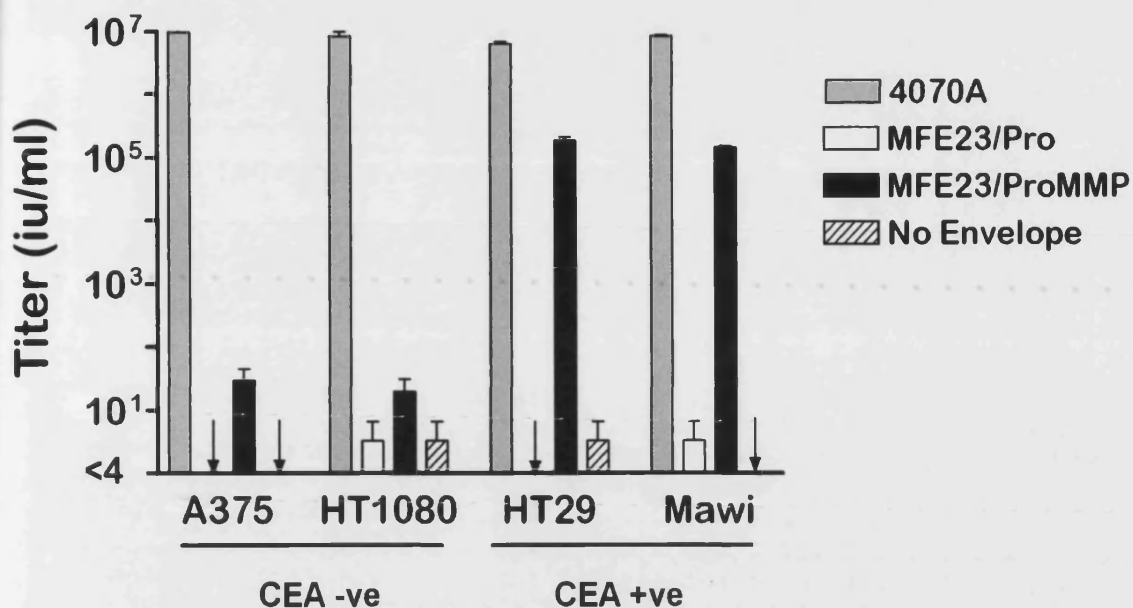
Viruses were harvested from the selected producer cells, in some cases concentrated at 2,500 x g at 4<sup>0</sup>C for 12 hours and titered on the respective cell lines either immediately or having been frozen at -70<sup>0</sup>C. Figure 4.4 shows infection by viruses incubated with target cells for 4 hours at 37<sup>0</sup>C in the presence of Polybrene and then washed and analysed by X-gal (5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside)





**Figure 4.3** Protease expression by target cells.

Target cells were incubated with dansylated peptide DNP-PLGLWADR-NH<sub>2</sub>. The cleavage of the peptide at its MMP site separates the DNP group (which acts as a quencher) from the tryptophan, leading to an increase in fluorescence with excitation at 280nm. Substrate hydrolysis was determined by monitoring the increase in fluorescence emission at 346nm using an excitation wavelength of 280nm.



**Figure 4.4 Titers of targeted viruses.**

Viruses with MFE23-targeted chimeras, 4070A or no envelope were added (with 4ug of Polybrene/ml) to CEA -ve or +ve cell lines as indicated.

Target cells were infected with serial dilutions of virus. Titer was calculated from values in the range where the number of infectious events was directly proportional to the volume of virus added (as described in Materials and Methods).

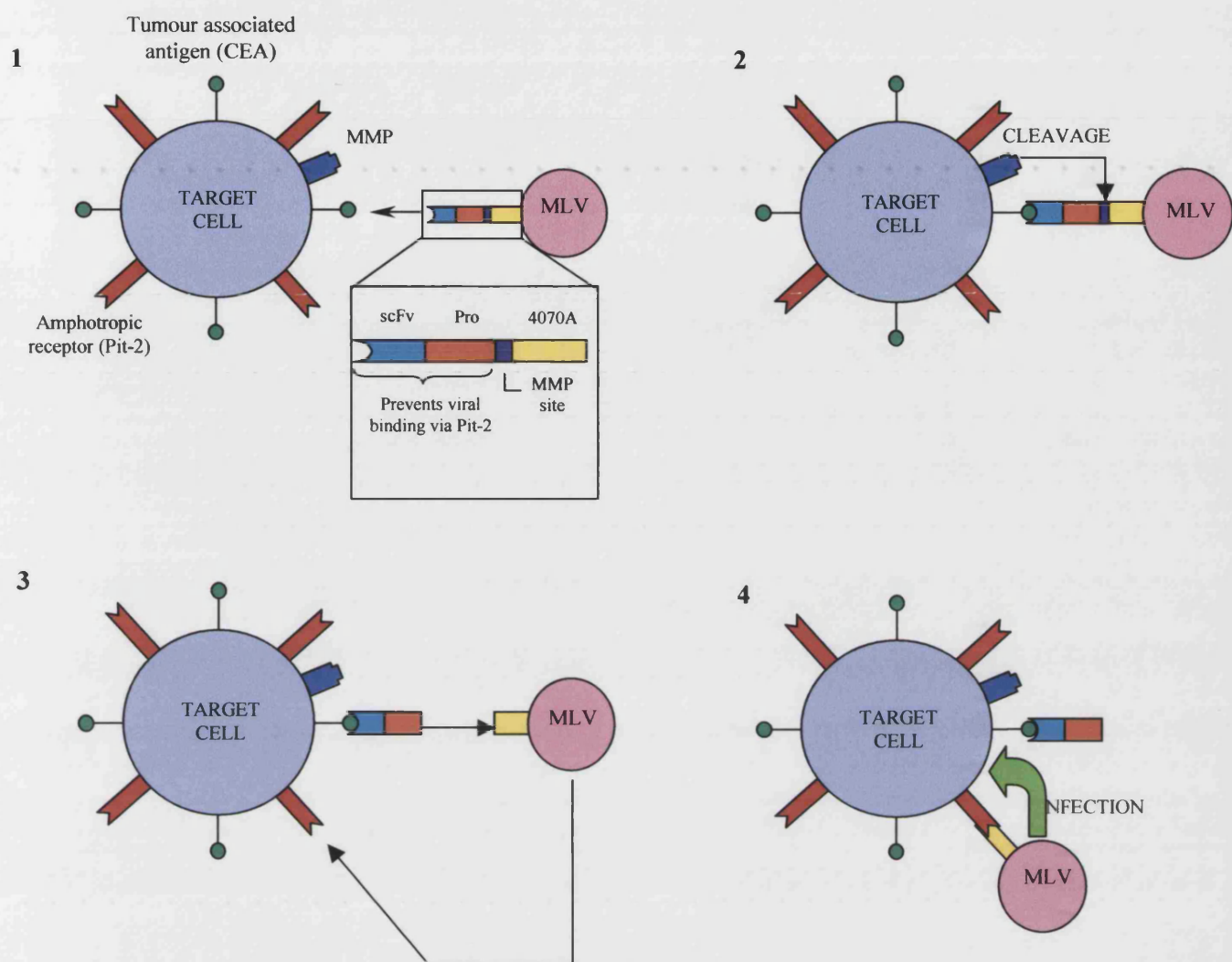
staining after 48 hours (Takeuchi *et al.*, 1994). This shows that viruses with the MFE23/ProMMP envelope were able to selectively infect CEA-positive cells. The highest titer was greater than  $10^5$  IU/ml and was approximately 10,000-fold more infectious on CEA-positive cells. MFE23/Pro-enveloped viruses were not infectious, with a titer less than 10 on all 4 cell lines that was even lower than virus with no envelope (supernatant from TELCeB6 cells). Viruses with the wild-type 4070A envelope were able to infect all 4 cell lines non-selectively at high-titer (ranging from  $10^6$  IU/ml to  $10^7$  IU/ml depending on cell line and infection conditions).

It appears from these experiments that the protease cleavage site is crucial in determining selective infection using this model as exemplified by the 10,000-fold or greater difference in titer seen between MFE23/Pro and MFE23/ProMMP, which differ solely by insertion of the MMP cleavage site in the latter envelope chimera. We propose that the incorporation of the single chain antibody and full-length proline linker into the retroviral envelope blocks the Pit-2 receptor binding domain of the wild-type 4070A backbone and prevents infection. In this model the binding of the scFv to the tumour antigen allows the chimeric envelope to undergo a conformational change that exposes the MMP cleavage site. Cleavage of the scFv and Pro reveals the 4070A Pit-2 receptor binding domain and allows subsequent fusion and infection. A schematic diagram explaining this mechanism is shown in figure 4.5. Even the MFE23/ProMMP chimera is 10-100 fold less infectious than the wild-type 4070A envelope, which implies that the process of binding and subsequent cleavage is not 100% efficient.

