EVALUATION OF SAFETY AND METHODS FOR PRODUCTION OF RETROVIRAL VECTORS FOR USE IN GENE THERAPY

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ABSTRACT

Retroviral vectors are replication-defective viral genomes, in which genes essential for viral replication have been replaced by therapeutic genes. Essential genes are provided *in trans* by a helper virus, or packaging cell line. Stable producer cell lines are selected following transfection or infection of the vector into a packaging cell line. Such cell lines produce infectious, but replication incompetent virus particles containing the therapeutic gene(s) of interest. A potential hazard in the adaptation of retroviruses for use as gene delivery vehicles is the potential for recombination between vector and packaging cell line sequences to produce replication-competent retrovirus (RCR). The primary objective of the present study was to validate and determine optimal conditions for assays employed in the detection of RCR. The results indicated that murine *Mus dunni* and feline PG4 S^+L^- cells were of similar sensitivity to infection by amphotropic murine leukaemia virus (A-MLV); three passages were found to be sufficient to detect the presence of RCR at a virus input of 10^{-1} infectious units/ml. Using co-cultivation procedures it was possible to detect 1 RCR in a total of 10^7cells. It was determined that the presence of replication incompetent vector reduced the sensitivity of direct PG4 S^+L^- assay to detect wild-type A-MLV. This effect was reduced in extended *Mus dunni* and PG4 S^+L^- assays while the presence of retroviral vector producing cells did not affect the detection of A-MLV in co-cultivation assays. Accordingly, it is recommended that co-cultivation and/or extended assays should be performed for detection of RCR.

In addition, studies were performed to evaluate the parameters which influence production of a selected retroviral vector, including determination of optimal cell culture and harvesting conditions, investigating scale-up of production, and assessing stability and methods of concentrating viral vector supernatants. Parameters for optimal cell growth coupled with maximum viral titre for a model producer cell line were defined using tissue culture flasks, and found to be suitable for scale-up without loss of viral titre into roller bottles or Fibra-Cel disks in a spinner culture flask. Producer cells could also be adapted to culture in serum-free media although cell expansion was slower and a decrease of approximately 1 $\log_{10}$ unit in viral titre

was observed. Ultrafiltration was assessed as a method for increasing viral titre, where it was demonstrated that viral titre could be increased by 1 $\log_{10}$ unit following concentration. The stability of vector supernatant was examined by assessing viral titre following storage at different temperatures in relation to wild-type A-MLV. Resulting viral titres indicated that supernatant could be stored at -80°C for six months without loss of titre, whereas at 4°C titre decreased within 5 days. At 37°C the half-life of viral vector was determined to be approximately 5 hours after which complete inactivation was observed. Although inactivation may have been expected to be complete within a short incubation period at 56°C, the results indicated that a fraction of the virus was resistant to inactivation following incubation for two hours.

DECLARATION

The studies presented in this thesis were performed in Q-One Biotech Ltd, Glasgow, between 1994 and 1997. All the work is my own, without exception. I confirm that no part of this thesis has been, or is being, submitted for any other academic award.

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ABBREVIATIONS

A-MLV	Amphotropic murine leukaemia virus
ALV	Avian leukaemia virus
ATCC	American Type Culture Collection
BLV	Bovine leukaemia virus
CBER	Center for Biologics Evaluation and Research
cfu	colony forming units
CPMP	Committee for Pharmaceutical Medicinal Products
DNA	Deoxyribonucleic acid
dpm	disintigrations per minute
E-MLV	Ecotropic murine leukaemia virus
env	retrovirus gene encoding SU and TM proteins
FACS	fluorescent activated cell sorting
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FITC	Fluorescein Isothiocyanate
ffu	focus forming units
FLV	Feline leukaemia virus
G418	Geneticin (G418)
gag	retrovirus gene encoding internal proteins
GaLV	Gibbon ape leukaemia virus
GTAC	Gene Therapy Advisory Committee
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
HTLV	Human T-cell leukaemia virus
ICH	International Conference on Harmonisation
IDUA	α-L-iduronidase
LTR	Long terminal repeat

MCF	Mink-cell focus-forming
MLV	Murine leukaemia virus
Mo-MLV	Moloney murine leukaemia virus
Mg^{2+}	Magnesium cation
Mn^{2+}	Manganese cation
MPS	Mucopolysaccharide
MWCO	molecular weight cut-off
neo	neomycin transferase
O.D.	Optical density
PALidSN	Retrovirus vector producer cell line
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PBL	peripheral blood lymphocyte
psi	packaging signal sequence
RCR	Replication competent retrovirus
RPMI	Roswell Park Memorial Institute
pol	Retroviral gene encoding RT
RT	Reverse transcriptase
RNA	Ribonucleic acid
SIN	Self-inactivating vector
SU	major retroviral envelope glycoprotein
TCID	tissue culture infectious dose
TM	minor retroviral transmembrane protein
TPA	12.0 tetradecanoyl-phorbol-13-acetate
VSV	vesicular stomatitis virus
X-MLV	Xenotropic murine leukaemia virus

1.0 INTRODUCTION AND REVIEW

1.1 Introduction to gene therapy

Gene therapy, or transfer of "foreign" genetic material into patients for treatment or prevention of disease has been accepted for many years. The aim of such therapy is to provide gene functions which have been lost through mutation or caused by a genetic disorder, or to introduce genes to serve a particular function, such as resistance to cytotoxic drugs, inhibition of viral replication, or elimination of tumour cells. It could be argued that gene therapy encompasses attenuated viral vaccination, whereby replication and expression of viral genes results in an immune response to the gene product. The earliest example of such gene therapy was the vaccinia vaccine for smallpox (Newson, 1988), and today, vaccination with attenuated rubella, polio, measles and mumps viruses is widespread (Murphy and Chancock, 1990). Another example of therapy involving gene transfer into patients are allogeneic organ transplants (Carpenter, 1990).

In order to effect gene transfer, genetic material of interest must be included in a construct which either integrates into the target genome, replicates autonomously, or is otherwise expressed. The basic methods of gene insertion include transduction (by viral vectors) or transfection (by DNA-mediated systems) of target cells (Miller, 1992; Ledley, 1994). Gene transfer as a therapeutic method for the treatment of genetic disease and tumours is now feasible owing to significant advances in the development of recombinant virus vectors that encode the therapeutic genes in place of the viral structural genes. Vectors based on various viruses have been developed, including adenovirus (Le Gal La Salle *et al.*, 1993), pox virus, such as vaccinia (Cooney *et al.*, 1993), insect baculoviruses (Barsoum *et al.*, 1997) and retroviruses. Retroviruses have received the greatest attention as vectors for gene therapy because they can infect a variety of cell types, integrate stably into the target cell and have simple, well-

characterised genomes (Coffin, 1992, 1996). The first retrovirus vector was described in 1981 (Wei *et al.*, 1981) and the first patient was treated for severe combined immunodeficiency syndrome (SCID) by transference of the gene encoding adenosine deaminase in 1990 (Anderson *et al.*, 1990).

1.2 Retroviruses

1.2.1 Classification

The retroviruses comprise a group of infectious agents, identified by a common virion structure and mode of replication. Retroviruses are unique owing to the ability of their single stranded RNA genome to direct the formation of a double stranded DNA provirus (Coffin, 1992, 1996). Retroviruses interact with host cells to cause a variety of diseases, the effects of which may range from benign to fatal. Until recently, classification of retroviruses was based primarily on virion structure and pathogenicity, however, recently acquired gene sequencing information reflecting fundamental relationships has led to an updated classification system. Seven major subfamilies of retrovirus are now recognised; mammalian type B, murine leukaemia virus (MLV)-related, human T-cell leukaemia virus (HTLV)-bovine leukaemia virus (BLV) group, mammalian type D, avian leukaemia virus (ALV)-related, lentiviruses, and spumaviruses (Coffin, 1992, 1996). The term oncovirus encompasses the first five subfamilies; oncoviruses are usually leukaemogenic viruses, and may remain latent in the host, such as HTLV-1, or replicate and interact with cellular proto-oncogenes to induce tumours, for example feline, avian, and murine leukaemia viruses. Lentiviruses, such as human and simian immunodeficiency virus (HIV and SIV), are responsible for the induction of a variety of non-neoplastic diseases in humans and animals, while spumaviruses, such as human, simian and feline foamy viruses, have not yet been linked to any known diseases. Classification may also be based on virion structure (oncoviruses may be subdivided into types A to D based on morphology and pattern of

virion released from infected cells), utilisation of specific cell receptors (Sommerfelt and Weiss, 1990), presence or absence of an oncogene, and mode of transmission (endogenous [integrated in the germline] or exogenous).

Vectors based on several different retroviruses have been developed, including HIV-1 (Jolly *et al.*, 1994) and gibbon ape leukaemia virus (GaLV) (Miller *et al.*, 1991), however, those based on MLV have been most widely used in gene therapy to date. Infection of cells by enveloped viruses depends on viral proteins which bind the virus to the target cell and promote fusion of the viral and cellular membranes. The envelope proteins (*env*) of MLV-related viruses can be divided into different classes based on the cell surface receptors with which they interact (Rein and Schultz, 1984), and the host-range or tropism of the viruses is limited to host species which express the appropriate receptor (Sommerfelt and Weiss, 1990). Various types of endogenous type-C MLV have been defined on the basis of their ability to infect and replicate in different types of cells: ecotropic MLV (E-MLV), such as Moloney-MLV (Mo-MLV), is infectious for murine cells only, whereas xenotropic MLV (X-MLV) is infectious for non-murine cells only. Other types of MLV-related sequences in cellular DNA exist, such as polytropic sequences, which are involved in the generation of recombinant mink cell focus-forming (MCF) viruses. MCF viruses are recombinants between ecotropic and xenotropic endogenous viruses, and infect cells of both murine and non-murine origin (Rein, 1982). A separate class of virus, amphotropic MLV (A-MLV), isolated from wild mice (Hartley and Rowe, 1975), is also replicative in both murine and non-murine species, and has been shown to induce a high incidence of myeloid leukaemia in mice (Wolff *et al.*, 1991). Amphotropic virus exhibits a host range similar to that of polytropic viruses, but is distinct from other MLV species with regard to antigenic and interference properties (Rein, 1982). Different strains of A-MLV differ in their interference capacity to superinfection by other MLVs (Chesebro and Wehrly, 1985).

1.2.2 Structure and Replication

All retroviruses are similar in virion structure, genome organisation, and mode of replication (Coffin, 1992, 1996). The virion is enveloped and approximately 100nm in diameter. The genome is diploid, consisting of two molecules of single stranded ribonucleic acid (RNA) (7-10kb in length) enclosed in an icosahedral core. The RNA is reminiscent of cellular messenger RNA (mRNA), modified by capping at the 5' end, and polyadenylation at the 3' end. The core is bridged to the envelope by a matrix protein. To initiate viral infection, the envelope glycoprotein (SU) binds to a receptor on the host cell surface and a transmembrane protein (TM) mediates virus entry into the host cell by fusion of the viral envelope and cell membrane. The virion is released into the cytoplasm, where the virally encoded enzyme, reverse transcriptase (RT), transcribes the virion RNA into deoxyribonucleic acid (DNA) by the process of reverse transcription. Reverse transcriptase uses a molecule of host-cell transfer RNA (tRNA) as a primer to copy the viral genome into DNA, from which a second strand of DNA is synthesised (Panganiban and Fiore, 1988; Varmus, 1983). The resulting linear duplex DNA provirus, consisting of an internal region encoding the virion proteins flanked by two identical long terminal repeats (LTR) is shown in Figure 1.1. These terminal non-coding regions are essential *in cis* for viral replication, and contain the R, U3, and U5 regions. The LTR represents the intact regulatory region, and includes all transcriptional sequences required for expression of the viral DNA genome. Between the LTRs are three main genes encoding structural proteins, the order of which is invariably *gag-pol-env* where *gag* encodes the internal proteins, *pol* encodes RT, and *env* the SU and TM protein.

The provirus produced by reverse transcription is circularised and is inserted into random sites in the genome of the host cell, with the aid of the integrase function of RT. The viral DNA serves as a primer for the synthesis of viral RNA. Transcription of the provirus is affected by the chromosomal position of integration, the cell type infected,

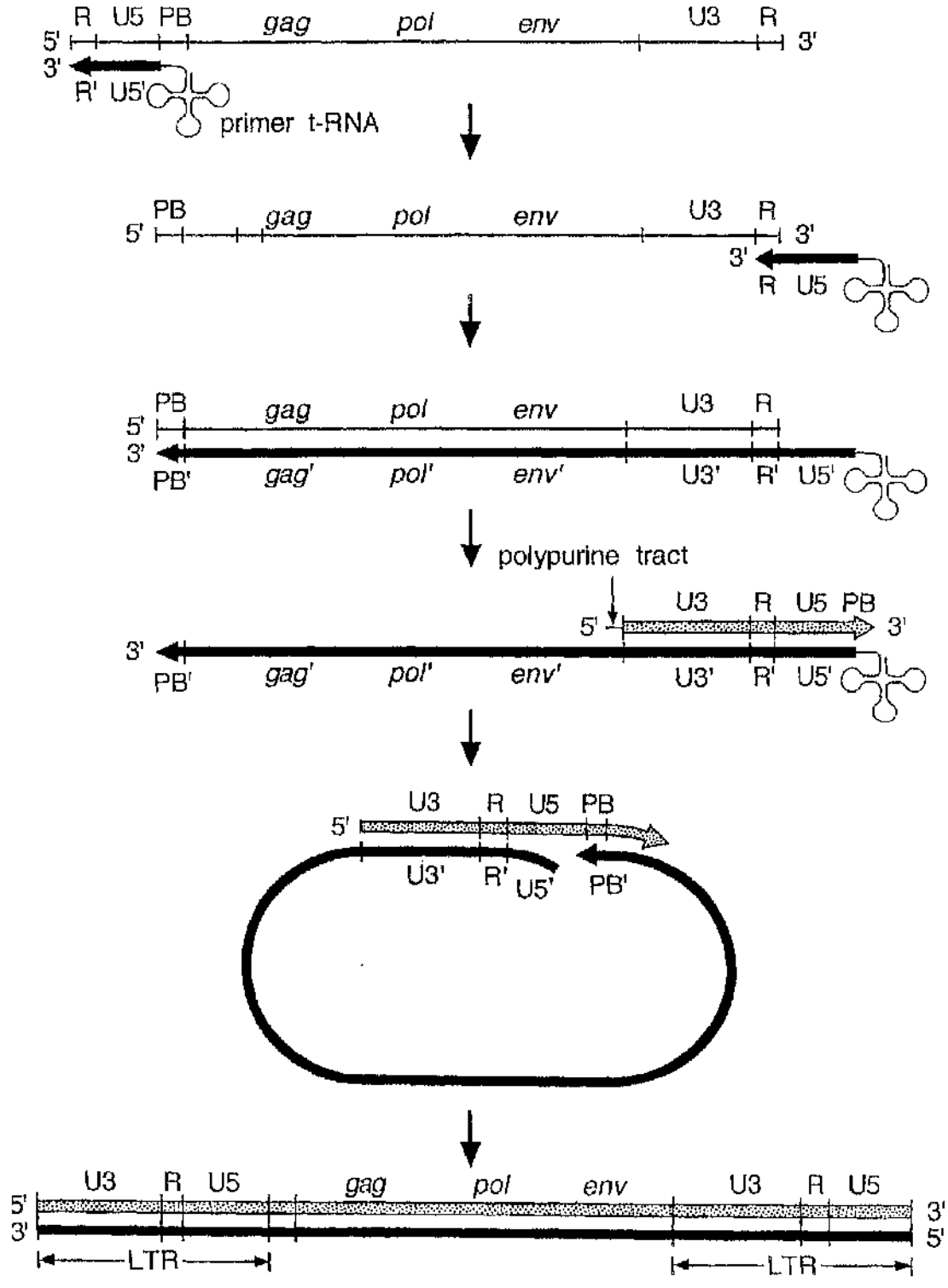

Figure 1.1 Double stranded DNA provirus

and the regulatory regions within the U3 region, which contains both the promoter and enhancer. The integrated DNA is normally transcribed by the host cell machinery into RNA that either acts as viral mRNA or is enclosed in a virion and released by budding from the host cell membrane. A less abundant mRNA produced by the action of the splice donor site in the 5' LTR region is used to express *env*. Translation of the full length genomic transcripts results in the expression of the *gag, pol,* and *env* proteins (Weiss *et al.*, 1983;1985). Packaging of the genomic transcripts into virions requires an RNA signal known as the *psi* sequence (Mann *et al.*, 1983) which lies 3` of the 5` LTR. This sequence is not present in spliced messages, therefore they cannot be packaged (Mann *et al.*, 1985).

1.3 Retroviruses as Vectors

Retroviruses which transform cells in culture are often defective for replication because essential replicative genes have been replaced by cellular genes. These cellular genes acquired by the virus genome are known as oncogenes, and confer upon the virus the ability to transform normal into malignant cells (Neil *et. al.*, 1983). In order to replicate, these viruses require the presence of a helper virus such as MLV, to supply the necessary functions for replication and packaging via functional *gag, pol,* and *env* genes. The defective, oncogenic viruses are called sarcoma viruses, and the replicating helpers, which are generally non-transforming, are leukaemia viruses.

Retroviruses which contain transforming oncogenes, such as *myc*, are natural vectors for the transmission and expression of foreign genes. Thus, retroviruses can be genetically manipulated to mimic natural modifications, and become vehicles for the delivery and expression of cloned genes in eukaryotic cells. The viral protein coding elements of a retrovirus can be deleted and replaced prior to packaging in a cell line which expresses the stuctural proteins, thus enabling infectious recombinant virus particles to be formed. Features of retroviruses favouring this purpose include their relatively small genome, which can be easily manipulated to introduce foreign genes,

such that resulting defects can be complemented for *in trans*, the high titres to which they grow in culture, and the potential of high efficiency of infection of target cells. Retroviruses carry powerful transcriptional enhancers that ensure a high level of gene expression. Infection leads to integration of retroviral DNA in the host cell genome such that virtually all infected cells can express the gene carried by the virus (Gluzman and Hughes, 1988). In addition, the broad tropism of retroviruses permits a wide range of cell types to be infected, including cells of the hematopoietic system. There are however, some limitations to the use of retroviral vectors. Retroviruses can only integrate into mitotically active cells (Roe *et al.*,1993), and the size of the DNA insert is limited owing to the small overall size of the retroviral genome. However, the main concern in the use of retroviruses as vectors for gene therapy is that of safety, based on the possibility that recombination events could result in the production of wild-type replication competent virus (See section 1.7 below).

1.4 Retrovirus Vector Systems

Retrovirus vectors should resemble natural defective transforming viruses where viral products essential for the replication and integration of transforming retroviruses are supplied *in trans* by a non-transforming, replication competent helper virus. Thus, a retrovirus vector system is designed to follow nature in having two elements: a replication-defective vector (section 1.4.1) and helper virus, or `packaging' cells (section 1.4.2). Retroviral vector particles lack coding sequences essential for generation of progeny while retaining the *cis*-acting elements essential for virus infection, reverse transcription, integration, and transcription in transduced cells. Packaging cells provide *in trans* the coding sequences for retroviral enzymatic and structural proteins.

1.4.1 Vector constructs

Retroviral vectors are constructed as part of plasmid DNA molecules which can be grown in bacteria and manipulated by recombinant DNA techniques (Miller *et al.*, 1993). Vectors are constructed from the DNA form of the retrovirus, which corresponds to the integrated provirus. The vector must behave as a viral genome to allow it to pass as a virus from the producer line, and therefore must retain the *cis*-acting sequences that allow it to replicate efficiently. These essential sequence elements are: the two LTRs, the packaging signal sequence (*psi*), which lies close to the 5` LTR, and the primer binding sites for reverse transcription, which lie adjacent to the LTRs. In retroviruses, the major determinant of RNA packaging is the *psi* sequence, which maps between the 5` LTR and the start of the *gag* coding sequence (Mann *et al.*, 1983). All the remaining sequences are dispensible, as their loss can be complemented for *in trans*. However, inclusion of a portion of the *gag* region into a vector construct has been shown to significantly increase viral titre, suggesting existence of an extended signal sequence which results in more efficient packaging (Bender *et al.*, 1987). Efficient vectors usually contain part of *gag* and have deleted splice donor sites and mutation of the *gag*$^+$ start codon. The *gag* sequence must be in its native position, adjacent to *psi*, to have an effect of 5-10 fold increase in viral titre (Morgenstern and Land, 1990). Thus, the structure of the provirus, with regulatory regions located at the ends of the genome, and structural genes occupying the central 8kb region, makes it relatively simple to replace the central structural genes (*gag, pol, env*) with therapeutic genes of interest. The loss of these sequences can be complemented *in trans* by a packaging cell line, the simplest of which consists of a provirus in which the *psi* sequence has been deleted (Mann *et al.*, 1983). When a recombinant provirus is introduced into packaging cells viral particles are produced which contain almost exclusively RNA genomes of the recombinant vector. Thus, the encapsulated recombinant is infectious and integrates into the host cell, but the RNA transcripts produced are not capable of self-replication, and cannot be packaged into viral particles.

A safe retrovirus vector should contain the minimal amount of retroviral sequence consistent with its ability to be packaged, undergo reverse transcription and integrate. Many vectors employed in gene therapy to date have been based on Mo-MLV, modified to produce either a single gene or two different genes (a therapeutic gene and a marker gene). Gene expression can be driven by the endogenous retroviral promoter (the LTR), or the second gene can be cloned in with its own promoter (commonly the SV40 or herpes simplex virus thymidine kinase (HSV-TK) gene promoters) to drive expression. A minimal vector generally contains only 10% of sequences found in a replication competent virus, as the viral structural genes are deleted and replaced with one or more restriction enzyme sites for the insertion of new sequences. This minimal vector is self-inactivating (SIN) (Yu *et. al.*, 1987) owing to the lack of a complete 3` U3 region. The proviral LTRs in infected cells are transcriptionally inactive, eliminating the possibility of transcriptional activation of adjacent genes. Although considered safer than double-gene vectors, SIN virus titres obtained to date have been low, in the region of 10^2 colony forming units per ml (cfu/ml) (Yee *et al.*, 1987). These simple vectors are limited in their applications because of the low viral titres obtained and their restriction to transmission of genes eliciting a clearly distinguishable phenotype owing to the lack of a suitable selectable marker. Double gene vectors include a gene encoding a selectable phenotype. Several selectable markers have been used for this purpose, including the genes encoding neomycin phosphotransferase (*neo*) (Cepko *et al.*, 1987) and thymidine kinase (TK) (Caruso *et al.*, 1993). A wide variety of vector constructions have been described, including those with LTRs as the major promoters (Cepko *et al.*, 1987), with internal promoters (Overell *et al.*, 1988) and with the gene of interest expressed in reverse orientation (Dzierzak *et al.*, 1988).

The more distal gene may be expressed from an internal promoter or from spliced subgenomic mRNA analogous to *env*. An example of a double gene splicing vector is pZIP Neo SV(X) (Cepko *et al.*, 1987) in which the gene encoding *neo*, which confers

resistance to the antibiotic Geneticin (G418) is inserted in the *env* equivalent position in the vector genome. A selected gene can be cloned into a BamH1 site at the *env* equivalent position. In this construction the LTR transcription signals drive expression of both genes. However, an internal promoter in either orientation relative to transcription may drive the more distal gene from the viral LTR. One commonly used internal promoter is the SV40 early promoter. This type of vector has possibilities for regulating the introduced gene by employing either inducible, cell-type specific, strong or weak promoters in vectors. A disadvantage to this type of vector is that an LTR-based promoter can exert a negative effect on promoters downstream of it, disturbing the relative levels of expression from the two promoters (Emerman and Temin, 1984). "Promoter selection" may be partially overcome by constructing double expression vectors with the internal promoter in the reverse orientation to the direction of transcription from the LTR.

1.4.2 Packaging cell lines

Recombinant vector constructs can be transcribed, processed, packaged, and replicated, but the viral functions necessary for the assembly of recombinant virus particles must be provided for *in trans* by a packaging cell line. Many MLV packaging cell lines are derived from adherent murine cell lines engineered to produce *gag*, *pol*, and *env* protein, but no full length genomic RNA or RNA which can be encapsidated. First generation packaging lines consisted of a provirus in which the *psi* sequence had been deleted (Mann *et al.*, 1983). However, recombination events between homologous regions of the vector and the packaging line resulting in the production of wild-type RCR has been observed in such cell lines (Miller, 1986, Otto *et al.*, 1994; Vanin *et al.*, 1994). In order to decrease sequence homology between the vector and packaging sequence, second generation packaging lines have been developed in which the 3' LTR was deleted as well as the *psi* sequence, and replaced with heterologous promoters and polyadenylation sites. Thus two recombination events between the

vector and packaging line are necessary in order to generate RCR. An example of a second-generation packaging line is the amphotropic retrovirus packaging cell line PA317 (Miller and Buttimore, 1986). PA317 cells were derived from NIH 3T3 thymidine kinase (TK)$^-$ cells by co-transfection with retrovirus packaging construct DNA (pPAM3) and the HSV TK gene. Introduction of retroviral vectors into these cells by transfection or infection results in the production of retrovirus virions with an amphotropic host range. However, even with deletions of *psi*, the 3' LTR, and part of the 5'LTR in second generation packaging cells, the possibility of producing RCR on introduction of a vector still exists (Danos and Mulligan, 1988). To further reduce the possibility of RCR production, third generation packaging cells have been developed, in which the *gag-pol* and *env* genes are introduced to the packaging cells on different DNA constructs (Morgenstern *et al.*, 1990). Sequential introduction reduces the high frequency of recombination that occurs during transfection. The amphotropic lines CRIP (Danos and Mulligan, 1988) and GP+*env*Am12 (Markowitz *et al.*, 1988) are two examples of packaging lines which, in addition to the deletions described above, contain viral DNA in which the *gag* and *pol* genes are on one plasmid and the *env* gene on another. Two independent rounds of transfection are employed to produce these cells: firstly, the *gag-pol* construct can be transfected and clones expressing *gag* and *pol* proteins selected for by reverse transcriptase assay. It is possible to determine the host range conferred by the packaging line in the second round of tranfection by choosing appropriate *env* gene constructs. Production of a functional envelope in secondary transfectants can then be tested for using a packaging assay. An advanced system has been developed involving the generation of the ΩE cell line, a high titre ecotropic packaging cell line (Morgenstern *et al.*, 1990). This line was developed with separate *gag-pol* and *env* constructs with minimum sequence overlap and decreased sequence homology achieved by 'codon wobbling'. The ΩE line was developed in conjunction with pBabe retroviral vectors, in which homologous *gag* coding sequences have been deleted. Together, the vector and packaging line reduce the risk of generating RCR through recombination events. These third generation lines increase

both safety and efficiency for gene transfer into cells, owing to the fact that three separate recombination events would be necessary to form an intact genome, which is likely to be a rare event. An estimate of the potential for recombination occurring would be difficult to estimate at present, owing to the frequency rate being thought to depend on the particular retroviral vector system.

1.5 Producer Cells

1.5.1 Introduction of vector into a packaging cell line

The introduction of a vector into a packaging cell line gives rise to a producer cell line which makes only replication-defective vector particles capable of introducing their genes into target cells. Producer cells release virus particles containing an RNA transcript of the vector including the *cis*-acting sequences necessary for its reverse transcription and integration. As many MLV packaging lines described to date are derived from murine NIH3T3 or BALB/c 3T3 cells, vector DNA can be introduced by transfection using a standard calcium phosphate precipitation method. However, enhanced virus titres are obtained if the vector is introduced by infection rather than by physical transfection techniques (Hwang *et al.*, 1984). Selecting for a dominant marker carried by the vector itself, or on a co-transfected plasmid, gives rise to virus produced from a stably transfected packaging line. Virus produced from transfection of one packaging line can be used to infect a second line. Packaging cells have immunity to infection by viruses that use the same cellular receptor; therefore a cell infected by one retroviral subgroup can only be infected by virus of a different subgroup (Chesebro and Wehrly, 1985). It is possible to employ a 'shuttle system' where a vector is transfected into one packaging line and shuttled into another by infection (Cepko *et al.*, 1984: Berger and Bernstein, 1985). For example, the vector could be transferred from an ecotropic to an amphotropic packaging line, or vice versa. Danos and Mulligan (1988) shuttled a vector between CRE cells (the ecotropic equivalent of CRIP) and either CRIP

or PA317 amphotropic lines. When the vector was psuedotyped several times to increase the possibility of detecting RCR, all virus stocks from the CRE/CRIP shuttling system were negative for the presence of RCR. Producer clones with titres of 10^6 cfu/ml were isolated by this method. However, recombination was apparent after the fourth and fifth round of infection in two separate experiments using the CRE/PA317 shuttling system. Advantages in sequential use of two different packaging lines over single-step transfection include a 100-fold increase in viral titre and infected colonies which are genetically more uniform, owing to individual copies of the proviral DNA being integrated in a precisely defined manner (Danos and Mulligan, 1988).

1.5.2 Production of vector

Initially, the producer cell line should be cultured, and a series of growth curves constructed to determine the optimal media formation and cell seed concentration for maximum cell growth. Media components may have an adverse effect on culture of producer cells, for example, it may desirable to adapt cells to serum-free media, as *in vivo* studies have shown that retroviral vectors are inactivated by serum in an antiviral immune response (Hodgson, C., 1995). Kotani *et al.* (1994) described the production and optimisation of transduction in twenty-two retroviral vector producer lines. They compared vector production at 32°C and 37°C in PA317 producer cells and found that although vector particle growth was slightly reduced at 32°C, the stability and titre of the vector were substantially higher. Therefore, the temperature at which retroviral vectors are produced may be important in the transduction efficiency of retroviral vectors. Assays should be also performed to determine the optimal titre obtainable for the viral vector. Small-scale evaluation studies should be performed to optimise vector production, and set the parameters for scaled-up production. Systems for culturing producer cell lines include tissue culture flasks, roller bottles, and the CellCube Bioreactor [The area of one Cellcube is equivalent to 25 roller bottles ($850cm^2$)]. Large scale production of retroviral vectors would be greatly improved if producer lines could

be grown as single cell suspensions, and other factors which influence large-scale production of retroviral vectors need to be defined.

1.5.3 Concentration of retroviral vector supernatant

Membrane technologies such as ultrafiltration may be used to concentrate retroviral particles using membranes with molecular weight cut-off (MWCO) values of 100 or 300kDaltons. Kotani *et al.* (1994) reported an approximate 20-fold increase in vector titre with over 90% recovery of infective particles using a Pellicon tangential flow concentrator using a 300kDalton MWCO membrane, generating viral vector titres of around 10^7cfu/ml. In another study, Saha *et al.* (1994) reported a 30-fold increase in concentration of MLV without loss of infectivity using a 100 kDalton MWCO.

1.5.4 Storage and stability of retroviral vectors

Recombinant retrovirus stocks are obtained from producer lines by harvesting culture medium when the cells are growing exponentially (Morgan *et al*, 1995, Roe *et al.* 1993). One method is to add fresh culture medium to producer cells cultured in tissue culture flasks at 37°C on reaching at 80-90% confluence and harvest the supernatant 24 hours later (Andreadis and Palsson, 1997). The half-lives of retroviral vectors cultured at 37°C have been measured to be 5 hours (Sanes *et al.*, 1986), 3.5-6.5 hours (Paul *et al.*, 1993) and 6-8hours (Chuck *et al.*, 1996). The length of time for which a retroviral vector can be maintained in culture under conditions that produce quality commercial products has yet to be fully established.

Retrovirus stocks should remain stable stored at -70°C in harvested tissue culture medium containing foetal bovine serum (FBS). Lyophilisation with storage at -20°C may be accomplished relatively simply, resulting in a long half-life (>200 days). Kotani *et al.* (1994) used a combination of additives including 50% glucose, 50% sorbitol, and

25% gelatin in phosphate buffered saline (PBS) resulting in a recovery rate of 64-83%. Retrovirus vectors have been described as displaying stability to variations in pH, ionic strength, and shear forces (Naussbaum *et al.*, 1993). Kotani *et al.* (1994) used another approach to enhance transduction efficiency in NIH-3T3 cells. Centrifugation of vector supernatants onto target cells to concentrate the viral particles directly onto the surface of cells increased transduction efficiency as measured by vector titration for Geneticin resistance, fluorescence-activated cell sorting (FACS), and polymerase chain reaction (PCR) analysis.

1.5.5 Determination of vector titre

Some retroviral vectors contain no selectable marker, and determination of vector titre is based on a measure of the gene product, for example, enzyme activity or protein concentration. These factors can vary widely between producer clones and data must be generated through extensive evaluation studies. Viral titre can be determined most accurately when a selectable marker is present in the producer line (Vile, 1991). For example, inclusion of the HSV-TK gene in producer cells which are treated with a nucleoside analogue such as ganciclovir means that dividing cells are killed, owing to the phosphorylated nucleoside acting as a chain terminator (Caruso *et al.*, 1993). Cells deficient in the expression of the TK gene can be grown in HAT (hypoxanthine/aminopterin/thymidine) medium. Selection must be positive, so that the cells of interest remain alive. The pBabe retroviral vector constructs transmit inserted genes at high titre from the Mo-MLV LTR. These vectors have been constructed with four different dominantly acting selectable markers, allowing the growth of infected mammalian cells in the presence of Geneticin, hygromycin B, bleomycin/phleomycin or puromycin. High titres are obtained from a third generation ecotropic packaging line (ΩE) designed in conjunction with pBabe vectors (Morgenstern *et al.*, 1990). Titration by non-selectable gene expression requires a method tailored to suit the specific producer line concerned. For example, a retrovirus vector coding for human α-L-

iduronidase has been prepared for use in a clinical trial for treatment of human mucopolysaccharide disease (MPS), using a neo-organ approach as used for the MPS VII disorder in mice (Moullier *et al.*, 1993). This vector is titrated by assaying the level of enzyme in supernatant from vector-infected MPS I cells using a fluorimeter. Another commonly used titration method to determine the quantity of viral genomic RNA in supernatant is hybridisation with a ^{32}P-labelled DNA probe. This is performed using a standard RNA dot-blot procedure after concentrating the virus. A quantitative determination is obtained by comparing hybridisation intensities with an accurately titrated control virus.

1.6 Method of delivery

Application of retroviral vectors may be applied in treatment of disease such as cancer whereby the transduced gene expresses an enzyme capable of converting a pro-drug into a cytotoxic drug, induces an immunological response, confers drug resistance, or restores tumour supressor genes (Jasmin, 1993). Treatment of the patient may be in the form of viral supernatant harvested from the producer cells, or direct injection of the producer cells themselves. For application in early stage clinical trials, where the target cells are haematopoietic stem cells or lymphocytes, or other easily isolated cell types, administration of retroviral vectors is achieved *ex vivo*. This is achieved by removing a biopsy from the patient, and expanding the cells prior to transduction *in vitro* with retroviral vector. Doses are then stored frozen and re-administered by injection to the patient. This can be an expensive procedure, but overcomes the potential problem of low infectivity of retroviruses, as it is possible to perform the transduction in a way in which infection can be increased or stimulated. An alternative method of application to patients is direct administration of retroviral vectors. Producer cell lines secreting retroviral vectors encoding HSV-TK have been injected into brain tumours (Culver *et al.*, 1992) and intramuscular injection of HIV-1 vectors expressing *env* have been employed to stimulate cellular immune responses to HIV-1 (Jolly *et al.*, 1994). The

ideal method of gene delivery for general clinical use is the direct (or *in vivo*) approach, where highly concentrated and purified retrovirus vectors can be prepared as ready-to-inject doses, and directly administered to a large patient base.