Table 4.1 shows that lipofectamine could enhance the efficiency of transduction of CEA-positive cells by MFE23/ProMMP-enveloped virus without affecting its specificity as described for other scFv-targeted viruses (Martin *et al.*, 1999; Martin *et al.*, 2003).

#### **4.5 Requirement of tumour antigen for infection**

The mechanism of infection proposed above is dependent on the chimeric envelope binding to CEA before undergoing a conformational change that allows proteolytic cleavage to unmask the Pit-2 RBD and allow subsequent fusion and infection. To



**Figure 4.5 Proposed mechanism of infection of MFE23/ProMMP.**

1. Retargeted chimeric envelope binds to CEA expressed on target cell surface.
- 2 + 3. MMPs on cell surface remove the scFv and Pro by cleavage at the MMP cleavage site.
4. Vector binds and enters target cell via Pit-2.

	A375			HT29		
	Nil	PB	LIP	Nil	PB	LIP
No Env	<4	<4	1.1x10 <sup>2</sup>	<4	5.0x10 <sup>1</sup>	1.2x10 <sup>2</sup>
MFE23/Pro	<4	<4	9.0x10 <sup>1</sup>	<4	<4	6.0x10 <sup>1</sup>
MFE23/ProMMP	1.2x10 <sup>2</sup>	3.0x10 <sup>1</sup>	1.5x10 <sup>2</sup>	1.1x10 <sup>5</sup>	1.8x10 <sup>5</sup>	6.3x10 <sup>5</sup>
4070A	6.0x10 <sup>6</sup>	1.0x10 <sup>7</sup>	1.4x10 <sup>7</sup>	1.4x10 <sup>6</sup>	6.3x10 <sup>6</sup>	9.0x10 <sup>6</sup>

**Table 4.1 Enhancement of protease targeting.**

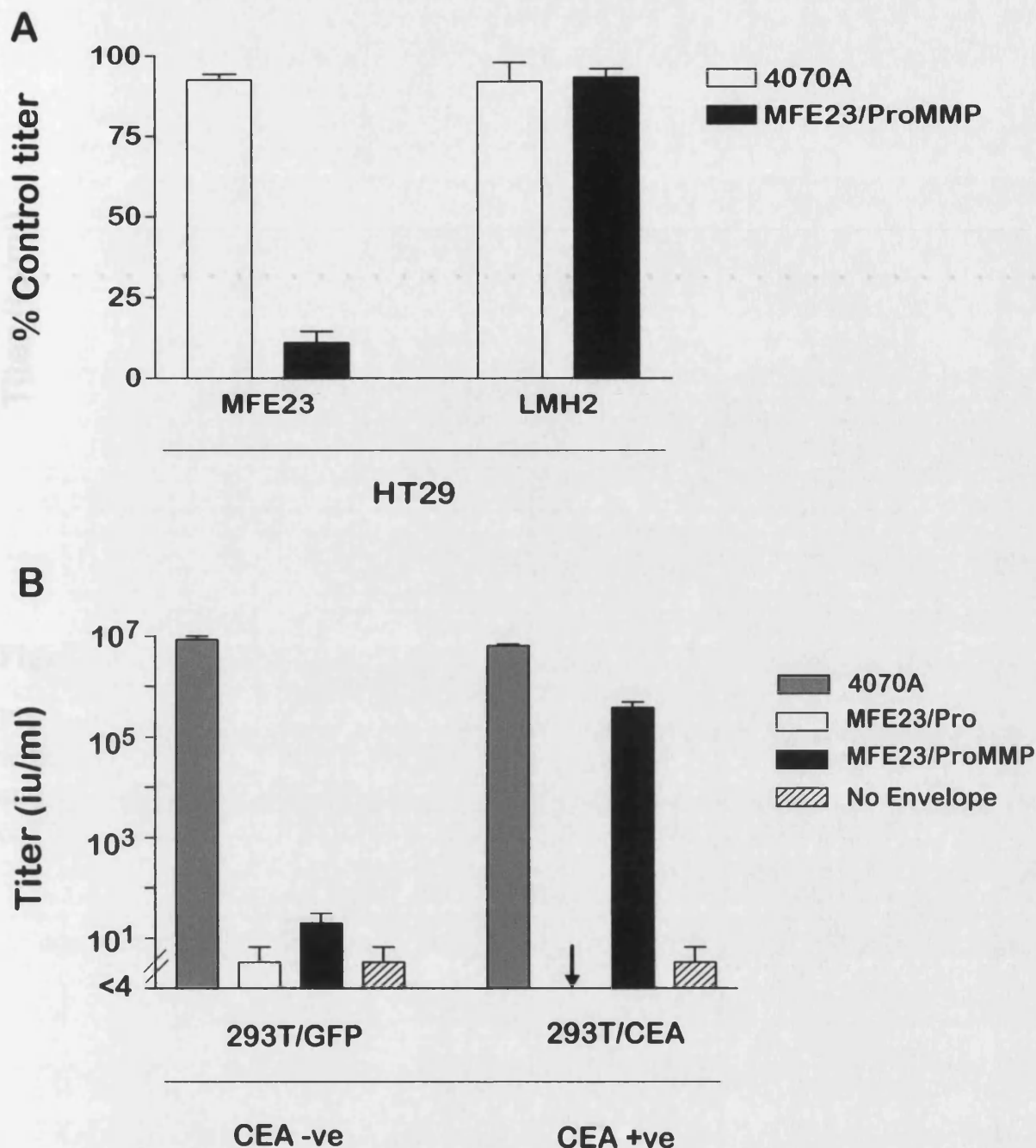
MFE23 targeted vectors were incubated with 4ug of Polybrene (PB)/ml or 10ug of lipofectamine (LIP)/ml or with no addition (Nil) for 10 min at room temperature before addition to the target cells. Results are indicated in IU/ml.

demonstrate that infection required initial binding to CEA blocking experiments were performed using either the relevant scFv (MFE23) or an irrelevant control (LMH2). Figure 4.6A shows that the addition of the appropriate, but not the irrelevant, scFv could inhibit targeted infection. Inhibition of infection was approximately 10% of control titer using MFE23. As previously stated blocking with the monovalent scFv is incomplete, presumably because of the higher avidity of viral binding. No significant effect on the titer of 4070A-enveloped viruses was seen showing that neither scFv affects the interaction of the 4070A RBD with its Pit-2 receptor.

Further evidence to support the requirement of CEA expression by target cells for infection by MFE23/ProMMP was provided by the infection of 293T cells transfected with pHR'-CMVCEA-IRESGFP or pHR'-CMVv-FLIP-IRESGFP (vector control) as described in chapter 3. Figure 4.6B shows that the titer of MFE23/ProMMP was significantly increased (greater than 10,000 fold) on 293T cells expressing CEA compared to 293T cells not expressing CEA. The titer of MFE23/ProMMP on the CEA-expressing 293T cells was similar if not greater than that seen on cells that endogenously express CEA (HT29 and Mawi). The titer of non-enveloped, 4070A- and MFE23/Pro-enveloped viruses was unaffected by transfection with either of the plasmids (Figure 4.6B). Figure 4.3 shows that 293T, 293T/GFP and 293T/CEA cell lines all express similar levels of protease and thus the differential infection seen with MFE23/ProMMP is dependent on CEA expression rather than any other feature of the colorectal cells or the transfection procedure itself.