1.7 Targetting of retroviral vectors

The host range of MLV-based vectors can be extended by pseudotyping, which is achieved by packaging the vector genome in a virion particle bearing envelope proteins from another virus (Kasahara *et al.*, 1994.) The vectors currently used in clinical trials are made in hybrid systems, where the host range of ecotropic Mo-MLV is altered by combining the *gag-pol* gene from Mo-MLV with an *env* gene from another source (pseudotype formation). For example, combination with MLV strain 4070A, which has an amphotropic envelope, allows the vector to infect a variety of cell types, including human cells (Porter *et al.*, 1996). Approaches employed in targetting specific host cell types include the use of tissue specific enhancers and promoters, alteration of the binding site of the *env* gene for attachment to specific cell receptors, and incorporating new ligands into the vector membrane which bind to specific cell receptors. However, such targeting approaches are still under development (Gunzburg *et. al.*, 1995).

1.8 Safety Considerations

The main concern in the development of biopharmaceutical applications is the production of safe products. Regulatory authority guidelines outlining quality and safety testing strategies are in place, advising on procedures recommended for a biotechnology derived product to obtain market authorisation. For any cell-based production process, full characterisation and testing of the master and working cell banks, intermediate harvests, the end of production cells, and the final product itself, is recommended by the authorities (CBER, 1997; ICH Q5A, 1996). Examples of testing recommended for the presence of adventitious agents include;

- mycoplasma and sterility screening
- identity testing
- *in vitro* assay (inoculation of supernatant onto different detector cell lines, including human and primate, in order to detect a range of viral contaminants)
- *in vivo* assay (inoculation of sample into animal systems in order to detect a range of viral contaminants which are not easily detected *in vitro*)
- testing for the presence of retrovirus contamination using a variety of techniques
- analysis by electron microscopy
- testing for the absence of infectious murine viruses produced from murine cell lines
- testing for the presence of bovine and porcine viruses (if exposure to serum and trypsin from these sources has occurred, this is essential).

In addition to virus safety testing, validation of a down-scaled version of the manufacturing process used for production is required, to demonstrate that any virus which was inadvertently present in the manufacturing facility would be removed or inactivated by the production process (CPMP, 1996). This provides additional confidence in the safety of the final product.

In addition to the above testing recommendations, specific guidelines have been implemented with regard to gene therapy studies, and in particular RCR testing. At present, production and purification processes used for retroviral vector systems do not contain process steps that will eliminate contaminants, therefore it is not feasible to perform virus validation studies to provide additional data concerning the safety of the product. Thus, thorough characterisation of the producer cells, and validation of both the packaging cell line and the recombinant gene constructs is essential, involving DNA fingerprinting, sequence analysis, and restriction mapping.

It is clear that safety issues are paramount in the use of retroviral vectors for treatment of disease by gene therapy (Smith *et al.*, 1996, Ostrove, 1994, Anderson *et al.*, 1993, Cohen-Haguenauer, 1995). In Europe, the Committee for the Proprietary Medicinal Products (CPMP) issued guidelines addressing quality aspects with regard to materials used for production of gene therapy products (CPMP, 1995). The Center for Biologics Evaluation and Research (CBER) of the Food and Drug Administration (FDA) have established guidelines for screening post-production vector producing cell lines and vector-containing supernatant used in production of retroviral vector products for human application, for the presence of RCR (CBER, 1991,1996, 1998). Vector-producing cell lines and culture supernatant require sensitive screening for the presence of RCR prior to use in the clinic, owing to the fact that recombination events between homologous sequences in the vector and packaging cell line may lead to the generation of RCR (Otto *et al.*, 1994, Vanin *et al.*, 1994). Otto *et al.* (1994) used retroviral-specific primer pairs to demonstrate that recombination, rather than a contamination, created a novel virus in retoviral supernatant harvested from PA317/G1Na producer cells.

Knowledge of the effect of MLV infection in primates is limited. Although MLV has been reported to be of limited pathogenicity in rhesus monkeys (Cornetta *et al.*, 1990, 1991), A-MLV was reported to induce T-cell lymphomas in three out of ten immunosuppressed macaques (Donahue *et al.*, 1992): In the latter study, exposure of RCR following ex-*vivo* transduction of bone marrow cells has been proven to be the cause of disease. The monkeys that died had prolonged retroviremia, and murine RCR sequences were detected in the lymphomas (Vanin *et al.*, 1994, Purcell *et al.*, 1996). This finding has increased concerns about the risk of MLV exposure in humans.

The need for safer retroviral vector systems which decrease the possibility of RCR generation has resulted in advances in vector design, and the development of third generation packaging cells, which require three separate recombination events in order

to generate RCR. This significantly reduces the frequency of recombination between vector and packaging sequences, and necessitates the employment of RCR detection assays of high sensitivity.

CBER recommend that product release testing for retroviral vector use in gene therapy includes amplification of 5% of the total vector-containing supernatant volume on an MLV replication-permissive cell line, for example, *Mus dunni* cells (Lander *et al.*, 1984), and 1% of the total post-production vector producing cells (or 10^8 cells, whichever is less) by co-cultivation with an MLV replication-permissive cell. Supernatants generated from extended *Mus dunni* and *Mus dunni* co-cultivation assays should be tested for the presence of RCR using the PG4 S^+L^- (PG4) assay. This is a simple S^+L^- assay using feline PG-4 S^+L^- cells to detect the presence of xenotropic or amphotropic retroviruses (Bassin *et al.*, 1982). PG-4 S^-L^- cells contain a defective murine sarcoma virus genome (sarcoma positive, leukaemia negative [S^+L^-]). Superinfection of the PG-4 S^+L^- cells with RCR results in rescue of the murine sarcoma virus. Replication of the murine sarcoma virus results in the transformation of MLV-infected PG-4 S^+L^- cells, and thus the formation of foci, which can be visualised via low-power light microscopy (Bassin *et al.*, 1982). An alternative to the PG4 S^+L^- assay is the marker rescue assay (Forestell *et. al.* 1996), which is acceptable to the authorities (CBER, 1996), if validated, and of sensitivity comparable to the PG4 S^+L^- assay. The detection assays are required to be of high sensitivity, as high levels of replication-incompetent particles have been reported to interfere with the detection of RCR in vector supernatants (Printz *et al.*, 1995). Therefore, assay sensitivity should be determined in the presence of high titre retroviral vector.

Testing at various stages of production is required owing to limited knowledge of the risks of retrovirus exposure and the possibility of generating recombinant RCR at any point in the production process (CBER, 1996). An improvement in the safety of retrovirus vectors may be achieved by incorporation of the packaging constructs into

cells that do not contain endogenous retroviruses. Most amphotropic packaging lines are based on NIH 3T3 cells which express endogenous VL30 retroviral related sequences. These sequences contain LTRs, and could act as insertional mutagens if packaged into virions. Therefore, safety could be improved in packaging lines by the use of non-murine cells, such as canine or human cells, in which endogenous retroviral elements are defective. A human-derived packaging cell line based on 293 cells was recently reported to transduce target cells with high efficiency (Davis *et al.*, 1997).

An additional safety issue in the use of retroviral vectors in gene therapy is that insertional mutagenesis may induce oncogenesis, either by disrupting a gene involved in growth suppression, or by activating a proto-oncogene. Therefore, a goal of current retroviral vector technology is to develop site-specific integration.

1.9 Summary

The safety of a viral vector for gene therapy is a combination of good vector design, controlled production and extensive evaluation and testing, which can be displayed as a 'safety triad' (Smith *et al.*, 1996; Figure 1.2). Retroviruses are well characterised and are suitable as vectors for gene delivery owing to the unique characteristics of the virus replication cycle.

Advances in the development of packaging cell lines and vector constructs should permit safer and more efficient gene transfer. Recombinant virus titres have been improved by the inclusion of *gag* sequences in the vector construct and, by introduction of the vector construct to the packaging cell by infection, rather than transfection. Packaging cell lines have been constructed in which the *gag/pol* and *env* products are produced from separate transcriptional units, which both increases safety, and allows the construction of pseudotyped vectors.

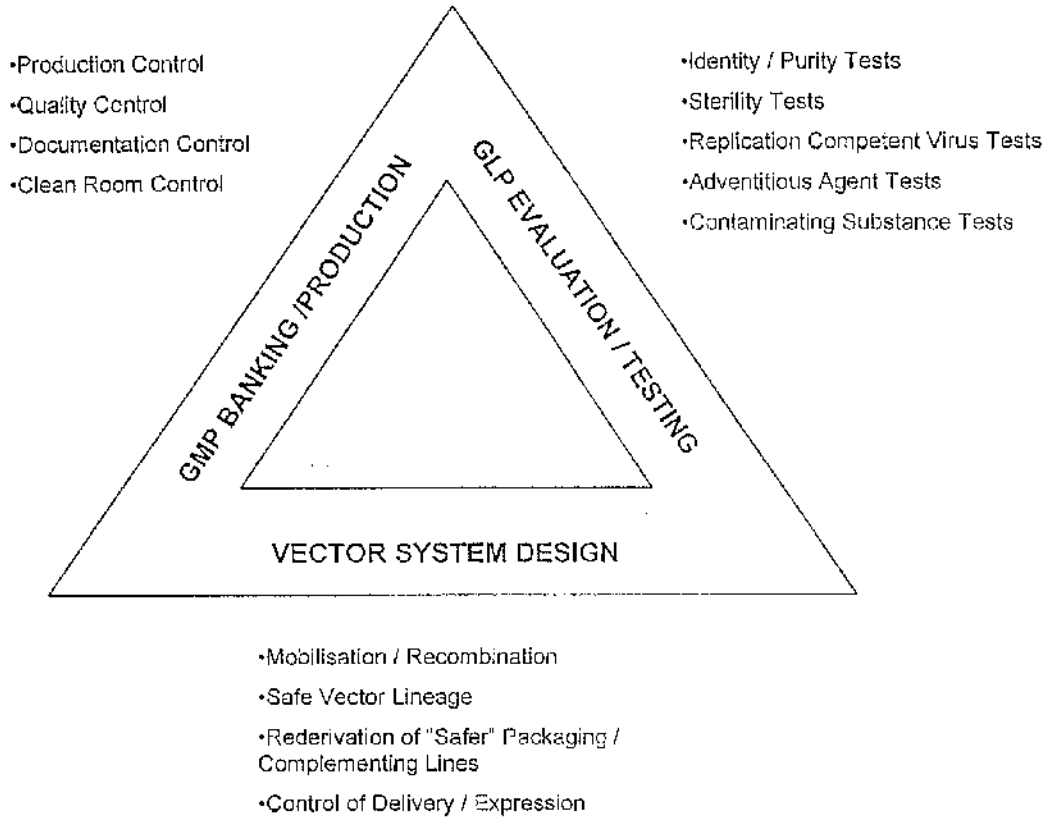

Figure 1.2 Safety considerations in production of retroviral gene therapy products

Retroviral vectors bind to a host cell and insert the vector sequence, resulting in stable integration of the genetic material into the host genome without detrimental effects on the cell. However, the random way in which the provirus integrates into the host genome is undesirable owing to the potentially variable level of gene expression, and the potential for insertional mutagenesis to occur. Other limitations in the use of retroviral vectors include the inability to transduce non-dividing cells, production of low-titre stocks (rarely greater than 10^6 units/ml), and an upper limit to the inserted gene size of approximately 9kb. Gene expression from retrovirus vectors can be achieved in a broad range of tissues, though many applications have targetted haematopoetic stem cells, which are easily accessed, and infection can be performed *ex vivo*.

The ideal retrovirus vector should have the following characteristics; reproducibly high-titre stock; tissue-specific delivery; optimal and regulated expression level of the transgene; and site-specific integration. However, tissue specific vectors with regulated, stable gene expression have yet to be developed. Production of retroviral vectors should be evaluated to provide optimal small-scale culture conditions that will provide stable, high titre vector stocks for use in clinical trials. One study has indicated that growth of cells at 32°C results in increased viral titre in producer cell stocks (Kotani *et al.*, 1994). For clinical trials, large volumes of vector are required at the highest possible titre. Production parameters identified in the evaluation phase would be used to scale-up production to the most suitable and cost-effective technology, for example, roller bottles, the CellCube system or the Brunswick Celligen system. Recent advances in vector production technology include optimisation of vector production by concentration and lyophilisation, as well as large-scale production of producer cells for the treatment of brain cancer. Various techniques, such as ultrafiltration can be used to concentrate large volumes of viral vector.

Retroviral vectors for use in gene therapy should be produced safely and efficiently, free from contaminating adventitious agents. The potential risk of exposure of patients to RCR is of concern. Development of complementing packaging cell lines and vector constructs with minimum sequence homology has increased the safety of retroviral vector systems. Third generation packaging cell lines have been developed to reduce homology between the vector construct and packaging cell line so that three separate recombination events would be required to generate RCR. A further increase in safety is manifest in currently used packaging cell lines which express the *gag* and *pol* proteins from one inserted provirus construct, and the *env* proteins from a second. The development of non-murine packaging cells would confer further advances in the safety of retroviral gene therapy products.

2.0 VALIDATION OF ASSAYS FOR DETECTION OF REPLICATION COMPETENT RETROVIRUS

2.1 Introduction

Many gene therapy protocols employ recombinant retroviral vectors, which are replication-defective retroviruses designed for gene transfer. Most retroviral vector systems are based on replication defective murine retroviruses, and consist of two components, a packaging cell line, and the vector (as discussed in section 1.4). The *env* component of the vector has been most commonly based on A-MLV, which was isolated from wild mice (Hartley and Rowe, 1975). The *env* component from other retroviruses, for example GaLV, has also been incorporated into vector constructs (Lam *et al.*, 1996). Transfecting or transducing packaging cells with a defective retrovirus vector incorporates the vector RNA into virus particles. These virions are capable of infection of target cells to integrate a DNA copy of the vector genome into the target cell, but are incapable of further replication.

A potential problem in using retroviral vectors is the possibility of generating replication competent retrovirus (RCR) through recombination events between the vector and the packaging sequence. The potential hazards of the presence of A-MLV are not easily predicted. One study indicated that A-MLV was not an acute pathogen in rhesus monkeys, as viraemia was not observed following administration of high titre RCR (Cornetta *et al.*, 1990). In contrast, another study reported the development of T-cell lymphomas in immunosuppressed monkeys following exposure to high levels of helper virus during retrovirus mediated gene transfer (Donahue *et al.*, 1992). However, the differences in these results may be partially explained by the fact that Cornetta injected the RCR intravenously, resulting in virus being lysed through complement inactivation, whereas Donahue's group transduced the cells *ex vivo*. Transient transfections of

vector constructs into packaging cell lines can result in generation of wild type virus in harvested supernatant, therefore development of safer systems has been a focus of research. Although the packaging cells and vector constructs have been modified to reduce the possibility of recombination occurring (Danos and Mulligan, 1988), safety testing for detection of RCR is of utmost importance. Current recommendations for the detection of RCR in retroviral vector products are set by the FDA Center for Biological Evaluation and Research (CBER, 1991, 1996, 1998) in the United States, and the CPMP (1995) in the European Union. As for all biopharmaceuticals, a system of Master Cell Bank (MCB) and Working Cell Bank (WCB) should be set up to provide fully-characterised starting material for the clinical lot (CBER, 1993). Testing at various stages of production is required owing to limited knowledge of the risks of retrovirus exposure and the possibility of generating recombinant RCR at any point in the production process. The required tests include both an amplification assay and co-cultivation assay with a cell line permissive to A-MLV infection. A commonly used cell line for RCR detection is derived from the tail fibroblasts of the Asian wild mouse, *Mus dunni,* which is permissive for replication of all classes of MLV (Lander and Chattopadhyay, 1984). The supernatant resulting from amplification or co-cultivation assay should be tested by feline PG-4 S^+L^- assay (Bassin *et al.*, 1982). PG-4 S^+L^- cells contain a defective murine sarcoma virus genome (sarcoma positive, leukaemia negative [S^+L^-]). Superinfection of the PG-4 S^+L^- cells with RCR results in rescue of the murine sarcoma virus. Replication of the murine sarcoma virus results in the transformation of MLV-infected PG-4 S^+L^- cells, and thus the formation of foci, which can be visualised via low-power light microscopy (Bassin *et al.*, 1982). *Mus dunni* cells are highly permissive for all MLV types and lack sequences homologous to known murine retroviruses, and are therefore unlikely to generate RCR spontaneously (Lander and Chattopadhyay, 1984). Thus, any RCR detected can be presumed to have originated from the sample being tested. The guidelines recommend testing 5% of the total supernatant from a cell bank by *Mus dunni* infectivity assay, and 1% of total pooled cells (or 10^8 cells, whichever is less) by co-cultivation with *Mus dunni* cells (CBER,

1996, 1998). Supernatants generated from both extended *Mus dunni* and *Mus dunni* co-cultivation assays are tested by PG4 S^+L^- assay. Additional recommendations have recently been published which further clarify regulatory opinion with respect to experience gained since the initial guidelines were implemented (Wilson *et al.*, 1997). This paper states that if appropriate, an alternative marker rescue assay is acceptable, if validated, and of sensitivity comparable to the PG4 S^+L^- assay (Forestell *et al.*, 1996).

This chapter presents results of studies designed to optimise the procedures used for detection of RCR. In the currently approved assay system, *Mus dunni* cells are infected or co-cultivated with vector supernatant or the producer cell line and passaged five times in order to amplify any infectious virus. Supernatants are tested at passages 1 and 5 for infectivity by PG4 S^+L^- assay and at passage 5 for reverse transcriptase activity. To validate and determine optimal conditions for the extended assay, *Mus dunni* cells were infected with varying multiplicities of MLV (both A-MLV and X-MLV, to assess the effect of virus variation on the assay) and the culture supernatants harvested at each passage for both PG4 S^+L^- and RT assay. The experiment was performed in parallel using PG4 S^+L^- cells, in order to compare the sensitivity of the assays. Similar experiments were performed for co-cultivation assays, where *Mus dunni* cells were co-cultivated with MLV-infected cultures containing varying percentages of infected cells, and supernatants harvested at each passage for PG4 S^+L^- and RT assay. An additional study was performed to determine if the presence of retroviral vector supernatant or vector producing cells (non-RCR) in excess in culture supernatant would reduce the sensitivity of the assay for detection of RCR. This was performed by titration of A-MLV through culture supernatant containing either vector or ecotropic murine leukaemia virus (E-MLV) prior to assay on *Mus dunni* or PG4 S^+L^- cells.

2.2 Materials and Methods

2.2.1 Culture medium, cells, viruses and antibodies

Cell lines were grown at 37°C in a mixture of 5% CO_2 and 95% air in Dulbecco's modified Eagles medium (DMEM) (*Mus dunni* and PALidSN), RPMI 1640 (mink ($MiCl_1$) S^+L^-), or McCoy's medium (PG4 S^+L^-) supplemented with 10% foetal bovine serum (FBS), L-glutamine, antibiotics, and vitamins (all reagents supplied by Life Technologies Ltd., Paisley, UK). *Mus dunni* (CRL2017), NIH/3T3 (CRL1658), $MiCl_1$ mink S^+L^- (CCL64.1), and PG4 S^+L^- (CRL2032) cells were obtained from the American Type Culture Collection (ATCC). A murine fibroblast based producer cell line (PALidSN) was obtained from Dr L. Fairbairn (Paterson Institute for Cancer Research, Manchester). The A-MLV, strain 292A (VR884) was obtained from the ATCC. E-MLV strain mov3 (Moloney, 1960) and feline leukaemia virus (FeLV, strain FeLV-C) were supplied by Professor D.E. Onions (Department of Veterinary Pathology, University of Glasgow). Anti-MLV monoclonal antibody (34C) was undiluted supernatant of cell line 34C (CRL1889) obtained from ATCC.

2.2.2 Infectivity assays

2.2.2.1 Introduction

The infectivity assays were performed in a direct and extended format. In direct assays a given volume of sample was inoculated onto the cells in the presence of polybrene and the virus titre determined directly by enumeration of resulting foci of infection or by indirect immunofluorescence. Low levels of infection, which may not be detected by direct assays, may be amplified by serial passage of inoculated cells (Morse and Hartley, 1986). Hence, extended assays were performed in which inoculated cultures were passaged five times. Extended assays are not quantitative, but in validation studies performed at Q-One Biotech Ltd (Glasgow, Scotland), they have been found to be of greater sensitivity than direct assay by up to a factor of one $\log_{10}$

2.2.2.2 *Mus dunni* assays

In the direct *Mus dunni* assay, cells were seeded onto Labtek 2 chamber slides (NUNC Inc. Napaville, Illinois USA) at a dilution of $2x10^4$ cells/well and inoculated the following day with 0.25ml/well of vector supernatant and 0.25ml/well polybrene (effective concentration of 10µg/ml). Four days later the cultures were fixed in cold acetone. Indirect immunofluorescence was performed using the murine anti-MLV monoclonal antibody 34C (Cheesebro *et al.*, 1983) and anti-murine FITC conjugated antibody (Sigma) as the second antibody. Foci were identified and enumerated by fluorescent microscopy (Olympus). Negative control (culture medium) and positive control (A-MLV) cultures were tested in parallel. In the extended assay *Mus dunni* cells were seeded into duplicate $25cm^2$ tissue culture flasks at a concentration of $2.5x10^5$cells/flask and inoculated the following day with 0.5ml/flask of supernatant and 0.5ml/flask polybrene (20µg/ml). The cultures were passaged every 3-4 days for a total of five passages. At each passage supernatant from the cultures was harvested and tested for A-MLV by PG-4 S^+L^- assay. Negative (culture medium) and positive (A-MLV) controls were tested in parallel.

2.2.2.3 S^+L^- assays

In the direct mink S^+L^- assay cells were seeded onto duplicate 60mm dishes (NUNC) at a dilution of $2.5x10^5$cells/plate and inoculated the following day with 0.5ml/plate of supernatant and 0.5ml/plate polybrene (8µg/ml polybrene). The cells were examined daily for formation of foci and focus forming units (ffu) counted. Samples with fewer than 10ffu/ml were tested by the extended assay by passaging every 3-4 days for a total of five passages and foci counted as before. Negative (culture medium) and positive (FeLV) controls were tested in parallel.

In the PG4 S^+L^- assays cells were seeded onto duplicate 60mm^2 dishes at a concentration of 2x10^{5}cells/plate and inoculated the following day with 0.5ml/plate of supernatant and 0.5ml/plate polybrene (20µg/ml polybrene). The cells were examined daily for focus formation and ffu counted (direct assay). The cells were then passaged every 3-4 days for a total of five passages and foci counted as before (extended assay). Negative (culture medium) and positive (A-MLV) controls were tested in parallel.

2.2.3 Reverse transcriptase (RT) assay

The purpose of this assay was to determine if reverse transcriptase activity was present in supernatant and to aid in the discrimination between viral and cellular DNA polymerase activities by using two different templates. The test was based on RNA-dependent DNA polymerase (RT) activity packaged in extracellular retroviral particles. This sensitive assay is useful for the detection of retrovirus production by cultured cells. The assay quantitated the incorporation of radiolabelled nucleotides into cDNA that is precipitated and bound to Whatman GC filters. If retrovirus was present in a sample, radiolabelled nucleotides incorporate into DNA which is copied from a synthetic viral template-primer [poly (rA)- oligo (dT)]. Use of a second template-primer [poly (dA)-oligo (dT)] provided a means to measure the endogenous cellular DNA polymerase activity of the test article (Kornberg, 1980). If RT was not present in the test article, synthesis from the viral template would not occur, and no labelled DNA would be precipitated and bound (Kacian and Splegelman, 1974, Temin and Baltimore, 1972).

10 mls of the test samples were clarified at 11,000 x g for 10 minutes. The samples were concentrated by centrifugation at approximately 100,000 x g for 1hour and each pellet was solubilised in 180µl of disruption buffer (40mM Tris pH8.1; 50mM KCl; 20mM DTT; 0.2% NP40). An aliquot of 25µl was then added to an equal volume of each of six reaction mixtures. All reaction mixtures contained 40mM Tris pH8.1, 50mMKCl, 25µCi [methyl-^{3}H] TTP (Amersham) with poly (rA) (0.05A_{260} units) or oligo (dT)

($0.05A_{260}$units) or 2mM Tris pH8.1/30mM NaCl with 2mM $MnCl_2$, or poly (rA) (0.1 A_{260} units) or oligo (dT) (0.1 A_{260} units) or 4mM Tris pH8.1/60mM NaCl with 20mM $MgCl_2$. The combined sample and reaction mixtures were incubated at 37°C for 1hour and the DNA or RNA templates precipitated by 10% trichloroacetic acid (TCA), 1% sodium pyrophosphate, onto GFC filters (Whatman). The ^{3}H-TTP incorporated into DNA or RNA templates and the background activity (no-template mixes) with either the Manganese (Mn^{2+}) or Magnesium (Mg^{2+}) cation was measured in Ecoscint in a scintillation counter (Beckman). The Mn^{2+} cation is preferred by the Type C oncoviruses belonging to the MLV and FeLV paradigm group. The Mg^{2+} cation is preferred by Type C retroviruses like bovine leukaemia virus (BLV) and human T-cell leukaemia virus (HTLV), Type B retroviruses like mouse mammary tumour virus (MMTV), Type D retroviruses like squirrel monkey retrovirus (SMRV) and the lentivirus group such as Maedi-visna virus (ovine lentivirus; MVV) and Human Immunodeficiency virus (HIV). If RT is not present in the test article, synthesis from the viral template will not occur, and no labelled DNA should be precipitated and bound. Results were presented as disintegrations per minute (dpm). A positive result was concluded when the poly (rA) incorporation exceeded 2000dpm with the poly (rA) incorporation at least twice the poly (dA) incorporation and at least four times the negative control poly (rA) incorporation. A negative result was concluded when the poly (rA) incorporation was less than 2000dpm. Any other result was regarded as equivocal. Negative control (disruption buffer) and positive controls (E-MLV for Mn^{2+} dependent incorporation and bovine leukaemia virus (BLV) or Maedi Visna virus (MVV) for Mg^{2+} incorporation) samples were tested in parallel.

2.2.4 Immunofluorescence assay

Cells were washed with Phosphate buffered saline (PBS), then centrifuged at 1000g for 5 minutes before resuspending at 10^6 cells per ml in PBS. 10µl of cell suspension was added to each well of Flow 8-well multitest slides and allowed to air dry. Slides were

then fixed by immersing in cold (-20°C) acetone (Phillip Harris) for 15 minutes. The slides were either tested immediately, or stored at -80°C. To perform the indirect immunofluorescence test, 10µl of undiluted anti-MLV monoclonal antibody 34C (Cheesebro *et al.*, 1983) in PBS was added to the fixed cells and incubated at 37°C in a humidified chamber for 30 minutes. 10µl of PBS was added to act as a negative control well. After incubation, the slides were washed in PBS for 5 minutes, washed in distilled water, and dried. Next, 10µl of fluorescein isothiocyanate (FITC)-conjugated anti-murine antiserum at a 1 in 20 dilution in PBS containing 0.01% Evans blue (Sigma) was added to each well. The slides were incubated at 37°C in a humidified chamber for 30 minutes then washed once in PBS and once in distilled water before drying. The slides were mounted in glycerol/PBS (1:1) under a glass coverslip, and examined using a Olympus BX fluorescence microscope with a mercury lamp source and an NB filter. Infected cells appear fluorescent green, and non-infected cells dark red or invisible.

2.2.5 Optimisation of infectivity assays

To validate and determine optimal conditions for the *Mus dunni* assay, *Mus dunni* cells were infected with varying titres of A-MLV and the cultures passaged five times. To evaluate the sensitivity of *Mus dunni* and PG4 S^+L^- cells to known inputs of A-MLV, the assay was performed in parallel using PG4 S^+L^- cells. Culture supernatants were harvested at each passage and tested for the presence of infectious MLV by PG4 S^+L^- assay. Supernatants from selected samples were also tested by RT assay. To evaluate the relative sensitivities of various cell lines to infection by A-MLV, known inputs of virus were titrated by direct assay onto *Mus dunni*, S^+L^-, and PG4 S^+L^- cells.

2.2.6 Optimisation of co-cultivation assays

Co-cultivation of producer cells with *Mus dunni* cells and subsequent passage of *Mus dunni* cells was performed in order to amplify MLV that may have been present at low levels in producer cells. After passage 5, cell culture supernatant was assayed by PG4 S^+L^- focus forming assay for the presence of X-MLV and A-MLV. Presence of retrovirus at passage 5 was also determined by RT assay. To validate and determine optimal conditions for the co-cultivation procedure, *Mus dunni* cells were co-cultivated, either by direct co-culture or using transwell dishes (Costar, Cambridge, MA) with MLV-infected cultures containing varying percentages of infected cells. Cultures were incubated for 5 passages and tested for infectious MLV by PG4 S^+L^- assay and also by RT assay. In order to ensure that the cell count dilutions were accurate, dilutions were plated in quadruplicate onto a 24-well plate, which was examined daily for cell growth, and the supernatants examined for viral detection by assay on PG4 S^+L^- cells. Also, the percentage of infected cells in the input sample was determined by indirect immunofluorescence, as described in section 2.2.4.

The use of direct co-cultivation of detector cells and test article cells presents the problem that all the test article cells must be removed or selectively killed by the end of the assay so that any retrovirus present in culture supernatant was from infected detector (*Mus dunni*) cells and not from test article cells. In addition, if test cells were not removed then they may outgrow detector cells. One method that obviates mixing of detector and test article cells is the use of transwell dishes. These dishes consist of a 90mm plate into which *Mus dunni* detector cells were seeded. A second dish with a base plate consisting of a 0.45μm membrane was inserted and test article cells seeded onto this. Retroviruses range in diameter from approximately 80-120nm (Coffin, 1992), thus any retrovirus being produced from the test article cells should pass through the membrane pores and be present in the culture supernatant surrounding the detector cells. Following a 4-5 day period of co-cultivation the upper transwell dish (and

consequently all test article cells) was removed and the detector cells passaged to amplify any low-level infection. Although this method does not permit cell to cell contact, which for some retroviruses aids virus infection, the test article cells and *Mus dunni* detector cells were seeded subconfluently and maintained in the same culture fluid for a period of 4-5 days. During this period both cell lines were in mitosis which is optimal for retroviral production and infection (Morgan *et al.*, 1995, Roe *et al.*, 1993).

2.2.6.1 Direct co-culture

Mus dunni cells were plated in $75cm^2$ tissue culture flasks at a dilution of $1X10^6$ cells in total. After 24 hours, A-MLV infected *Mus dunni* cells serially diluted in non-infected *Mus dunni* cells were inoculated onto the *Mus dunni* monolayer in the presence of 8μg/ml polybrene. The total number of cells inoculated was kept constant at a concentration of $1X10^6$ cells, with a ratio of infected: uninfected *Mus dunni* cells ranging from $0:10^6$ to $10^6:0$ at 10 fold intervals. The A-MLV infected *Mus dunni* cells were diluted out to a concentration of 10^{-3}. The cell count of infected *Mus dunni* cells was demonstrated to be accurate by plating each of the dilutions from 10^{-3} to 10^3 in a 24-well plate. The proportion of *Mus dunni* cells expressing virus in the infected culture was determined by indirect immunofluorescence using the 34C monoclonal antibody to MLV. The cells were co-cultured in flasks for four days, after which the cells were passaged five times and supernatants harvested after each passage and tested for the presence of retrovirus by direct PG4 S^+L^- assay. Negative (culture medium) and positive (A-MLV infected *Mus dunni* cells) controls were tested in parallel.

2.2.6.2 Co-culture using transwell dishes

Mus dunni cells were plated at a dilution of $1x10^6$ cells/plate in the lower dish of 90mm transwell dishes with pore size 0.4μM (Costar, Bucks., UK). In separate experiments, A-MLV infected *Mus dunni* cells or the amphotropic retroviral vector producer cell line

PALidSN, were serially diluted in non-infected *Mus dunni* cells. The dilutions were inoculated onto the upper plate of the transwell, as described above, in the presence of 8μg/ml polybrene. The total number of cells inoculated was kept constant at a concentration of $1x10^6$ cells, with a ratio of infected:uninfected *Mus dunni* cells ranging from $0:10^6$ to $10^6:0$ at 10 fold intervals. The A-MLV infected or producer cell *Mus dunni* cells were diluted out to a concentration of 10^{-2}. The cell count of infected *Mus dunni* cells was shown to be accurate by plating each of the cell dilutions from 10^3 to 10^{-3} in a 24-well plate as above (2.2.6.1). The proportion of *Mus dunni* cells expressing virus in the infected culture was determined by indirect immunofluorescence using a monoclonal antibody to MLV. The cells were co-cultured in transwells for four days after which the upper transwell dish containing either infected cells or producer cells was discarded. The cells from the lower transwell were passaged five times to amplify any low level infection, and supernatants harvested after each passage and tested for the presence of retrovirus by PG4 S^+L^-assay.

Negative controls were mock-infected *Mus dunni* cells. Cell culture supernatants were harvested at each passage, and tested for A-MLV by PG-4 S^+L^- assay. The presence of retrovirus was also detected in supernatants by RT assay.

2.2.7 The effect of non-RCR on infectivity and co-cultivation assay

2.2.7.1 The effect of non-RCR on direct PG4 infectivity assay

To evaluate the effect of retroviral vector supernatant on detection of A-MLV, virus was titrated through various concentrations of retroviral vector supernatant before direct assay on PG4 S^+L^- cells. Vector supernatant with known titre of $4x10^5$ colony forming units per ml (cfu/ml) was serially diluted in culture medium to give supernatant titres of $4x10^4$ and $4x10^3$ cfu/ml. A-MLV with a known titre of between $2\text{-}5x10^5$ ffu/ml was titrated through culture medium, the three concentrations of vector supernatant, and E-

MLV with a known titre of $1.4x10^5$ ffu/ml. E-MLV utilises a different cellular receptor from A-MLV, therefore the presence of E-MLV should have negligible effect on A-MLV titre (Somerfelt and Weiss, 1990).

2.2.7.2 The effect of non-RCR on the *Mus dunni* co-cultivation assay

Retroviral vector producer cells were directly co-cultivated with *Mus dunni* cells in the presence of known quantities of *Mus dunni* cells infected with A-MLV. Cells were passaged five times, and supernatant from each passage harvested for titration on PG4 S^+L^- cells. Cultures without producer cells, containing *Mus dunni* cells infected with known A-MLV concentration were set up in parallel.

Throughout the studies, the negative control was *Mus dunni* cultures mock-infected with tissue culture medium. The positive controls were *Mus dunni* cultures co-cultivated with MLV-infected *Mus dunni* cultures or *Mus dunni* or PG4 S^+L^- cultures infected with A-MLV (Strain 292A, ATCC VR-884).