#### **4.6 Requirement of Pit-2 expression for infection**

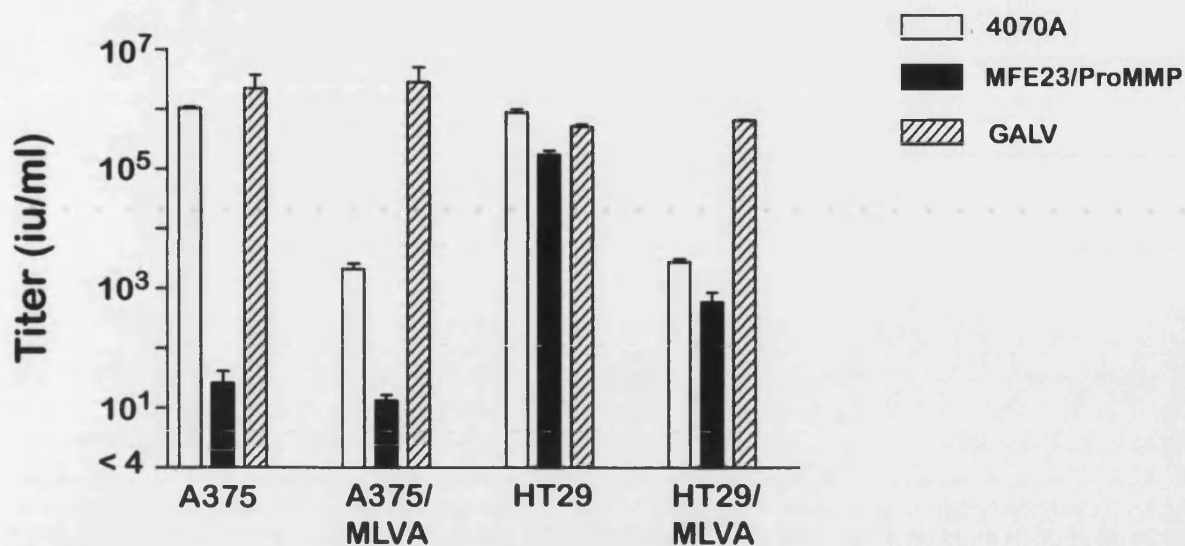
The mechanism of infection proposed is also reliant on the RBD of the 4070A backbone of the envelope chimera mediating infection via its Pit-2 receptor after the blocking domain has been proteolytically cleaved. To show that Pit-2 was required for infection interference experiments were performed as described in chapter 3. Receptor interference assays were performed using HT29 (CEA-positive) cells and A375 (CEA-negative) cells 'chronically infected' (two weeks of passage) with replication-competent 4070A MLV (Jobbagy *et al.*, 2000). Figure 4.7 shows that transduction of 4070A-infected HT29 cells by MFE23/ProMMP was reduced by approximately 500-fold, similar to the reduction of infection by viruses carrying the unmodified 4070A



**Figure 4.6 Targeted infection requires tumour antigen.**

A. Antigen blocking. CEA+ve cells (HT29) were treated with LMH2 or MFE23 scFv prior to infection. Infection by viruses with 4070A or MFE23/ProMMP envelopes (with 4  $\mu$ g Polybrene/ml) was then measured. Titer is expressed as a percentage of that of untreated cells.

B. Transfection of CEA. 293T cells were transfected with a GFP or CEA expressing plasmid. 48 hours after transfection viruses with MFE23-targeted chimeras, 4070A or no envelope were added (with 4ug of Polybrene/ml) to each target cell.



**Figure 4.7 Targeted infection requires Pit-2.**

Pit-2 blocking. HMWMAA +ve (A375) and CEA +ve (HT29) cells were infected with wild type amphotropic MLV. After 2 weeks A375, HT29, infected A375/MLVA and HT29/MLVA cells were infected by viruses expressing 4070A, MFE23/ProMMP or GALV envelopes (with 4 µg of Polybrene/ml) and their titers determined.



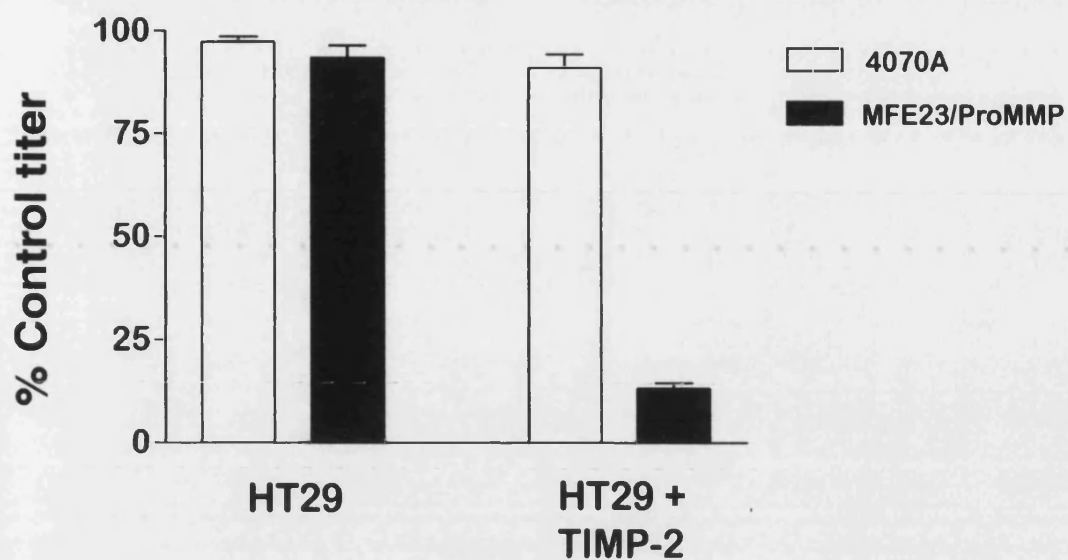
envelope. The titer of amphotropic MLV pseudotyped with the GALV envelope, which recognises a different surface receptor Pit-1 (Lam *et al.*, 1996), was unaffected indicating that it is the effect on Pit-2 that affects the titer of the targeted viruses and viruses carrying the unmodified 4070A envelope.

#### **4.7 Protease inhibition**

After binding to CEA, MMP cleavage of the chimera is necessary to expose the RBD of the 4070A backbone. To demonstrate that protease cleavage was required for gene delivery, infections were performed in the presence of tissue inhibitor of metalloproteinase (TIMP)-2 (Sternlicht and Werb, 2001). TIMP-2 (at a final concentration of 5µg/ml) was added to the viral supernatant prior to infection. Figure 4.8 shows that the inhibition of MMP activity by TIMP-2 decreased the titer of the MFE23/ProMMP-enveloped viruses to approximately 15% of control titer. No significant effect on the titer of 4070A-enveloped viruses was seen showing that MMP activity is not required for their infectivity and that treatment with TIMP-2 does not affect the permissiveness of the target cells.

#### **4.8 Infection of mixed cell populations**

In order to see whether MFE23/ProMMP-enveloped viruses could discriminate between CEA-positive and CEA-negative cells in a heterogeneous mixture of cells the following experiments were performed. 293T cells were transfected with pHR'-CMVCEA-IRESGFP or pHR'-CMVv-FLIP-IRESGFP (vector control). The efficiency of transfection was determined by analysing the percentage of green cells, as both plasmids harbour green fluorescent protein (GFP). The aim of transfection was to achieve an efficiency of 50% and thus a mixed cell population. This was achieved by titrating the amount of plasmid transfected and determined by counting four separate microscopy fields. Infection was carried out 48 hours post-transfection. On the same day as the infection the cell mixture was separated by flow cytometry into GFP-positive and -negative populations. The cells were replated and X-gal staining performed 48 hours later.



**Figure 4.8 Targeted infection requires MMP activity.**

MMP inhibition. CEA +ve cells (HT29) were treated with TIMP-2 at the time of infection. Infection by viruses with 4070A or MFE23/ProMMP envelopes (with 4  $\mu$ g Polybrene/ml) was then measured. Titer is expressed as a percentage of that of untreated cells.

Expression of CEA was confirmed by Western blotting of lysates of cells transfected with the relevant plasmids. Lysates were performed 48 hours post-transfection and confirmed that the 293T cells transfected with the CEA expressing plasmid pHR'-CMVCEA-IRESGFP expressed a high level of CEA comparable to that of CEA-positive colorectal carcinoma cell lines (Figure 3.3). 293T cells transfected with the vector control showed no evidence of CEA expression.

Table 4.2 shows that the unmodified 4070A-enveloped viruses infected all of the cell lines without selectivity. The MFE23/ProMMP-enveloped viruses infected 36% of the CEA-expressing 293T cells but less than 0.1% of the CEA-negative 293T or 293T/GFP cells. Greater selectivity was seen with the MFE23/ProMMP-enveloped viruses than when EGF-displaying or CD40L-displaying vectors were used to infect cell mixtures (Peng *et al.*, 1999). These experiments are important because they imply that MFE23/ProMMP viruses become activated at the CEA-positive target cell membrane resulting in infection of these cells but not neighbouring cells. Thus, commitment to a particular target cell precedes cleavage activation by the cell associated protease.