2.3 Results

2.3.1 Infectivity assays

2.3.1.1 Direct infectivity assays

Table 2.1 presents the relative sensitivities of *Mus dunni*, PG4 S^+L^-, and mink S^+L^- cells to infection by A-MLV when compared by direct assay. The viral titres resulting from known inputs of virus were converted to log values and plotted. Linear regression graphs indicate the relationship between the direct assays (Figure 2.1). The results indicated that *Mus dunni* and PG4 S^+L^- cell lines were of similar sensitivity to infection by A-MLV, and that both cell lines were of higher sensitivity to A-MLV infection than

mink S^+L^- cells by approximately 1 log_{10}.

Table 2.1 **Comparison of sensitivities of *Mus dunni*, PG4 S+L-, and mink S^+L^- cells to infection by A-MLV by direct assay**

Virus Input[1]	Cell Types					
	Mus dunni		PG4 S^+L^-		Mink S^+L^-	
	Titre[(1)]	Log titre	Titre[(2)]	Log titre	Titre[(3)]	Log titre
10^4	4×10^4	4.6	1.8×10^4	4.3	4×10^3	3.6
10^3	4×10^3	3.6	5×10^3	3.7	1×10^3	3.0
10^2	4×10^2	2.6	3×10^2	2.5	1×10^2	2.0
10^1	4×10^1	1.6	1×10^1	1.0	0	0
10^0	0	0	0	0	0	0
10^{-1}	0	0	0	0	0	0

1. titre measured by *Mus dunni* assay (fluorescent ffu/ml).
2. titre measured by PG4 S^+L^- assay (ffu/ml)
3. titre measured by mink S^+L assay (ffu/ml)

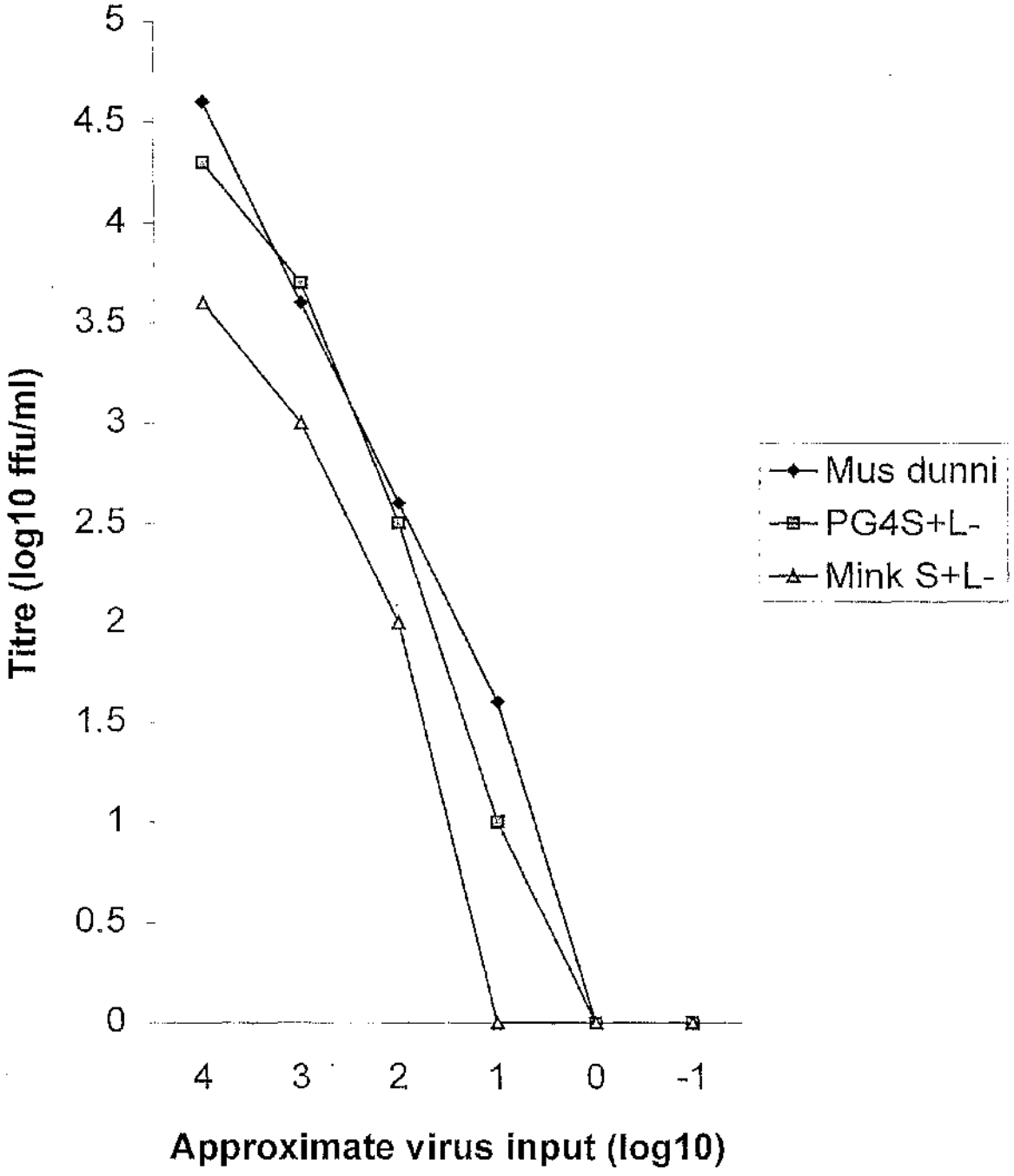

Figure 2.1 Comparative titration of varying quantities of amphotropic murine leukaemia virus by *Mus dunni*, PG4 S^+L^- and mink S^+L^- assays

2.3.1.2 Extended infectivity assays

The results of the evaluation of the sensitivity of the extended *Mus dunni* and PG4 S^+L^- assays are presented in Table 2.2. *Mus dunni* and PG4 S^+L^- cells infected with varying dilutions of A-MLV were passaged five times, and the supernatants harvested at each passage for PG4 S^+L^- assay. The number of foci observed at each passage when tested by PG4 S^+L^- assay is presented in Table 2.2 The results indicate that *Mus dunni* and PG4 S^+L^- cells are of similar sensitivity to A-MLV infection by extended assay. Overall, both cell lines showed similar sensitivity to infection by A-MLV, and virus was detected following inoculation of a dilution of 10^{-1} infectious units of A-MLV. However, there appeared to be differences in the dynamics of infection in the early passages following inoculation. At this dilution (10^{-1}) virus was detected at passage 1 in *Mus dunni* cells, and at passage 2 in PG4 S^+L^- cells. Inoculation of A-MLV diluted to low multiplicity (10^{-1} infectious units) resulted in confluent foci in the duplicate cultures by passage 2 of *Mus dunni* assay, and by passage 4 of PG4 S^+L^- assay. At lower dilutions of A-MLV, virus was not detected in either cell line.

Table 2.2 **Detection of A-MLV by PG4 S^+L^- assay in supernatants of *Mus dunni* or PG4 cells infected with known virus concentration**

Virus Input[1]	Passage number									
	P1		P2		P3		P4		P5	
	PG4 S^+L^-	*Mus*[2] *dunni*	PG4 S^+L^-	*Mus dunni*	PG4 S^+L^-	*Mus dunni*	PG4 S^+L^-	*Mus dunni*	PG4 S^+L^-	*Mus dun*
0	0/0[3]	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
10^{-3}	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
10^{-2}	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
10^{-1}	0/0	+[4]/63	+/0	+/+	+/0	+/+	+/+	+/+	+/+	+/+
10^{0}	9/46	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
10^{1}	+/55	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
10^{2}	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
10^{3}	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+

1. titre measured by PG4 S^+L^- assay (ffu/ml).
2. titre measured by *Mus dunni* assay (fluorescent ffu/ml).
3. 0 symbolises no foci were observed.
4. + symbolises observation of confluent foci.

Reproducibility of the results for the *Mus dunni* assay presented in Table 2.2 was determined by re-titrating A-MLV in *Mus dunni* cells, passaging 5 times, and assaying supernatant from each passage on PG4 S^+L^- cells. Table 2.3 presents the results obtained on repetition of the amplification assay. Again, inoculation of A-MLV at low muliplicity (10^{-1} infectious units) resulted in confluent foci in the duplicate cultures by

passage 2 of *Mus dunni* assay, and focus forming virus was not detected at lower A-MLV dilutions. The results, therefore, were similar to the previous experiment.

Table 2.3 Detection of A-MLV by PG4 S^+L^- assay in supernatants of *Mus dunni* cells infected with known virus concentration

Virus Input[1]	Passage Number				
	P1	**P2**	**P3**	**P4**	**P5**
10^{-3}	0/0[2]	0/0	0/0	0/0	0/0
10^{-2}	0/0	0/0	0/0	0/0	0/0
10^{-1}	10/7[3]	+/+	+/+	+/+	+/+
10^{0}	54/55	+/+[4]	+/+	+/+	+/+
10^{1}	+/+	+/+	+/+	+/+	+/+
10^{2}	+/+	+/+	+/+	+/+	+/+
10^{3}	+/+	+/+	+/+	+/+	+/+

1. titre measured by PG4 S^+L^- assay (ffu/ml)
2. 0 symbolises no foci were observed.
3. ffu/ml
4. + symbolises observation of confluent foci

Supernatants generated from each passage of the *Mus dunni* cells infected with the lowest A-MLV dilution at which virus was detected (10^{-1}) by direct PG4 S^+L^- assay were assayed for the level of RT activity. This was performed for both *Mus dunni* titrations, and the resulting RT activities are presented in Table 2.4. (x representing duplicate samples). RT activity was detected in only one of the duplicate samples in the first titration at passage 1, but by passage 2, activity increased and duplicate samples in

each titration were positive for RT activity. In both experiments RT activity rose to a peak by passage 3, after which the level remained at a plateau through to passage 5.

Table 2.4 **Reverse transcriptase activity (Mn^{2+}dependent) in supernatants of *Mus dunni* cells infected with A-MLV at low multiplicity (10^{-1} infected cells)**

	1st titration (Table 2)		2nd titration (Table 3)	
virus input	10^{-1}	10^{-1X}	10^{-1}	10^{-1X}
Passage 1	8679[1]	1070	497	546
Passage 2	201277	14054	4671	4394
Passage 3	1106148	1078935	261161	193161
Passage 4	765041	401403	249851	228341
Passage 5	1196963	869724	204059	237858

1. Numbers indicate the disintegrations per minute (dpm) in reverse transcriptase reactions with a poly rA template in the presence of the manganese cation (Mn^{2+}). A positive result is indicated by a value greater than 2000dpm. The supernatants tested above were negative (less than 2000dpm) for reverse transcriptase activity dependent on the presence of magnesium cations (Mg^{2+}).

Figure 2.2 presents the Mn^{2+}-dependent RT results converted to log values for cells in both *Mus dunni* assays inoculated with A-MLV at a dilution of 10^{-1} infected cells. RT enzyme levels increased from passage 1 to passage 3, with enzyme activity reaching a peak at passage 3, and remaining at a high level through to passage 5.

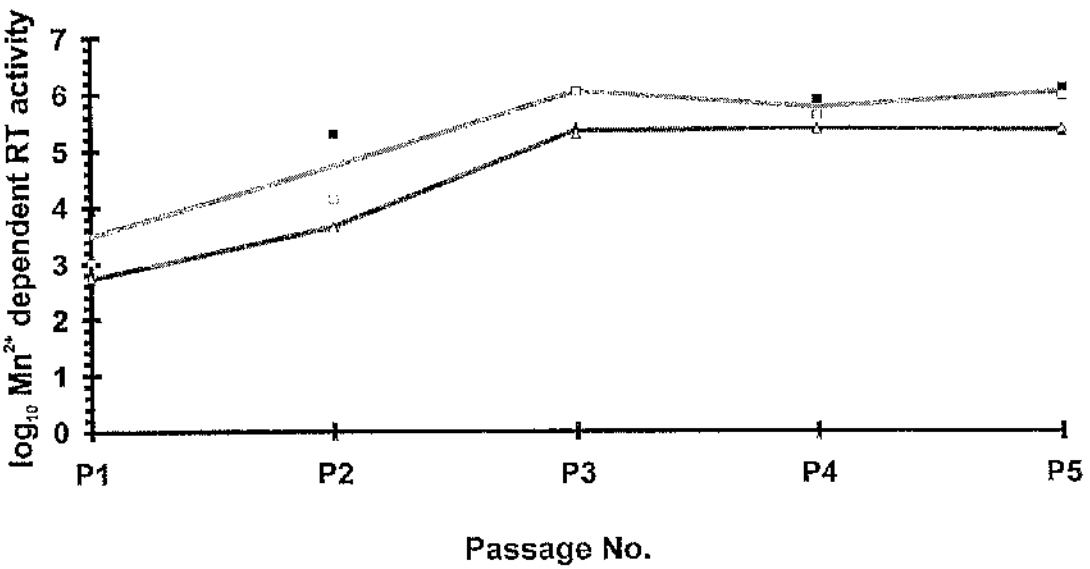

Figure 2.2 Reverse transcriptase activity in supernatants generated from *Mus dunni* cells inoculated with A-MLV at low multiplicity

2.3.2 Co-cultivation assays

To validate and determine optimal conditions for the co-cultivation procedure, *Mus dunni* cells were co-cultivated, either directly or indirectly using transwell dishes, with MLV-infected cultures containing varying percentages of infected cells. The proportion of *Mus dunni* cells expressing virus in the stock infected culture used in these experiments was 10%; determined by indirect immunofluorescence using anti-MLV monoclonal antibody 34C as described in section 2.2.4. Following co-cultivation, cultures were incubated for 5 passages and tested for infectious MLV by PG4 S^+L^- assay.

A culture of *Mus dunni* cells infected with A-MLV was used to inoculate the co-cultivation assays. In order to ensure that the cell count dilutions made were accurate, dilutions were plated in quadruplicate onto a 24-well plate, which was examined daily for cell growth. The cell counts were found to be accurate, with confluent cell growth being observed in the wells inoculated with 100, 10 and 1 virus-infected cells. Three out of the four wells of the 0.1 infected cell inoculation also displayed confluent cell growth, whereas no cell growth was observed in the 0.01 or 0.001 cell dilutions. This was confirmed by inoculating supernatants from each well of the 24-well plate onto a 24-well plate of PG-4 S^+L^- cells. The number of well displaying foci which were observed by PG-4 S^+L^- assay of the *Mus dunni* /A-MLV cell supernatants are presented in Table 2.5.

Table 2.5 **PG4 S^+L^- assay of serially diluted A-MLV-infected *Mus dunni* cell supernatants inoculated into a 24-well plate**

	Number of Infected Cells Plated			
Well number	10	1	0.1	0.01
1	+[1]	+	+	0[2]
2	+	+	+	0
3	+	+	+	0
4	+	+	0	0

1. + symbolises observation of confluent foci
2. 0 symbolises no foci were observed

2.3.2.1 Direct co-cultivation assay

The results of the PG4 S^+L^- assays on supernatants harvested from each passage after direct co-cultivation are presented in Table 2.6. Confluent foci were observed after inoculation of A-MLV at low multiplicity (10^{-1} infectious units) in the duplicate cultures by passage 2, and focus forming virus was not detected at lower A-MLV dilutions. Confluent focus forming virus was detected from passage 1 onward when 1 infected cell per 10^6 cells was co-cultivated with *Mus dunni* cells. The sensitivity of the assay was at a detection level of 1 infected cell per 10^7 cells. RT assay was not performed in this study, as all direct co-culture supernatants would be expected to be positive owing to the presence of virus-producing cells in all cultures.

Table 2.6 **Detection of A-MLV by PG4 S^+L^- assay in supernatants of *Mus dunni* cells directly co-cultivated with cells infected with known virus concentration**

No. of *Mus dunni* / MLV infected cells[1]	Passage number				
	P1	P2	P3	P4	P5
0	0/0[2]	0/0	0/0	0/0	0/0
10^{-3}	0/0	0/0	0/0	0/0	0/0
10^{-2}	0/0	0/0	0/0	0/0	0/0
10^{-1}	18/11[3]	+/+[4]	+/+	+/+	+/+
1	+/+	+/+	+/+	+/+	+/+
10^1	+/+	+/+	+/+	+/+	+/+
10^2	+/+	+/+	+/+	+/+	+/+

1. titre determined by immunofluorescence using a MAb to MLV
2. 0 symbolises no foci were observed.
3. ffu/ml
4. + symbolises observation of confluent foci

2.3.2.2 Co-cultivation assay using transwell dishes

The results of PG4 S^+L^- titre measured by PG4 S^+L^- assay of supernatants harvested from each passage following co-cultivation using transwell dishes are presented in Table 2.7. *Mus dunni* cells were plated in the lower dish of the transwell plate and *Mus dunni* cells with varying proportions of A-MLV infected cells were seeded onto the upper membrane base as described in section 2.2.6.2.

Table 2.7 **Detection of A-MLV by PG4 S^+L^- assay in supernatants of *Mus dunni* cells co-cultivated with cells infected with known virus concentration using transwell dishes**

No. of *Mus dunni*-MLV infected cells[1]	Passage Number				
	P1	**P2**	**P3**	**P4**	**P5**
0	0/0[2]	0/0	0/0	0/0	0/0
10^{-3}	0/0	0/0	0/0	0/0	0/0
10^{-2}	0/0	0/0	0/0	0/0	0/0
10^{-1}	0/1[3]	0/+[4]	1/+	+/+	+/+
1	+/+	+/+	+/+	+/+	+/+
10	+/+	+/+	+/+	+/+	+/+
10^2	+/+	+/+	+/+	+/+	+/+
10^3	+/+	+/+	+/+	+/+	+/+
10^4	+/+	+/+	+/+	+/+	+/+

1. titre determined by immunofluorescence using a MAb to MLV
2. 0 symbolises no foci were observed.
3. ffu/ml
4. + symbolises observation of confluent foci

The number of foci observed after inoculation of A-MLV at low multiplicity (10^{-1} infected cells) reached confluency in the duplicate cultures at passage 4. At higher concentrations, the confluent foci were observed in the duplicate cultures by passage 1. Virus was not detected in dilutions lower than 10^{-1} infected cells. Co-cultivation using transwell dishes was of similar sensitivity to direct co-cultivation (detection of 1 infected cell in 10^7cells).