## **4.9 *In vivo* experiments**

### **4.9.1 Targeting of tumour xenografts**

The ultimate aim of gene therapy is the development of efficient, targeted vectors capable of *in vivo* gene delivery. The efficient *in vitro* targeting seen with the MFE23/ProMMP-enveloped vector led us to believe that it would be suitable for *in vivo*. LMH2/ProMMP had previously been tested in tumour xenografts grown in nude mice (Martin *et al.*, 2002) and this series of experiments were highly relevant to the design of *in vivo* experiments for MFE23/ProMMP.

The feasibility of *in vivo* targeting by LMH2/ProMMP was first assessed by cell mixing experiments. Here HMWMAA-positive or -negative tumour cell lines were mixed with lethally irradiated producer cells (producing amphotropic vectors, LMH2/ProMMP-enveloped vectors or unenveloped vectors). These cell mixtures were injected intradermally into nude mice and after 2 weeks the tumours were

**TRANSFECT :**

	293T	293T/GFP	293T	293T/GFP/CEA
<b>4070 A</b>	<b>92%</b>	<b>97%</b>	<b>94%</b>	<b>96%</b>
<b>MFE23/ProMMP</b>	<b>0.05%</b>	<b>0.02%</b>	<b>0.03%</b>	<b>36%</b>
<b>No envelope</b>	<b>0.04%</b>	<b>0.08%</b>	<b>0.1%</b>	<b>0.07%</b>

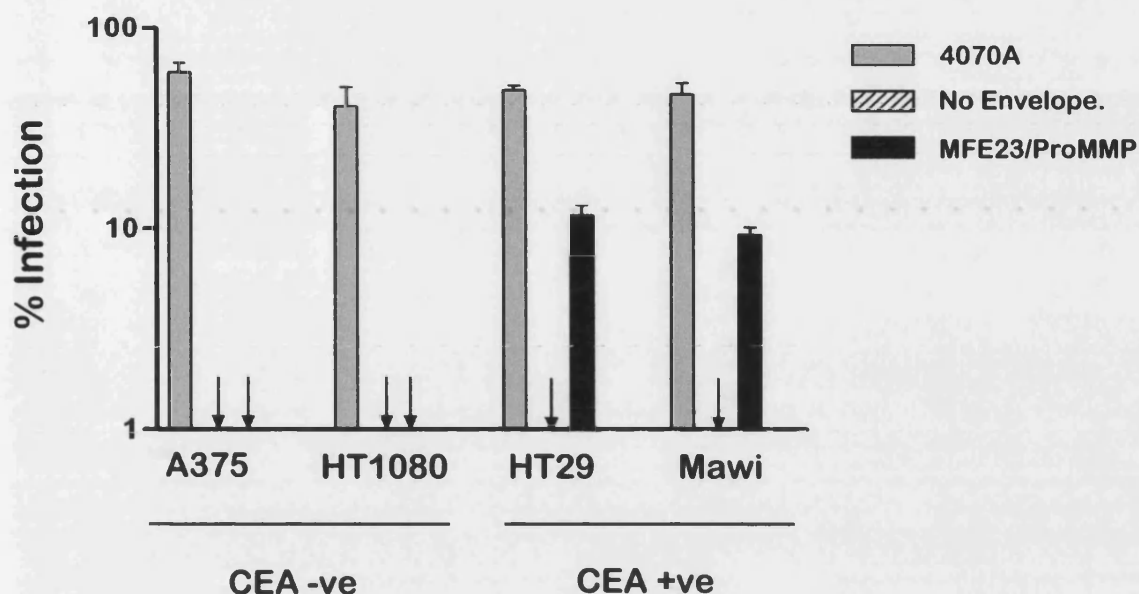
**Table 4.2 Infection of mixed cell populations.**

Cell mixtures were obtained by transfection of 293T cells with HR/IRESGFP or HR/CEA/IRESGFP plasmids (as described in Materials and Methods). Cell mixtures were infected by viruses expressing 4070A, MFE23/ProMMP or no envelope (with 4  $\mu$ g Polybrene/ml) and then separated by flow cytometry into GFP-positive and -negative populations. The cells were replated and X-Gal staining performed 48 hours later.

excised, disaggregated and analysed for  $\beta$ -galactosidase expression. The rationale for this experimental approach is that it allows optimised conditions for vector delivery as the vector continues to be delivered as the tumour develops. Also as the cells are interspersed it aids vector spread throughout the tumour. It is a useful screening method to assess the potential of vectors although it obviously has little clinical relevance for macroscopic or metastatic disease. Amphotropic vectors showed a very high efficiency of transduction (up to 95% of the tumour) for both HMWMAA-positive and -negative cell lines. LMH2/ProMMP vectors only transduced the HMWMAA-positive tumours with an efficiency of transduction of approximately 10% of that seen with wild-type amphotropic envelope (Martin *et al.*, 2002). The encouraging results from these cell mixing experiments led to the next series of experiments where the retroviral vectors were injected directly into established tumour xenografts. Again the LMH2/ProMMP vectors only transduced the HMWMAA-positive tumours. However the level of transduction was only approximately 3% of the tumours, compared with 10-20% when the same tumours were injected with the amphotropic vectors.

These results showed that targeting could be maintained *in vivo* using this model. However, the level of transduction seen by direct intratumoural injection of the targeted vector was disappointingly low. In an attempt to improve transduction efficiency whilst maintaining relevance to clinical applications it was decided to inject lethally irradiated producer cells directly into pre-established tumour xenografts. CEA-positive and -negative tumour xenografts were established in nude mice. When the tumours had reached 5-7 mm in diameter they were injected with lethally irradiated producer cells (producing 4070A-enveloped, MFE23/ProMMP-enveloped or unenveloped vectors). One week after producer cell injection the tumours were excised, disaggregated and analysed for  $\beta$ -galactosidase expression.

Figure 4.9 shows the percentage of infections seen in the tumour xenografts after injection with the different producer cell lines. The 4070A-enveloped vectors showed a very high level of transduction in all four cell lines. MFE23/ProMMP vectors maintained their selectivity for CEA-positive tumours (HT29 and Mawi) with no/minimal infection of the CEA-negative tumours (A375 and HT1080). The



**Figure 4.9 In vivo targeting.**

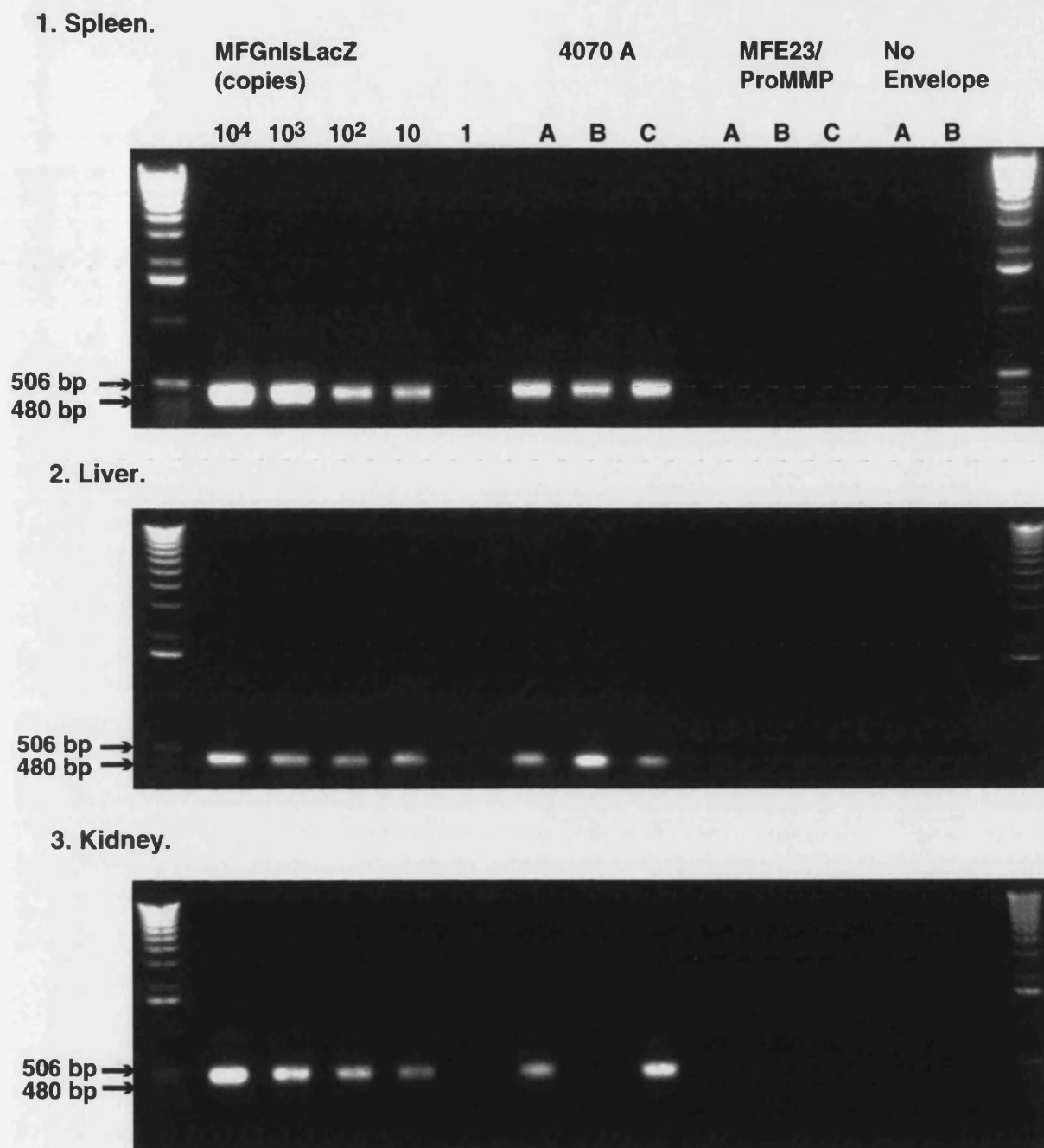
CEA-negative and -positive tumours were established in nude mice. Lethally irradiated producer cells producing unmodified 4070A, MFE23/ProMMP or unenveloped vectors were injected into these tumour xenografts. Transduction efficiency is shown as a percentage of tumour cells expressing the  $\beta$ -galactosidase marker gene after counting approximately  $10^5$  cells.