Supernatants from cell cultures co-cultivated in transwell dishes with A-MLV at low multiplicity (0.1 infectious units) were tested by RT assay, the results of which are presented in Table 2.8. RT results correlated with PG4 S^+L^- results in that RT activity was not observed at passage 1. At passages 2 and 3 only one of the duplicate cultures was positive and at passages 4 and 5 both duplicates were positive for RT activity with Mn^{2+} preference (the cation of preference for MLV).

Table 2.8 Reverse transcriptase activity (Mn^{2+}-dependent) in supernatants of *Mus dunni* cells co-cultivated with A-MLV infected cells at low multiplicity (0.1 infectious units)

	RT activity in duplicate cultures	
Passage number	**Sample 1**	**Sample 2**
P1	503[1]	482
P2	2455	1052
P3	53845	591
P4	22768	3525
P5	34030	31997

1. Numbers indicate the disintegrations per minute (dpm) in reverse transcriptase reactions with a poly rA template in the presence of the Mn^{2+} manganese cation. A positive result is indicated by a value greater than 2000dpm. The supernatants tested above were negative (that is, less than 2000dpm) for reverse transcriptase activity with preference for the Mg^{2+} cation.

2.3.3 The effect of non-RCR on infectivity and co-cultivation assays

2.3.3.1 The effect of non-RCR on the PG4 S^+L^- infectivity assay

To evaluate the effect of retroviral vector supernatant on detection of A-MLV, virus was titrated through various concentrations of retroviral vector containing supernatant before direct assay on PG4 S^+L^- cells, the results of which are presented in Table 2.9. To determine if any effect observed was specific, A-MLV was also titrated through E-MLV. The titre of the E-MLV stock used in the assay was $1.4x10^5$ plaque forming units per ml (pfu/ml) as determined by the XC plaque assay (Klement *et al.*, 1969), as performed at Q-One Biotech Ltd. The results indicated that there was a loss of sensitivity of approximately 1 $\log_{10}$ in the presence of higher titre retroviral vector ($4X10^5$ffu/ml). Vector supernatant of lower titre had no effect on PG4 S^+L^- assay sensitivity (ie. variability of the assay was less than 0.5 $\log_{10}$ ffu). The presence of E-MLV also had no effect on A-MLV titre.

Table 2.9 The effect of the presence of vector supernatant or E-MLV on detection of A-MLV by PG4 S^+L^- assay

A-MLV titrated through:-	No. of ffu[3] detected at 10^{-4}	No. of ffu[3] detected at 10^{-5}	Calculated Titre[4]
Vector Supernatant (4×10^5)[1]	1/1	0/0	2.0×10^4
Vector Supernatant (4×10^4)[1]	15/11	1/1	2.6×10^5
Vector Supernatant (4×10^3)[1]	11/12	0/1	2.3×10^5
Ecotropic MLV (1.4×10^5)[2]	11/13	2/0	2.0×10^5
Culture media	22/25	2/1	4.7×10^5

1. colony forming units per ml (cfu/ml)
2. plaque forming units per ml (pfu/ml)
3. focus forming units (ffu)
4. ffu/ml of inoculum

2.3.3.2 The effect of non-RCR on the *Mus dunni* co-cultivation assay

Retroviral vector producer cells were directly co-cultivated with *Mus dunni* cells in the presence of known quantities of A-MLV infected *Mus dunni* cells. Cells were passaged five times, and supernatant from each passage harvested for titration on PG4 S^+L^- cells. Assays where no producer cells were present were inoculated in parallel as a control. The results showing the effect of vector cells on detection of A-MLV by *Mus dunni* assay are presented in Table 2.10. Supernatants generated from co-cultivation in the

presence or absence of producer cells produced similar results by titration on PG-4 S^+L^- cells. The results were also similar to previous co-cultivation and infectivity titrations in that virus was not detected in supernatants harvested from cultures infected with 0.01 or less infected *Mus dunni* cells, whereas confluent focus-forming virus was observed at all passages when 1 or more infected cells were inoculated. At passage 1, following low multiplicity of infection (0.1 infected cell), 19 foci were detected in the absence of producer cells and 18 foci in the presence of producer cells. For both titrations, confluent foci were observed from passage 2 through to passage 5. Thus the effect of the presence of producer cells on indirect co-cultivation assay was negligible with regard to RCR detection, with the sensitivity of the assay remaining at 1 infected cell in 10^7 cells in the presence or absence of producer cells.

Table 2.10 **Detection of A-MLV by PG4 S$^+$L$^-$ assay in supernatants of *Mus dunni* cells after direct co-cultivation with *Mus dunni* or retroviral vector producing cells seeded with varying dilutions of A-MLV infected *Mus dunni* cells**

No. of *Mus dunni* / MLV infected cells	Passage Number									
	P1		P2		P3		P4		P5	
	Mus dunni	Vector	*Mus dunni*	Vector	*Mus dunni*	Vector	*Mus dunni*	Vector	*Mus dunni*	Vector
0	0/0[1]	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
10^{-3}	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
10^{-2}	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
10^{-1}	18/1[2]	15/3	+/+[3]	+/+	+/+	+/+	+/+	+/+	+/+	+/+
1	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
10	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
10^2	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+

1. 0 symbolises no foci were observed.
2. titre measured by PG4 S$^+$L$^-$ assay (ffu/ml).
3. + symbolises observation of confluent foci

2.4 Summary

This study determined the limits of sensitivity of infectivity and co-cultivation assays used for the detection of RCR, and investigated the effect of the presence of non-RCR on RCR detection.

2.4.1 Infectivity and co-cultivation procedures

Relative sensitivities of *Mus dunni*, mink S^+L^- and PG4 S^+L^- cells to infection by A-MLV were assessed by titrating known inputs of virus by direct assay onto each cell line. (Table 2.1). The results indicated that *Mus dunni* and PG4 S^+L^- cells were of comparable sensitivity to infection by A-MLV, whereas mink S^-L^- cells were approximately 1 $\log_{10}$ less sensitive to A-MLV infection.

The sensitivity of *Mus dunni* and PG4 S^+L^- cells to A-MLV infection by extended assay was evaluated (Tables 2.2 and 2.3). The results indicated that both cell types were similar in sensitivity for detection of A-MLV. Manganese-dependent RT activity in supernatants from each passage of *Mus dunni* cells infected with A-MLV at low multiplicity (10^{-1} infectious units) was measured (Table 2.4). The results indicated that 3 passages are adequate for viral detection with respect to both PG4 S^+L^- assay results and RT activity detected (Figure 2.2). Infectivity results by PG4 S^+L^- assay of *Mus dunni* supernatants displayed no change in virus titre from passage 2 onwards (a virus input of 10^{-1} infected cells resulted in confluent foci), indicating the high sensitivity of the assay.

Validation of co-cultivation procedures demonstrated that the presence of 1 RCR in 10^7 cells could be detected by direct co-cultivation in tissue culture flasks or indirect co-cultivation using transwell dishes (Tables 2.6 and 2.7).

2.4.2 Effect of non-RCR on RCR detection

The results indicated that there was a loss in sensitivity of the direct PG4 S^+L^- assay in the presence of high titre retroviral vector (Table 2.9). This result was shown to be specific for vector supernatant, as viral titre was not reduced by titration of A-MLV in the presence of E-MLV. E-MLV uses a different viral receptor to A-MLV (Somerfelt and

Weiss, 1990), therefore the A-MLV receptor can be blocked by vector but not by E-MLV. The presence of vector producer cells in the co-cultivation assay had no effect on detection of A-MLV (Table 2.10).

3.0 STUDIES ON RETROVIRAL VECTOR PRODUCTION IN A MODEL CELL LINE

3.1 Introduction

In order to achieve optimal viral vector production from producer cells, it is important to establish specific growth parameters for the particular cell line, as maximum retrovirus production is attained when cells are rapidly dividing (Roe *et al.*, 1993, Morgan *et al.*, 1995). In this study, growth characteristics of a model producer cell line were investigated, including the effect of culture in media containing varying concentrations of either foetal or newborn bovine serum, glucose, and serum-free media. Optimal growth of producer cells in flasks, roller bottles and Fibra-Cel® disks in a Techne spinner flask was assessed. Optimal culture conditions were established to assess specific cell growth parameters that provide retroviral vector of the highest possible titre. The effect of culturing producer cells at different temperatures, and in the presence of inducing agents was examined. In addition, studies were performed to determine the effect on retroviral vector by concentration of supernatant by filtration.

The cell line used to produce the model retrovirus vector for these experiments was PALidSN cells, which were obtained from Dr. L.. Fairbairn from the Paterson Institute of Cancer Research, Manchester. PALidSN is a murine fibroblast cell line which expresses the α-L-iduronidase (IDUA) gene in a vector used for the treatment of Hurler's syndrome. PALidSN is a vector construct consisting of the genes encoding α-L-iduronidase, the SV40 promoter and *neo* marker flanked by two LTRs (LidSN) which is packaged in the amphotropic retrovirus vector packaging cell line PA317 (Miller and Buttimore., 1986).

LTR --------- IDUA -------- SV/neo -------- LTR **LidSN**

Geneticin (G418-sulphate, or G418) is an antibiotic that is toxic to both eukaryotic and prokaryotic cells (Vile, 1991). The gene encoding neomycin transferase *(neo)* confers resistance to G418 by converting the toxic drug into tolerable byproducts. The presence of *neo* in eukaryotic cells provides a dominant selectable marker that allows cell growth in media containing G418. PALidSN cells encode the *neo* gene therefore are permitted growth in media containing G418 as the antibiotic is rendered non-toxic. Thus the titre of the vector can be obtained by assessing the number of living colonies of PALidSN cells when titrated in media containing G418 (Vile, 1991).

Retroviruses can only infect growing cells, as integration of viral DNA is dependent on mitosis (Roe *et al.*, 1993, Morgan *et al.*, 1995). Mammalian cells can be characterised in culture by four growth phases: lag, log, plateau and decline. In lag phase the cell culture is in a 'resting' state and growth is negligible. The log phase of cell growth is when exponential growth occurs and the proportion of cells in cycle is high. For adherent cells such as PALidSN, the plateau is normally reached when confluence is achieved. Division slows down and cell growth is balanced by cell death if the culture is supplied with fresh culture media. However, if the medium is unchanged, cell death will exceed cell growth and the culture will decline. A series of growth curves for PALidSN cells were established in this study and optimal cell growth conditions established in relation to viral vector production and IDUA enzyme activity in culture supernatants.

One study reported higher transduction efficiency of retroviral vector, resulting in higher titre virus stock, when producer cells were cultured at 32°C rather than 37° (Kotani *et al.*, 1994). Therefore, viral titre of PALidSN cells was assessed at both temperatures to determine if a similar effect was observed. Producer cells were also exposed to inducing agents which have been reported to increase vector titre. One such agent is sodium butyrate (NaB), which affects expression of a number of viral and cellular genes, and has been reported to increase production of virus from retroviral-derived producer cell lines by between 20-fold to 1000-fold (Olsen and Sechelski, 1995). Another is the phorbol ester

12-O tetradecanoly-phorbol-13-acetate (TPA), an inducing agent which can increase viral production from infected cells by promoting latent viral infections to the lytic cycle (Zur Hausen *et al.*, 1978).

Chemically defined culture medium such as Dulbecco's Modified Eagle's Media (DMEM) used in this study normally requires the addition of a serum base such as foetal or newborn bovine serum. There are disadvantages to using a serum-based system in that serum is potentially cytotoxic to certain cell types owing to the presence of selective inhibitors, bacterial toxins and lipids. Batch to batch variations in the composition of sera necessitate extensive screening, and the presence of serum in supernatant may be a cause for safety concerns owing to the potential for contamination of cell lines with bovine adventitious agents (McLean *et al.*, 1996). Thus, in this study, adaptation of PALidSN cells to serum-free media and the resultant level of viral vector production was examined by assessing cell growth and viral titre in cells both during adaptation and in completely serum-free media.

As larger quantites of gene therapy material are required will become necessary to scale up the production process. This may involve different culture systems such as the Costar Cellcube system or the New Brunswick Celligen fermenter system. In the study presented in this report, the feasibility of scale-up for retroviral vector production by PALidSN cells was determined using both roller bottles and the Celligen fermenter system, which was used to evaluate cell growth and vector production on Fibra-Cel® disks in a Techne spinner flask.

As higher titres of vector material are required for use in gene therapy protocols, methods for concentrating retroviral vector stocks are being studied. In this study, the potential for viral vector supernatant concentration was examined by filtration using SARTACON MICRO 100KD filters.

3.2 Materials and Methods

3.2.1 Assay of retroviral titre in vector supernatant

Murine NIH/3T3 cells were seeded at $3X10^4$ cells/well in 24 well plates (NUNC Inc. Napaville, Illinios USA), and inoculated in quadruplicate the following day with 0.25ml/well of vector supernatant and 0.25ml/well (4µg/well) polybrene (Sigma), with one row of wells serving as negative control (culture medium). The medium was replaced after 6 hours exposure to virus, with fresh medium containing G418 at a concentration of 600µg/ml (Life Technologies Ltd. After 14 days of selection, with media replaced every 3-4 days, the negative control cells were non-viable. The titre of vector was obtained by counting the number of colonies in the wells containing the lowest dilution of vector supernatant which gave rise to G418 resistant colonies.

3.2.2 Assay of enzyme activity in vector supernatant

Retroviral vector activity was determined by infection of human fibroblast MPSI cells (supplied by Dr Jean-Michel Heard, Institut Pasteur, Paris) followed by preparation of cell extracts in which IDUA enzyme activity was measured by fluorimetry. MPSI cells were seeded into 90mm plates (NUNC) at $2X10^5$ cells/plate. The following day, retrovirus vector supernatant was 0.45µM filtered and 4mls inoculated onto subconfluent MPSI cells in the presence of 8µg/ml polybrene. Plates were incubated at 37^0C for 2 hours, after which 6mls medium was added to each plate. The supernatant was replaced after 24 hours. When the MPSI cells reached confluence after 4-5 days, they were harvested with trypsin, washed in PBS and resuspended in lysis buffer. The cells were lysed by three freeze/thaw cycles and centrifuged at 1012g for 5 minutes. The supernatant was harvested and the protein concentration measured using a Sigma micro protein determination kit. Enzyme activity was measured by

reaction of 0.25mg/ml cellular extract with the substrate (4-methyl umbelliferyl IDUA) (Sigma) and detection of product by fluorimetry. The optical density (O.D.) value from a reaction mix containing cellular extract was read and calculated by the formula O.D. x 1.2 = units/mg protein. For infection of MPSI cells, the negative control was mock-infected MPSI cells, and the positive control was culture supernatant from a vector containing producer line. For the enzyme assay, the negative control was the extract from mock infected MPSI cells, and the positive control was the extract from MPSI cells infected with CRIP-ID15 supernatant (supplied by Dr. Jean-Michel Heard, Institut Pasteur, France).

3.2.3 The effect of serum and glucose content in culture media on cell growth and viral vector production of PALidSN cells in tissue culture flasks

In order to determine the optimal media for PALidSN cell culture, cells were cultured in DMEM supplemented with 4500mg/L or 1000mg/L glucose containing either 10% foetal bovine serum (FBS) or 10% newborn bovine serum (NBS), and a series of growth curves established to provide information on doubling times and cell yields. PALidSN cells grown in each of the media types were seeded at $1.0X10^6$ viable cells in each of ten $75cm^2$ flasks. The flasks were cultured over a period of 168 hours and two flasks from each culture system harvested per 24 hour time period (with the exception of the 120 and 144 hour timepoints). Viable cell counts were calculated at each timepoint using a haemocytometer, and all supernatants were clarified by centrifuging at 1012g for 10 minutes, stored at -70^0C for analysis of viral vector titre (section 3.2.1) and IDUA enzyme activity (section 3.2.2).

A study was performed to compare viral vector titres for PALidSN cells cultured in high glucose DMEM containing either the standard FBS supplement of 10% or a lower concentration of 5%. PALidSN cells seeded at $1.0X10^6$ viable cells per $T75cm^2$ flask were cultured over a period of 6 days and each culture system harvested per 24 hour time

period. Supernatants were clarified by centrifuging at 2000rpm for 10 minutes and stored at -70^{0}C for analysis of viral vector titre (section 3.2.1).

3.2.4 The effect of temperature and inducing agents on viral vector production of PALidSN cells in tissue culture flasks

PALidSN cells were seeded at 1.0X10^6 viable cells into each of eight 75cm^2 flasks. TPA (Sigma) at a concentration of 20ng/ml was added to two of the flasks, 5mM NaB (Sigma) was added to two flasks, and a combination of TPA and NaB at the same concentrations were added to another two cultures. The additional two cultures served as controls with no inducing agent added. A control and one of each of the induced cultures were incubated at 32°C, and the remaining four cultures at 37C. The flasks were cultured over a period of 3 days after which supernatants were harvested. Supernatants were clarified by centrifuging at 1012g for 10 minutes and stored at -70^{0}C for analysis of viral vector titre

3.2.5 Cell culture in serum-free media

The process of adaptation of PALidSN cells to serum-free media was investigated. The media used were as follows; Ultraculture (Biowhittaker Inc., Walkersville, MD), CHO-S-SFMII (Life Technologies Ltd,, Paisley, UK), QBSF51 (JRH Boiscience, Lenexa, KS) and QBSF56 (JRH Bioscience, Lenexa, KS). PALidSN cells cultured in high glucose DMEM containing 10% FBS were harvested and each of four 25cm^2 flasks seeded with 6 x 10^5 viable cells, in media formulations of 90% DMEM (as above)/10% serum-free media. All cultures were examined daily. When the cultures became confluent they were passaged into media formulations of 80% DMEM (as above)/ 20% serum-free media (as above) and seeded at 2x10^6 viable cells/flask. The cultures were passaged with progressive reduction of serum concentration until the PALidSN cell cultures were growing in 100% serum-free media. In order to determine the growth characteristics of the PALidSN cell line, cultured in the media named above, three growth curves were generated to provide

information on doubling times and cell yields.

PALidSN cells cultured in 100% Ultraculture, QBSF51 and QBSF56 were seeded at $1.0x10^6$ viable cells per T75cm^2 flask, into 5 flasks for each medium studied. These flasks were cultured over a period of 168 hours with one flask from each system harvested per 24 hour time period. (The Ultraculture system was not harvested at the 120 and 144 hour time point. QBSF51 and QBSF56 were not carried beyond the 72-hour time point.) The condition of the PALidSN cell culture in each of the specified media was determined by microscopic examination provided total viable counts and trypan blue staining. Supernatants generated from the Ultraculture system were clarified by centrifuging at 2000rpm for 10 minutes and stored at -70^{0}C for analysis of viral vector titre.

3.2.6 Viral vector production by PALidSN cells cultured in roller bottles in DMEM containing 1% or 10% FBS

Titres of viral vector produced from PALidSN cells in roller bottles were determined by assay of supernatants harvested from cultures of PALidSN cells in media containing 10% serum. The aim was to optimise the production of viral vector in roller bottles over 15 days to determine the viral titre and stability of the viral vector at 37^{0}C. PALidSN cells cultured in high glucose DMEM containing 10% FBS were harvested and 3 x 850cm^2 roller bottles initiated at $1.1x10^7$ viable cells/roller in 150ml of medium. Roller bottles were designated numbers 1, 2 and 3. Roller bottle 1 was incubated for the first 4 days at 37^{0}C. On day 4 the supernatant was harvested, clarified by spinning at 1012g for 10 minutes, aliquoted and frozen at -80^{0}C. Fresh growth medium was then added to the roller culture. This process was repeated daily from days 4 to 15. Roller bottle 2 was harvested in the same manner from days 1 to 15 and roller bottle 3 was incubated for the first 7 days and then harvested daily until day 15. Samples of the supernatants harvested were assayed for viral vector titre on NIH/3T3 cells as described in section 3.2.1.

In a separate study, the effect of decreasing the serum concentration from 10% to 1% FBS in cells cultured in a roller bottle was determined. PALidSN cells cultured in DMEM containing 10% FBS were harvested and an 850cm^2 roller bottle initiated at 1.5x10^7 viable cells in 150ml of medium. The roller bottle was incubated for 5 days at 37^{0}C. On day 5 the supernatant was harvested, clarified by spinning at 2000rpm for 10 minutes, allquoted and frozen at -80^{0}C. 150ml of fresh DMEM containing 1% FBS was added to the roller culture. This process was repeated daily until day 10 when the maintenance medium volume was reduced to 75ml. Daily harvests then continued until day 14.

3.2.7 Scale-up of producer cells; PALidSN cells grown on Fibra-Cel® disks in a Techne spinner flask

The growth of PALidSN cells and viral vector production was determined in cells grown on Fibra-Cel® disks in a Techne spinner flask. A spinner flask containing 575mg of Fibra-Cel® disks was prepared and autoclaved. The Fibra-Cel® disk culture system was seeded with 1.0x10^7 viable PALidSN cells in 12.5ml growth medium and allowed to adhere for 2 hours at a spinner speed of 20 rpm with stirring for 2 minutes every 30 minutes. At the end of this time period the medium was made up to 50ml with prewarmed growth medium (DMEM, 10% FBS) and the vessel was returned to the incubator spinning continuously at a spinner speed of 20 rpm. The culture system was sampled at 24, 48, 120, 144 and 168 hours and nuclei counts taken using 0.1M citric acid (Sigma) containing 0.1% crystal violet (Sigma). Medium replacement was carried out after the 48-hour harvest and 120 hour harvest. The PALidSN spinner supernatant harvested at 120 hours was assayed for retroviral vector titre by titration on NIH/3T3 cells as described in section 3.2.1.

3.2.8 Concentration of vector supernatant by fitration

In order to concentrate large volumes of vector supernatant, the supernatant was initially filtered using a MILLIPAK-40 0.45μM filter unit. A Watson-Marlow peristaltic pump was used to exert a low membrane feed pressure to draw the supernatant through the filter unit into a sample collection bag which was kept on ice at all times. The filtered supernatant was concentrated using SARTOCON MICRO 100KD filters. The membrane filters were flushed through with HBSS before addition of the supernatant. The filtered supernatant was pumped through two parallel SARTOCON filters as part of a cyclical system. Virus-free filtrate was drawn from the system by creating backpressure on the retentate line, thus forcing the supernatant through the filter units, by using a restriction clamp. The system was pre-washed with sterile HBSS (Gibco) before passing the supernatant through the system. Filtrate was drawn off at a flow rate of approximately 4.5ml/minute. The starting material, filtrate and retentate were assayed for viral vector titre by titration on NIH/3T3 cells as described in section 3.2.1.

3.2.9 Calculation of exponential growth/doubling time/cell yield

Using the data from viable cell counts over a period of time, various phases of cell growth can be plotted. Exponential growth and doubling times were determined using the following equations (Dawson, 1992).

$$\log_{10}(\text{mean})N = \log_{10}(\text{mean})N_0 + x\log_{10}2$$

where, mean N= average final cell number, mean N_0= average initial cell number, and

x= number of generations of exponential growth.

$$\text{Doubling Time} = \frac{\text{Total time elapsed}}{\text{Generations of exponential growth}}$$

Cell yield/cm^2 = No. viable cells(T75cm^2 flasks used)

75

3.3 Results

3.3.1 The effect of serum and glucose content in culture media on PALidSN cell growth and vector production in tissue culture flasks

3.3.1.1 Effect of serum type and glucose content on viable cell count

Initially, a series of growth curves were established for PALidSN cell culture in high or low glucose DMEM containing 10% FBS or 10% NBS. Table 3.1 presents viable cell counts obtained over a 168-hour period after seeding 1X10^5 viable PALidSN cells in tissue culture flasks in each of the above media.

From the data presented in Table 3.1 a series of growth curves were established. The viable cell counts in relation to time (hours) for cultures of PALidSN cells in each of the media above are presented in Figure 3.1. The curves indicate that cells cultured in media supplemented with 1000mg/L glucose rapidly cease growing as glucose is depleted from the media. This result contrasts with cells cultured in media supplemented with 4500mg/L glucose, where the decrease observed in viable cell count following 96 hours culture is less. Exponential growth and cell doubling time were calculated using the equations as in section 3.2.6 (Dawson, 1992), and these values are summarised in Table 3.2. The cell yield per cm^2 was similar with each of the media, ranging between 2.2X10^5 and 3.1X10^5 cells. However, the doubling time of 13.99 hours for cells cultured in DMEM with 4500mg/L glucose containing 10% FBS is lower than cells cultured in media containing 1000mg/L glucose with 10% NBS and 4500mg/L or 1000mg/L glucose containing 10% NBS by at least 6 hours.

Table 3.1 The effect of serum type and glucose content on total viable cell counts for PALidSN cells cultured in tissue culture flasks

TIME (hours)	4500mg/L glc/10% NBS	1000mg/L glc/10% NBS	4500mg/L glc/10% FBS	1000mg/L glc/10% FBS
0	$1X10^6$	$1X10^6$	$1X10^6$	$1X10^6$
24	$1.9X10^6$	$6.9X10^5$	$4.9X10^5$	$1.6X10^6$
48	$4.6X10^6$	$7.2x10^6$	$1.9X10^6$	$6.4X10^6$
72	$7.5X10^6$	$12.5X10^6$	$7.6X10^6$	$15.1X10^6$
96	$16.8X10^6$	$2.35X10^6$	$19.8X10^6$	$18.8X10^6$
168	$13.5X10^6$	$7.7X10^5$	$17.6X10^6$	$1.5X10^5$

Table 3.2 Effect of serum type and glucose concentration on doubling times and cell yields for PALidSN cells

	4500mg/L glc/ 10% NBS	1000mg/L glc/10% NBS	4500mg/L glc/10% FBS	1000mg/L glc/10% FBS
Doubling time (hours)	22.64	28.24	13.99	20.17
cell yield/cm^2	$2.2X10^5$	$3.1X10^5$	$2.64X10^5$	$2.5X10^5$

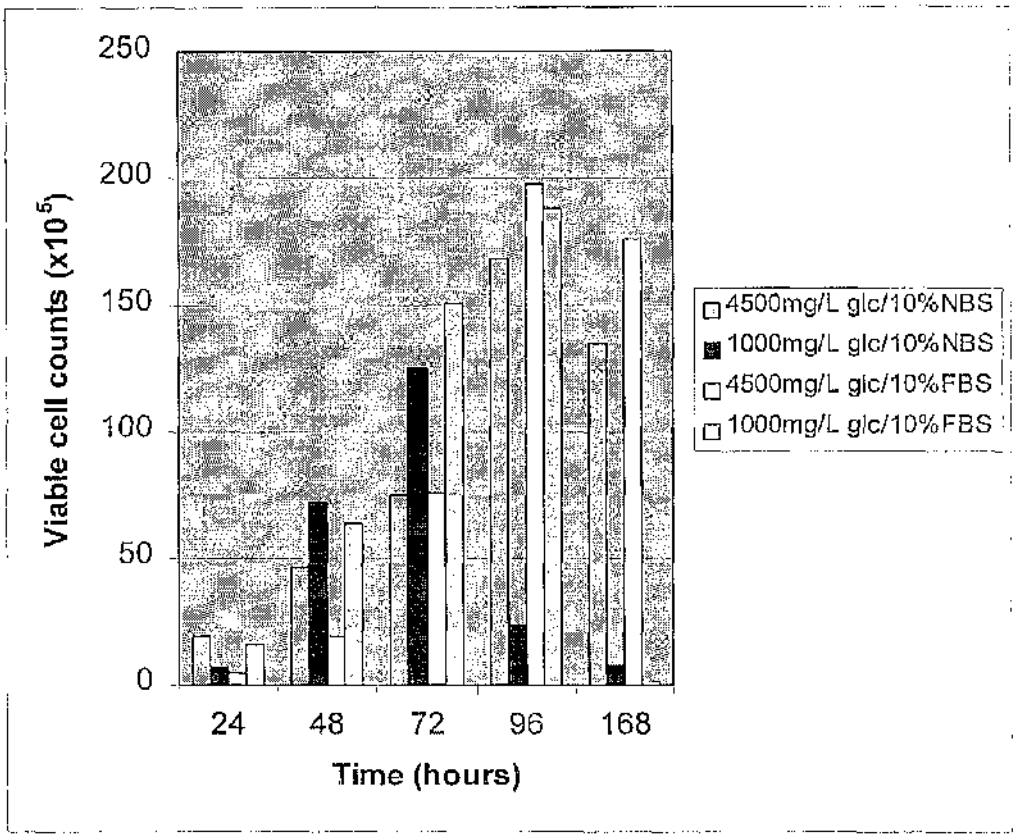

Figure 3.1 **The effect of media serum type and glucose content on viable cell counts during PALidSN cell culture**

3.3.1.2 Effect of media serum type and glucose content on IDUA enzyme activity in PALidSN supernatants

Table 3.3 presents IDUA enzyme activity in supernatants generated over a 168-hour period from the cell cultures above. Figure 3.2 presents this data graphically. Enzyme activity was greater in culture supernatants harvested from cells grown in high glucose DMEM. Activity reached a peak of 10,968 U/mg after 72 hours culture in NBS, and 14,940 U/mg after 96 hours culture in FBS. Enzyme activity was lowest in media with lower glucose concentration containing FBS, where activity reached a peak of 6,924 U/mg after 72 hours and declined rapidly to 42 U/mg after 168 hours. In lower glucose media containing NBS the enzyme activity was an average of 5,472 U/mg over the 168 hours. Thus, the results showed enzyme activity to be greatest (14,940 U/mg) after 96 hours culture in high glucose DMEM with 10% FBS.

Table 3.3 The effect of media serum type and glucose content on IDUA enzyme activity in PALidSN supernatants

Time of Harvest (hours)	1000mg/L glc/ 10% FBS	4500mg/L glc/10% FBS	1000mg/L glc/10% NBS	4500mg/L glc/10% NBS
24	772[1]	NA[2]	4380	8244
48	2916	7332	7584	7500
72	6924	4764	6552	10968
96	571	14940	4908	6504
168	42	12000	3936	5088

1. IDUA enyme activity (units/mg protein)
2. not applicable
3. Note: assay negative control 021/031, assay positive control 1496/1680

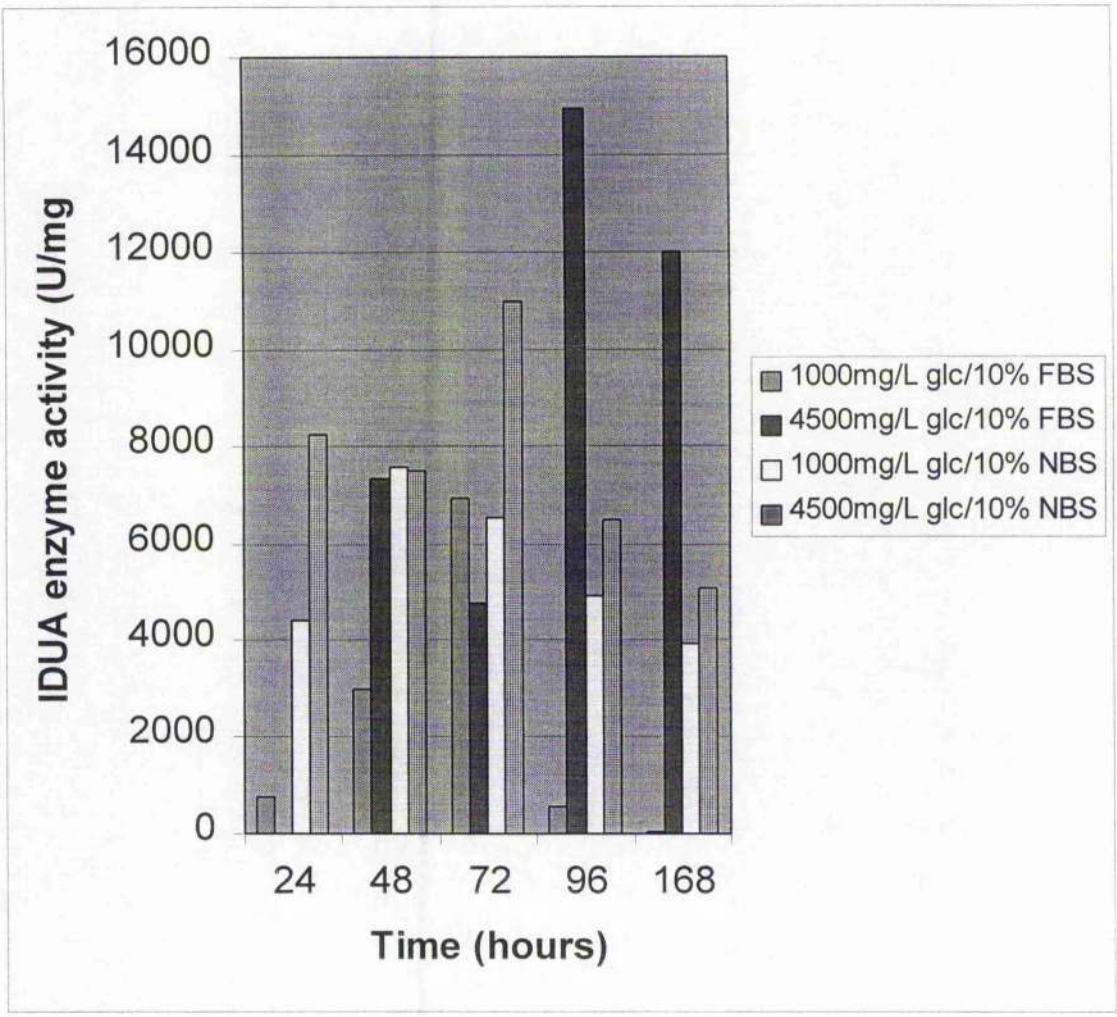

Figure 3.2 **The effect of media serum type and glucose content on IDUA enzyme activity during PALidSN cell culture**

3.3.1.3 Effect of serum type and glucose content on viral vector production in PALidSN supernatants

The viral vector titres as determined by *neo* resistance in supernatants generated over a 168-hour period from the cell cultures above are presented in Table 3.4 and Figure 3.3. Viral titres were greater in culture supernatants harvested from cells grown in high glucose DMEM, which correlates with the enzyme activity values obtained above. The titre reached a peak of $2.0x10^4$ cfu/ after 168 hours culture in NBS, and $1.0x10^5$ cfu/ml after 168 hours culture in FBS. Viral titre was lowest in media with lower glucose concentration containing FBS or NBS, where the maximum titre obtained was in the order of 10^3 cfu/ml. The results showed titres to be greatest ($7X10^4$ and $1X10^5$ cfu/ml) after 96 and 168 hours culture in high glucose DMEM with 10% FBS. Thus, the optimal enzyme activity and viral titre was obtained when PALidSN cells were cultured in high glucose DMEM containing 10% FBS and culture supernatant harvested after 96 hours.