transduction efficiency was approximately 10% of the targeted tumours. Thus, MFE23/ProMMP vectors are able to maintain their specificity and efficiency *in vivo* with transduction efficiencies of approximately 20% of that seen with the amphotropic vectors. This was achieved without enhancement agents, such as Polybrene or Lipofectamine, which is advantageous as the use of cationic liposomes *in vivo* would be undesirable due to complement activation (Chonn *et al.*, 1991).

#### **4.9.2 Analysis of vector distribution**

Targeting using the MFE23/ProMMP model aims to deliver genes selectively to CEA-positive tumours and the previous results show that this is achieved with direct injection of tumour xenografts. This model also aims to block infection of non-target cells, which could be potentially harmful and would deplete the pool of vector particles. The results show that MFE23/ProMMP does not infect CEA-negative tumour xenografts, but it is necessary to examine other non-target organs using a technique that is more sensitive than staining for  $\beta$ -galactosidase. It was initially planned to examine organs from the animals infected as above by PCR using LacZ1/LTR1 primers that amplify a vector fragment. However, a previous study had shown that transduced tumour cells are likely to be spread to other organs as micrometastases (Martin *et al.*, 2002) and thus this method is unable to differentiate vector spread from metastasis in tumour-bearing animals.

An alternative method was developed using irradiated producer cells injected intraperitoneally (Martin *et al.*, 2002). Amphotropic, MFE23/ProMMP or unenveloped irradiated producer cells were injected intraperitoneally in nude mice. In order to compensate for the higher titer seen with amphotropic vectors ten times as many MFE23/ProMMP and unenveloped producer cells were injected. Two weeks after injection the mice were sacrificed and the DNA extracted from the spleen, liver and kidneys for nested PCR analysis. The nested PCR used primers that amplified a 480-bp DNA fragment located between the 3' end of the  $\beta$ -galactosidase gene and the 3' LTR of the integrated vector.



**Figure 4.10 Analysis of vector spread.**

Lethally irradiated producer cells expressing 4070A, MFE23/ProMMP or no envelope were injected intraperitoneally in nude mice. Proviral analysis was carried out by nested PCR using primers that amplify a 480 bp DNA fragment located between the 3' end of the  $\beta$ -galactosidase gene and the 3' LTR of the integrated vector. DNA from the spleens, livers and kidneys from the eight individual mice (labelled A-C) were analysed for proviral content. Plasmid DNA serial dilutions (from 10<sup>4</sup> to 1 plasmids) of an MFGnlsLacZ vector were used to determine sensitivity.



Figure 4.10 shows the results of the nested PCR for the three vectors in the three organs analysed. This shows that in all three mice injected with amphotropic producer cells detectable levels of proviral DNA were seen in the liver and spleen. When the kidneys from these mice were analysed they were shown to be positive in two of the mice. However, none of the mice injected with MFE23/ProMMP producer cells or unenveloped producer cells showed detectable levels of proviral DNA in the spleen, liver or kidney.

Semiquantitative analysis of proviral content in spleen, liver and kidney was performed to determine the level of amphotropic transduction in these organs. DNA from the mouse labelled A in the 4070A group (Figure 4.10) was used. Serial dilutions of DNA were used to carry out nested PCR using the same primers (Figure 4.11). The limit of detection of this assay is 10 copies as shown by the serial dilution of MFGnlsLacZ. Thus, from figure 4.11 the likely number of copies of proviral DNA per  $10^6$  cells can be calculated. For spleen, liver and kidney this is  $10^5$ ,  $10^4$  and  $10^3$  copies of proviral DNA per  $10^6$  cells respectively. No proviral DNA was detected in any organ from mice injected with MFE23/ProMMP (Figure 4.12) or unenveloped producer cells. Based on these results and taking into account that the limit of detection is 10 copies per  $10^6$  cells it appears that MFE23/ProMMP vectors are approximately  $10^4$  times less likely to spread than amphotropic vectors. It may be argued that this effect is partly due to their lower infectivity but this was addressed by injecting a higher number of MFE23/ProMMP producer cells into each mouse to compensate for their lower titer.

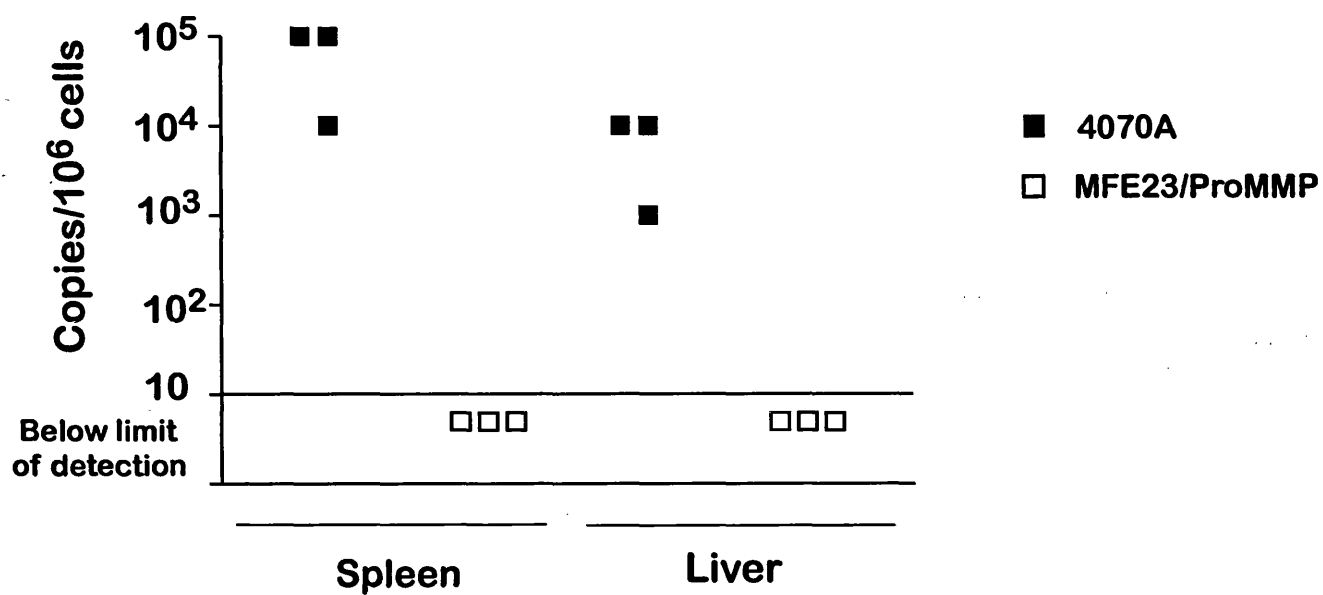
#### 4.10 Discussion

This series of experiments have shown that it is possible to achieve selective targeting to carcinoembryonic antigen (CEA) both *in vitro* and *in vivo* by tropism restriction of amphotropic MLV using this approach. Infection is dependent on cells expressing both a specific tumour antigen and a specific protease on their cell surface. This dual requirement provides an extra degree of specificity and hence safety as it decreases the likelihood that non-target cells will be transduced. The proposed mechanism of infection is that after binding to CEA the envelope chimera undergoes a



**Figure 4.11 Semi-quantitative analysis of proviral DNA content.**

Serial dilutions of DNA from the spleen, liver and kidney from mouse A injected with producer cells for 4070A-enveloped vectors were used for nested PCR reactions (as described in figure 4.10).



**Figure 4.12 Quantitative analysis of proviral DNA.**

Quantitation from all mice. Estimated proviral number was calculated based on the number of cells used in the last dilution where positive amplification was found and the sensitivity of the nested PCR reaction

conformational change that exposes the MMP cleavage site enabling cleavage to occur. Removal of the scFv and proline-rich linker exposes the Pit-2 receptor binding domain of the 4070A backbone allowing interaction with Pit-2 and efficient infection (Figure 4.5).

The titer achieved here for MFE23/ProMMP of greater than  $10^5$  IU/ml is approximately 1 log higher than that seen with MFE23/Pro2 as described in chapter 3. This is why this vector was chosen for the *in vivo* experiments that assessed targeting of tumour xenografts in nude mice. The titer achieved with MFE23/ProMMP is 2 logs higher than that seen in a previous study where MFE23 was fused to ecotropic MMLV and co-expressed with wild-type envelope (Konishi *et al.*, 1998). As stated previously, Khare and colleagues developed a chimera in which an scFv that recognises CEA is fused to ecotropic MMLV envelope and coexpressed with wild-type ecotropic envelope (Khare *et al.*, 2001). The titer achieved with this model was 1 log lower than that seen with MFE23/ProMMP in spite of the fact that co-centrifugation of virus and cells was used in addition to Polybrene to enhance infection. This vector was used to deliver a suicide gene to CEA-positive tumour xenografts in nude mice and was able to produce tumour suppression with a 70% reduction in tumour weight for the treated group as compared to the control group (Khare *et al.*, 2002). This would suggest that a useful therapeutic effect could be achieved by using the MFE23/ProMMP model to deliver suicide genes *in vivo*. Particularly as the effect of gene delivery may be augmented by the bystander effect a phenomenon by which the introduced gene can affect neighbouring cells in which it is not itself present (Mesnil and Yamasaki, 2000).

The results show that MFE23/ProMMP vectors are able to maintain their specificity *in vivo*. The use of direct injection of producer cells allows transduction of up to 10% of the tumour cells, which should be sufficient to produce a reduction in tumour size if a suicide gene can be delivered as effectively. This targeted strategy has the added advantage that reduced non-target delivery of targeted vectors has been demonstrated with a considerable gain in safety over non-targeted vectors. It also demonstrates a gain in safety over other targeted strategies. Targeting to HMWMAA using LMH2/ProMMP showed that the likelihood of spread was at least 1% of that seen with 4070A-enveloped vectors (Martin *et al.*, 2002). Thus, MFE23/ProMMP appears

to show at least a 100-fold gain in safety over this model. As stated earlier targeting using vectors blocking domains that only restrict infection on certain cells such as the EGF displaying chimeras will almost certainly infect non-target cells that do not express the relevant receptor such as EGFR. This model has shown selective infection of MMP-rich tumours *in vivo* but this study did not assess the extent of non-target cell transduction (Peng *et al.*, 1999). Another promising *in vivo* gene delivery system is based on matrix-targeted retroviral vectors (Gordon *et al.*, 2000; Hall *et al.*, 2000; Gordon *et al.*, 2001). This vector-escorting strategy incorporated matrix-targeting motifs (i.e. collagen-binding peptides) on amphotropic MLV vector particles. This approach demonstrated enhanced vector penetration and transduction of tumour nodules after local or systemic delivery with significant tumour regression. However, such preferential targeting is unlikely to be highly specific and further experiments of vector biodistribution are needed to establish whether the increase of affinity achieved is sufficient to significantly reduce the leak of infectivity to non-target cells.

Further modifications to improve efficiency could involve engineering scFvs to allow an improvement in envelope incorporation. Changes in scFv to CEA binding may allow more efficient post-binding conformational changes and hence improve MMP cleavage and infection. It is possible to optimise the MMP cleavage site, which may allow increased cleavage after binding to CEA. This can be achieved using retrovirus display libraries (Buchholz *et al.*, 1998). This approach was used to optimise the protease cleavage site in EGF-bearing chimeras by using replication-competent MLV incorporating EGF at the N-terminus of the envelope. A retrovirus display library was generated that diversified the seven-residue linker between the envelope glycoprotein and EGF. Selective pressure for EGF cleavage was applied by serial passage on EGF receptor rich cells. The selected viruses showed wild-type infectivity conferred by improved cleavage of their linkage sequences. It is possible that point mutations introduced into 4070A envelope may improve its stability and thus improve transduction efficiency as has been demonstrated for the ecotropic MLV envelope (Zavorotinskaya and Albritton, 2001).

## **Chapter 5**

### **Discussion**

The goal of cancer gene therapy is to safely deliver genes to a sufficient number of target cells that results in tumour regression. In practise considerable obstacles have emerged, most notably gene delivery. Thus far the problem has been an inability to deliver genes efficiently, safely and to obtain sustained expression. Gene therapy protocols would be improved by the availability of targetable vectors that could deliver genes to specific target cells or disease sites. Non-target cells should not be infected as gene delivery could be potentially harmful e.g. suicide genes, insertional mutagenesis and would deplete the pool of vector particles.

#### **5.1 Targeting by envelope modification**

This thesis has described work aimed at improving retroviral targeting by modifications in the retroviral envelope glycoprotein. In chapter 3 retroviral vectors were constructed to achieve selective infection of target cells by receptor cooperation. This series of experiments showed that it is possible to achieve selective targeting to cells expressing the tumour antigens HMWMAA or CEA by tropism restriction of amphotropic MLV. Infection was dependent on cells expressing both the tumour antigen and the amphotropic receptor Pit-2. Receptor cooperation was optimised by using differing proline-rich linkers between the amphotropic receptor binding site and the scFv targeted to the relevant tumour antigen. A 9 amino acid linker (Pro2) proved optimal in transiently masking the Pit-2 RBD, which becomes accessible after binding to the relevant tumour antigen via the scFv.

Both targeted vectors were able to selectively infect tumour antigen-expressing cells, with minimal infection of antigen-negative cells. The higher titer seen with MFE23/Pro2 than LMH2/Pro2 (10,000 IU/ml versus 1,000 IU/ml) probably reflected a higher level of envelope incorporation into virions. The level of infection seen with MFE23/Pro2 is characteristic of approaches that use retroviral envelope interaction with its natural receptor to trigger efficient fusion. Targeting by receptor cooperation

relies only on the expression of a target molecule and a retroviral receptor. This approach may provide a more general approach than more limited methods such as inverse targeting (Fielding *et al.*, 1998).

Future work with this model may initially try to maximise titer *in vitro*. Modifications to improve efficiency could involve engineering scFvs to allow efficient envelope incorporation. The level of incorporation of MFE23 was acceptable but the level of LMH2 was suboptimal and almost certainly had a detrimental effect on infection. It has been demonstrated that receptor cooperation can be achieved using different non-viral  $\beta$ -spiral peptides to act as the interdomain linker (Valsesia-Wittmann, 2001). Thus, modifications in composition and length of the  $\beta$ -turn helix may optimise conformational changes after initial binding. It is possible that point mutations introduced into 4070A envelope may improve its stability and thus improve transduction efficiency (Zavorotinskaya and Albritton, 2001). Once the envelope chimera has been optimised the next step will be to test targeting *in vivo* using human tumour xenografts in nude mice. The level of infection seen with MFE23/Pro2 is comparable to previous targeting models that showed adequate transduction efficiency *in vivo* (Martin *et al.*, 1999) and also models that showed a therapeutic benefit (Khare *et al.*, 2002).

In chapter 4 retroviral vectors were constructed to achieve selective infection of target cells by protease targeting. This series of experiments showed that it is possible to achieve selective targeting to CEA both *in vitro* and *in vivo* using this approach. Infection is dependent on cells expressing a specific tumour antigen, a specific protease and the amphotropic receptor Pit-2. This provides an extra degree of specificity and hence safety as it decreases the likelihood that non-target cells will be transduced.

The titer of MFE23/ProMMP-enveloped viruses on CEA-positive cells of  $10^5$  IU/ml is only 10-100-fold less efficient than viruses with unmodified 4070A (titre  $10^{6-7}$  IU/ml). This titer compares favourably with other CEA targeted models that have therapeutic effects *in vivo* (Khare *et al.*, 2002). The results using irradiated producer cells showed that this approach allowed the vectors to maintain their specificity *in vivo*. Direct

injection of tumour xenografts allowed transduction of up to 10% of tumour cells, which should be sufficient to produce a therapeutic effect if a therapeutic gene could be delivered as efficiently. Use of this targeted approach has the important advantage of a considerable gain in safety over non-targeted vectors. It appears that MFE23/ProMMP vectors are approximately  $10^4$  times less likely to spread than amphotropic vectors. This level of gain in safety has not been demonstrated with any other retroviral targeting approach (Peng *et al.*, 1999; Gordon *et al.*, 2000) and is a significant advantage of this system.

Further modifications to improve efficiency of the MFE23/ProMMP vector could involve engineering scFvs to allow an improvement in envelope incorporation. Changes in scFv to CEA binding may allow more efficient post-binding conformational changes and hence improve MMP cleavage and infection. It is possible to optimise the MMP cleavage site, which may allow increased cleavage after binding to CEA. This can be achieved using retrovirus display libraries (Buchholz *et al.*, 1998). Improvements in stability of the 4070A envelope may be achieved by point mutations in the envelope itself, which may also improve transduction efficiency (Zavorotinskaya and Albritton, 2001). The next key stage in the development of this vector will be to see if it is able to maintain its selectivity of transduction when delivering a therapeutic gene, most likely a suicide gene.

## **5.2 Cell surface targets**

To date, the only surface-targeting strategies that have allowed efficient infection by retroviral vectors *in vivo* are those in which the target cell expresses the receptor used by the retroviral surface protein (SU) backbone of the envelope chimera (Peng *et al.*, 1999; Gordon *et al.*, 2001; Peng *et al.*, 2001; Martin *et al.*, 2002). This allows successful fusion after retargeted binding. These approaches have limited the tropism of amphotropic murine leukaemia virus (MLV-A), which can infect cells of many mammals, by modification of the envelope glycoprotein.

The choice of targeted surface molecule appears to play a crucial role in determining the infectivity of the new chimera. At one extreme is the attachment of a natural viral receptor binding domain to the MLV-A backbone. This has been shown by fusion of



the ecotropic mCAT-1 binding domain to the N-terminus of MLV-A via differing proline spacers (Valsecia-Wittmann *et al.*, 1997). The new chimeras were able to efficiently infect cells displaying both mCAT-1 and Pit-2.

Some targeted cell surface molecules have the ability to abolish infection, even though the retroviral receptor (in this case Pit-2) is also displayed at the cell surface. This was first described for MLV-A displaying EGF, which were unable to infect human cells expressing EGF receptors and Pit-2 (Cosset *et al.*, 1995a). Inhibition of infection is specifically caused by the interaction with the high-affinity EGF receptor as competition with soluble EGF can restore infectivity via Pit-2. It is thought that some cell surface molecules, such as EGF, induce competitive virus sequestration, which abrogates infection. The receptors sequester and/or traffic bound retroviruses to cell compartments, which are not compatible with interaction with Pit-2. This phenomenon of 'inhibitory' receptors has been seen in retroviral vectors targeted against other surface molecules. For example, vectors displaying stem cell factor (Fielding *et al.*, 1998), insulin-like growth factor (Chadwick *et al.*, 1999) and the lymphocyte surface antigen CDw-52 9 (Russell and Cosset, 1999). Inverse targeting is a strategy that exploits receptor-mediated virus neutralisation allowing selective infection of cells not displaying the targeted receptor.

Another surface molecule that falls into the class of molecules that do not favour viral infection is the  $\alpha$  folate receptor. This is a high-affinity folate binding protein that is over-expressed in 90% of non-mucinous ovarian carcinomas. An scFv was fused at the N-terminus of the MLV-A envelope in attempt to target the  $\alpha$  folate receptor, however levels of infection were poor (Pizzato *et al.*, 2001). The results are similar to those seen MLV-A targeted to EGF. In this case a different mechanism may explain poor infectivity, as only 10% of receptors in ovarian cancer cells are internalised and thus sequestration would appear unlikely. It was felt that interaction between the scFv and  $\alpha$  folate receptor might interfere with subsequent binding of the MLV-A RBD or post-binding events that trigger fusion.

A third class of targeted cell surface molecules would appear to be 'neutral' for infection i.e. they allow binding but are unable to allow infection directly. Retargeted

retroviruses remain reliant on the natural retroviral receptor for entry. CEA and HMWMAA would both appear to belong to this category of surface molecules. Binding to either of them doesn't abrogate infection by sequestration or other methods. Retargeting to this class of receptor is reliant on transiently masking the RBD. In our case this was successfully provided by insertion of either a short proline linker or a protease cleavage site. Receptor interference studies showed that Pit-2 was necessary for infection for both receptor cooperation and protease targeting using this class of surface molecule.

### **5.3 Safety concerns**

Safety issues are currently at the forefront of viral gene therapy protocols. These first came to prominence after the death of Jesse Gelsinger, who died in September 1999 following the administration of a recombinant adenoviral vector containing the ornithine transcarbamylase (OTC) gene. The precise cause of his death remains unclear but it appears that it resulted from a systemic adenovirus induced shock syndrome, cytokine release, acute respiratory distress and multiorgan failure (Verma, 2000) In the 10 year old history of nearly 400 clinical gene therapy trials involving over 4000 patients this was the first death directly attributable to the gene delivery vehicle. Quite rightly this has lead to new regulations and guidelines proposed by the NIH and FDA to improve both the quality of clinical trials and to protect volunteers enrolled in trials particularly all gene therapy protocols.

Of more relevance to retroviral vectors has been the recent report of two cases of T-cell leukaemia in patients treated with retroviral gene therapy for X-linked severe combined immune deficiency (X-SCID) (2003). X-SCID is an inherited disorder characterised by an early block in T and natural killer (NK) lymphocyte differentiation. This results in the absence of both T and B cells leading to severe and recurrent infections that are usually fatal in the first years of life. Bone marrow transplantation (BMT) can be used to successfully treat X-SCID, but it works best when there is a fully compatible donor. Unfortunately this is the case in under one third of X-SCID children. In unmatched recipients, BMT carries the risk of graft failure, graft-versus-host disease, lymphoma and other medical problems.

The lack of therapeutic options in X-SCID led to the development of the gene therapy trial by Fischer and colleagues (Cavazzana-Calvo *et al.*, 2000). Bone marrow stem cells were obtained from the affected children, cultured with growth factors and transfected on three successive days with a Moloney derived retroviral vector carrying the  $\gamma_c$  gene. 10 out of the first 11 patients achieved effective and life-saving immune reconstitution (2003). Thus gene therapy was able to correct the disease phenotype and from a clinical perspective the patients may have been considered cured by this pioneering treatment.

However, 30 months after treatment one of the patients developed a monoclonal gamma-delta T-cell lymphoproliferative disorder (leukaemia like disorder). Subsequently a second child has developed a T-cell leukaemia. Both leukaemias appear to be caused by insertional mutagenesis i.e. retroviral activation of a cellular oncogene at the site of insertion. The gene LMO2, located on chromosome 11, is normally involved in the control of blood cell proliferation and differentiation and is known to be activated in certain types of T-cell leukaemias. Its expression has shown to be elevated in both cases.

Multiple factors may have contributed to the development of leukaemia in the patients involved in this trial. These include the high level of engraftment and expansion of genetically modified cells, unique properties of the haematopoietic stem and progenitor cells in the bone marrow of X-SCID patients, the immune deficiency of X-SCID patients and/or the transferred gene itself. The gene itself is one that lends itself perfectly to gene replacement in that it provides a stimulus to growth and survival but this may have contributed to the malignant transformation. Further use of current gene-transfer methods for the treatment of X-SCID poses a complex dilemma in the consideration of potential risks and benefits.

Insertional mutagenesis had always been considered a potential risk associated with the use of retroviral vectors. It is important to recognise that the risks could be different for each disease, each therapeutic gene and each class of patient and every subsequent gene therapy trial involving the use of retroviral (or other integrating vectors) should be preceded by a careful assessment of the risk-benefit ratio. The X-

SCID case highlights the importance of targeted gene therapy as it may well emerge that there are certain cell types that we do not wish to accidentally transduce such as haematopoietic stem cells. Thus, the selective transduction seen with the envelope targeted models, particularly the protease targeted model provides an important level of safety.

#### **5.4 Cancer gene therapy**

The success of gene therapy to treat cancer requires the delivery of genes specifically and accurately to sufficient numbers of tumour cells *in vivo*. To date the majority of animal studies using both viral and nonviral vectors have used direct intratumoural injection of vectors (Harrington *et al.*, 2002). The vector systems currently being developed for most gene therapy applications cannot be produced to levels that are sufficient to absorb the losses that will be incurred after systemic administration, which is especially true for retroviral vectors. Many vectors are first tested *in vivo* in immunodeficient mice that allow the establishment of human tumour xenografts. Even in this situation, in which most of the major immune effects that act to reduce viral titers *in vivo* are absent, the levels of virus that can be administered are often insufficient to achieve meaningful levels of tumour infection.

One of the ways in which viral titer is 'lost' *in vivo* is by non-specific binding and adhesion and this has led to extensive efforts such as ours to alter the viral tropism by alterations in the envelope glycoproteins (Russell and Cosset, 1999). However, it has been shown *in vitro* that non-specific adhesion of viral particles to the cell surface appears to occur before the envelope-receptor interaction (Pizzato *et al.*, 1999). Although this interaction does not result in productive infection if it were to occur *in vivo* the reduction in viral titer could be significant.

Thus despite significant advances in the engineering of tropism-determining proteins to target vectors for *in vivo* delivery, it remains to be shown that targeted, recombinant retroviral vectors can attain titers that will have a therapeutic value in patients with disseminated disease when administered systemically. It seems apparent that a means of protecting these fragile and vulnerable vectors is needed. Ideally the vectors should be carried directly to the vicinity of their sites of action and released in an

environment where the molecular alterations conferring tropism redirection have a realistic chance of working. The ideal candidates to deliver tumour-targeted vectors would be immune-invisible, tumour-homing cells.

For our tumour antigen targeted MFE23/ProMMP vectors we aimed to protect the vectors as well as producing a degree of amplification by using irradiated producer cells. The results using irradiated producer cells showed that this approach allowed the vectors to maintain their specificity *in vivo*. Direct injection of tumour xenografts allowed transduction of up to 10% of tumour cells, which should be sufficient to produce a therapeutic effect if a therapeutic gene could be delivered as efficiently.

This approach was initially used to treat brain tumours by direct injection of murine retroviral producer cells. The aim was to release retrovirus encoding the herpes simplex virus thymidine kinase (HSVtk) suicide gene, which would infect surrounding tumour cells (the only cells that should be replicating at that site and thus be susceptible to C-type retroviral infection) and render them susceptible to the prodrug ganciclovir (GCV) (Culver *et al.*, 1992). This would kill the retroviral producer cells, the infected cells and bystander tumour cells. This approach proved successful in the rat model (Culver *et al.*, 1992), however there was only limited success when used to treat human brain tumours (Ram *et al.*, 1997). There was some antitumour activity in five smaller tumours (1.4 +/- 0.5 ml) with one patient achieving a durable complete response (>220 days), but there appeared to be limited gene transfer to tumours suggesting that this effect was due to 'bystander' mechanisms from the vector-producing cells. The injected producer cells remained stuck in close proximity to the injecting needle and tk cDNA transfer by the retroviral vector was limited to a few cells away. The response of only very small tumours showed the feasibility of this approach, but highlighted the need to improve delivery of the therapeutic gene.

The packaging cell lines used in our experiments are developed from an allogeneic tumour cell line (TE671; a rhabdomyosarcoma cell line). Ultimately the aim is to use these producer cells to deliver our targeted retroviral vectors carrying a therapeutic gene, most likely a suicide gene. It is important that the transferred producer cells are eventually killed and in our case this should not be a concern as they had been lethally

irradiated (40 Gy) prior to injection. In future studies, producer cells are likely to carry a suicide gene and thus on administration of the prodrug will be killed themselves. A mechanism which may well augment the therapeutic effect. Also we are using allogeneic tumour cells that will be rejected by an immune competent host. The latter point is important as this may greatly reduce its value as a vector delivery vehicle and actually prolonging the survival of the allogeneic cell line may become an issue. It is important to note that irradiated allogeneic tumour cells are already clinically used as cancer vaccines (Harrington *et al.*, 2002), thus the concept of using tumour cells to treat tumours is not as radical as it may have appeared. This may make it easier for their introduction and acceptance into clinical practise.

The ultimate vector carrying cell should home preferentially to the tumour. Once at the tumour it should be activated to start vector production, which in turn should be targeted to the tumour cell. Such a system has recently been described by Chester and colleagues (Chester *et al.*, 2002). This model used T cells to deliver targeted retroviral vectors, which were protected until they could be released at high local concentrations. The T cells were targeted by complexing an scFv to CEA to the intracytoplasmic domain of the T-cell receptor (TCR/CD3). By using this signal from T-cell binding to initiate viral production appropriate temporal and spatial production of virus is achieved. The virus produced is transcriptionally targeted by placing a CEA promoter in the retroviral genome. In this case the HSVtk suicide gene was under control of this promoter. This system showed therapeutic, tumour-specific vector delivery in models of both local intratumoural and systemic delivery to both lung and liver metastases.

This study is not without its limitations. It was performed with human T cells attacking human tumour cells in an athymic mouse. An idealised mouse model with limited tumour burden was used whose applicability to human treatment remains to be established. For any clinical trial in humans, optimising the ratio of injected T cells to estimated tumour burden will prove challenging. Despite these drawbacks this is an elegant study that shows the potential of human T cells to be used as carriers of retroviral vectors.

Our own vector producing cells may have an element of intrinsic targeting as packaging cells express viral envelopes on their surface and thus the chimeric envelope that contains an scFv to CEA. Our model does not have the control of the timing of vector production as seen with the T cell model described above. However, it may be possible to overcome some of the limitations of this system. The CEA targeted models have shown selective targeting of colorectal cancer cell lines or tumour xenografts. Colorectal cancer is known to commonly metastasise to the liver with two-thirds of colorectal cancer patients developing liver metastases during the course of their disease (Bengtsson *et al.*, 1981). Patients with liver metastases have a poor prognosis with a median survival time of 9-11 months (Bengtsson *et al.*, 1981). The best treatment option for isolated metastases is surgery, which has the potential to cure the disease. However, only 20-25% of patients who present with hepatic metastases are suitable for resection and recurrence after surgery is common (Lorenz *et al.*, 2000). Knowing the likely pattern of relapse in this disease allows treatment when there is minimal residual disease and the highest chance of successful therapy.

Treatment options for this group of patients are limited and novel molecular therapies are appropriate. It is possible to deliver regional therapies using direct intratumoural injection or hepatic arterial infusion and this would be appropriate for cell-based vector carriers such as our packaging cell lines. A recent phase I trial in patients with metastatic colorectal adenocarcinoma in the liver has assessed the safety of using an adenoviral vector carrying HSVtk (Sung *et al.*, 2001). Sixteen patients received intratumoural injections of the adenoviral vector followed by intravenous ganciclovir. Toxicities were low and transient showing the feasibility of this approach.

Although gene therapy has failed to live up to initial expectations it still has the potential to become an important part of cancer treatment. It is important to consider gene therapy within the context and aims of treatment strategies. With current delivery systems, gene therapy is unable to clear large scale disease due to the inability to deliver therapeutic genes to sufficient cancer cells. Gene therapy is most likely to be effective as adjuvant therapy where it targets minimal residual disease. This is a common situation in colorectal cancer where after removal of the bulk disease (the primary colorectal tumour) surgically there is a risk of developing metastatic disease post-operatively. The liver is a common site for such disease as

explained earlier and the ideal time to deliver gene therapy may be at or shortly after surgery. This situation can be created easily in an animal model where the vector is given shortly after tumour cells have been seeded but before metastases develop.

Palliative treatment aims to relieve symptoms and improve the patients' quality of life. Unfortunately, unpleasant side-effects of treatment often outweigh any small benefits achieved as radiotherapy and chemotherapy do not specifically target tumour cells. Gene therapy may be particularly suited to palliative treatment as it is specifically directed at tumour cells and treatment toxicity is thus predicted to be low. Very few side-effects have been seen in patients treated with gene therapy to date (Somia and Verma, 2000).

If gene therapy for cancer has so far failed to live up to expectations, it is because these expectations have been unrealistic. Gene therapy is most likely to find a useful place in clinical practise as an adjuvant radical treatment or as a palliative treatment in advanced disease. Appropriate preclinical and clinical trials with realistic expectations and designed within the context of present treatment strategies should still allow gene therapy to show its true value.



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