Table 3.4 The effect of media serum and glucose content on viral vector titre in PALidSN supernatants

Time of Harvest (hours)	1000mg/L glc/ 10% FBS	4500mg/L glc/10% FBS	1000mg/L glc/10% NBS	4500mg/L glc/10% NBS
24	$5.0 \times 10^{2(1)}$	3.0×10^2	1.6×10^2	1.0×10^3
48	4.0×10^3	1.0×10^4	1.6×10^3	5.0×10^3
72	4.0×10^3	1.0×10^4	2.0×10^3	4.0×10^3
96	3.0×10^3	7.0×10^4	2.0×10^3	1.8×10^4
168	4.0×10^2	1.0×10^5	0	2.0×10^4

1. titres (cfu/ml) measured by NIH-3T3 titration

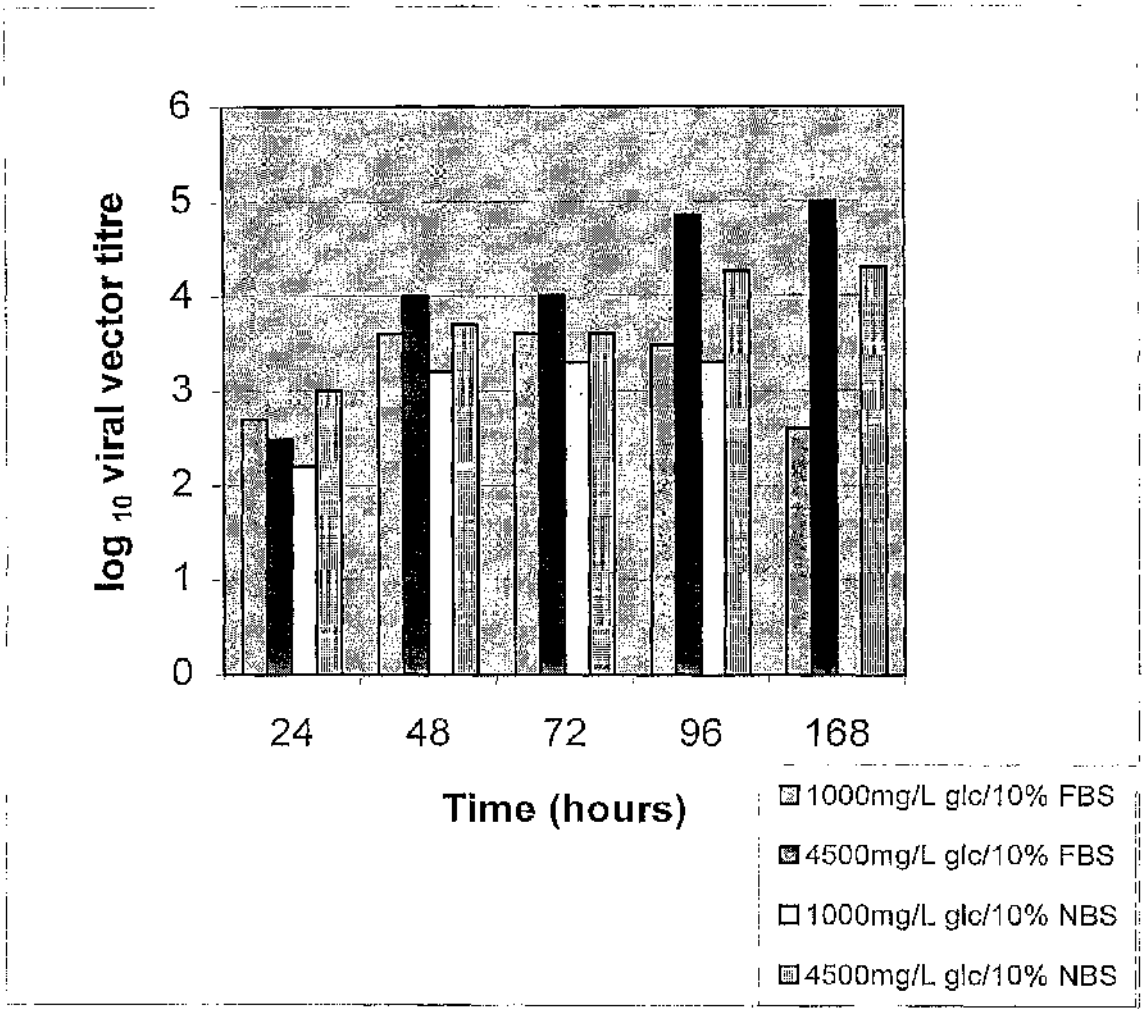

Figure 3.3 **The effect of media serum type and glucose content on viral vector titre during PALidSN cell culture**

3.3.1.4 Titres of vector supernatant from PALidSN culture in tissue culture flasks in high glucose DMEM containing either 5% or 10% FBS

Having established that high glucose DMEM with 10% FBS was the culture media of choice for PALidSN cells, a comparison of 10% and 5% FBS was made to determine if reduced serum concentration had an effect on titre. Table 15 presents viral titres determined by titration on NIH 3T3 cells. Titres were found to be similar throughout the 144 hour period, increasing from 10^3 to 10^5 cfu/ml over days 1 to 3, reaching a peak of $5x10^5$ cfu/ml at 96 hours, then declining to $5x10^4$ cfu/ml by 144 hours.

Table 3.5 The effect of media serum concentration on viral vector titre in PALidSN supernatants

TIME (hours)	5% FBS	10% FBS
24	$5.0 \times 10^{3(1)}$	5.0×10^3
48	2.5×10^4	3.5×10^4
72	1.5×10^5	1.5×10^5
96	5.0×10^5	5.0×10^5
120	1.0×10^5	1.0×10^5
144	4.5×10^4	5.0×10^4

1. titres (cfu/ml) measured by NIH-3T3 titration

3.3.2 The effect of temperature and inducing agents on viral vector production by PALidSN cells

Table 3.6 presents viral vector titres following 3 days incubation of PALidSN cells at 32°C or 37°C in the presence and absence of the inducing agents TPA and NaB. The results did not indicate a significant effect (ie. outwith 0.5 log $TCID_{50}$) on titre either by lowering incubation temperature or inducing cultures by the addition of TPA or NaB.

Table 3.6 The effect of temperature and by inducing agents on viral vector production during 3 days PALidSN cell culture

PALidSN culture	Titre at 32°C	Titre at 37°C
Control	$2.0X10^{5(1)}$	$5.0X10^{5}$
NaB-induced	$5.0X10^{5}$	$2.0X10^{5}$
TPA-induced	$5.0X10^{5}$	$7.0X10^{5}$
NaB and TPA-induced	$2.0X10^{5}$	$6.0X10^{5}$

1. titres (cfu/ml) measured by NIH-3T3 titration

3.3.3 Adaptation of PALidSN cells to culture in serum-free media

3.3.3.1 Viable cell counts on adaptation of PALidSN cells to serum-free media

The effect of adapting PALidSN cells to growth in serum-free media was determined as described in Section 3.2.3. Total viable cell counts were taken from cell cultures as they were gradually adapted from culture in high glucose DMEM containing 10% FBS to growth in four different types of serum-free media; Ultraculture, CHO-S-SFMII, QBSF51, and QBSF569. Counts were calculated by removing a cell sample from each flask, placing onto a haemocytometer, observing under a light microscope, and using a cell counter to count the number of cells within a field of vision. Viable cell counts from the adaptation of PALidSN cells to the four types of serum-free media over a 32-day period are summarised in Table 3.7. PALidSN cells adapted well to growth in Ultraculture, QBSF51 and QBSF56 serum-free media, and were confluent and healthy at the end of the culture period. Cell growth was initially very poor on addition of CHO-S-SFMII serum-free media, but by day 14 (90% original medium/10% serum-free medium) the cells reached confluency and the total viable cell count was found to be $7x10^6$ cells. However, further increasing the proportion of serum-free medium led to complete detachment of the cell sheet from the tissue culture flasks by day 21 (50% original medium/50% serum-free medium). The morphology of the cells appeared altered, and when the culture was further passaged it began to decline, resulting in complete cell death. Therefore CHO-S-SFMII serum-free media was not used in further studies.

Table 3.7 Cell counts on adaptation of PALidSN cells to serum-free media

	ULTRACULTURE		CHO-S-SFMII		QBSF51		QBSF56	
Day	**Form.[1]**	**Count[2]**	**Form.**	**Count**	**Form.**	**Count**	**Form.**	**Count**
7	90:10	$3.9X10^5$	N/A[3]	N/A	90:10	$8.7X10^5$	90:10	$8.7X10^5$
10	80:20	$3.2X10^6$	N/A	N/A	80:20	$8.9X10^6$	80:20	$5.9X10^6$
14	70:30	$6.5X10^6$	90:10	$7.0X10^5$	70:30	$6.7X10^6$	70:30	$1.6X10^5$
16	60:40	$3.4X10^6$	70:30	$3.1X10^5$	60:40	$3.9X10^6$	60:40	$2.8X10^6$
18	50:50	$3.6X10^6$	60:40	$2.7X10^5$	50:50	$6.1X10^6$	50:50	$4.2X10^6$
21	40:60	$5.8X10^6$	50:50[4]	$5.1X10^6$	40:60	$4.5X10^6$	40:60	$7.4X10^6$
23	30:70	$3.0X10^6$	N/A	N/A	30:70	$8.0X10^6$	30:70	$4.9X10^6$
25	20:80	$7.2X10^6$	N/A	N/A	20:80	$4.0X10^6$	20:80	$5.8X10^6$
28	10:90	$2.4X10^6$	N/A	N/A	10:90	$3.9X10^6$	10:90	$3.7X10^6$
32	0:100	$3.8X10^6$	N/A	N/A	0:100	$2.3X10^6$	0:100	$2.1X10^6$

1. media formulation (a:b), where a; original 10% medium and b; serum free medium
2. total viable cell counts
3. not available
4. complete detachment of cell sheet from flasks was observed

3.3.3.2 Viable cell counts for PALidSN cells cultured in serum-free media

Growth parameters for PALidSN cell culture in Ultraculture, QBSF51 and QBSF56 serum-free media are presented in Table 3.8. The data in Table 3.8 for growth of cells in Ultraculture medium was plotted as a function of viable cell count in relation to time (Figure 3.4). The lag phase of cell growth was 48 hours ($1.1x10^6$ viable cells) and the exponential phase of cell growth ended at 72 hours ($2.3x10^6$ viable cells). Exponential

growth and doubling time were determined using equations described by Dawson (1992). When cultured in QBSF51 or QBSF56 media the PALidSN cell culture began to decline at the 24-hour time period and did not recover. Therefore the exponential growth, doubling time and cell yield cannot be calculated.

Table 3.8 Total viable cell counts from growth of PALidSN cells in serum-free medium

Time (hours)	Ultraculture	QBSF51	QBSF56
0	$1.0X10^6$ (1)	$1.0X10^6$	$1.0X10^6$
24	$9.0X10^5$	$2.9X10^5$	$3.1X10^5$
48	$1.1X10^6$	$3.1X10^5$	$2.5X10^4$
72	$2.3X10^6$	$1.1X10^5$ adherent	$1.6X10^4$ adherent
		$9.0X10^5$ suspension	$8.0X10^4$ suspension
96	$2.1X10^6$	N/A(2)	N/A
168	$8.0X10^5$	N/A	N/A

1. total viable cell counts
2. not applicable

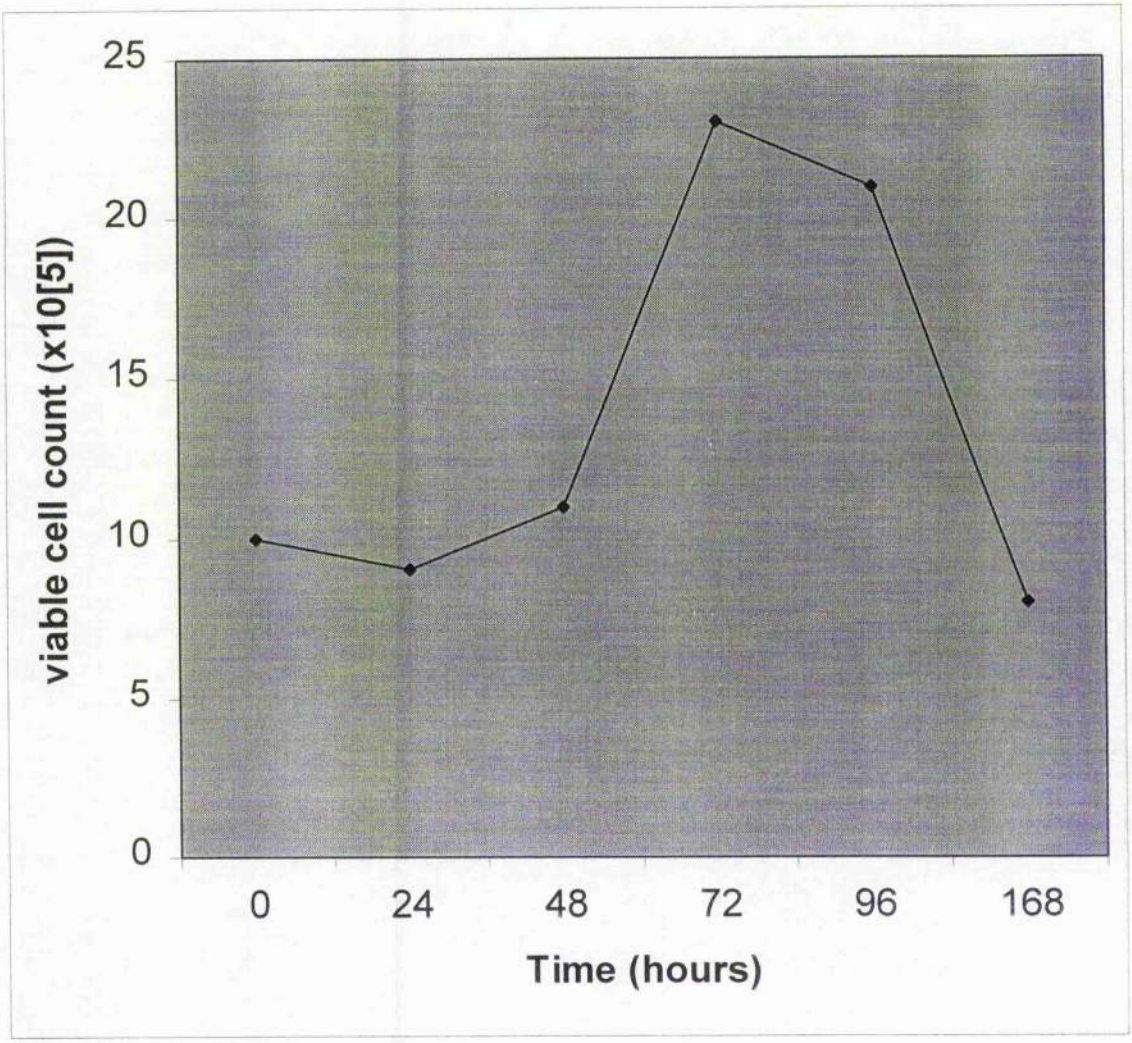

Figure 3.4 **Total viable cell counts during culture of PALidSN cells in Ultraculture serum- free medium**

3.3.3.3 Viral vector production for PALidSN cells cultured in serum-free media

The viral vector titres for three separate PALidSN cell cultures in Ultraculture serum-free media over a 15-day period are presented in Table 3.9 and Figure 3.5. Supernatants were harvested at various timepoints in each of the three flasks in order to determine the optimum conditions to produce high titre virus. In flask 1, the cultures were harvested periodically, and the results show a maximum titre of $4X10^5$ cfu/ml (flask 1, day 12). In flask 2, the expected optimum culture conditions resulting from studies in serum-containing media (that is, a 96-hour harvest) produced a titre of only $1x10^3$ cfu/ml. The maximum titre obtained in flask 2 was on day 7, when the titre was $2x10^5$ cfu/ml.

The lowest titres obtained were in flask 3, where the cells were left in culture for 7 days prior to harvesting. This produced low titre virus (10^2-10^3 cfu/ml) until day 11, after which the culture was not harvested until day 14. The titre was found to have increased by day 14 to $1x10^5$ cfu/ml. From these results, cells cultured in serum-free media appear to require a longer culture period, and more regular media replacement than cells cultured in serum-containing media.

Table 3.9 **Viral vector production from supernatants harvested over a 15 day period from PALidSN cells cultured in Ultraculture serum-free medium**

Day	Flask 1	Flask 2	Flask 3
1	6.0×10^{3}[1]	N/A[2]	N/A
2	1.0×10^{4}	N/A	N/A
3	N/A	N/A	N/A
4	N/A	1.0×10^{3}	N/A
5	1.0×10^{4}	N/A	N/A
6	2.0×10^{4}	N/A	N/A
7	5.0×10^{4}	2.0×10^{3}	2.0×10^{2}
8	1.0×10^{5}	4×10^{3}	7.0×10^{2}
9	1.0×10^{5}	2.0×10^{4}	7.0×10^{2}
10	N/A	3.0×10^{4}	5.0×10^{2}
11	N/A	7.0×10^{4}	3.0×10^{3}
12	4.0×10^{5}	N/A	N/A
13	1.0×10^{5}	N/A	N/A
14	1.0×10^{5}	4×10^{4}	1.0×10^{5}
15	1.0×10^{5}	1.0×10^{4}	3.0×10^{4}

1. titre measured by NIH-3T3 titration (cfu/ml)
2. not applicable

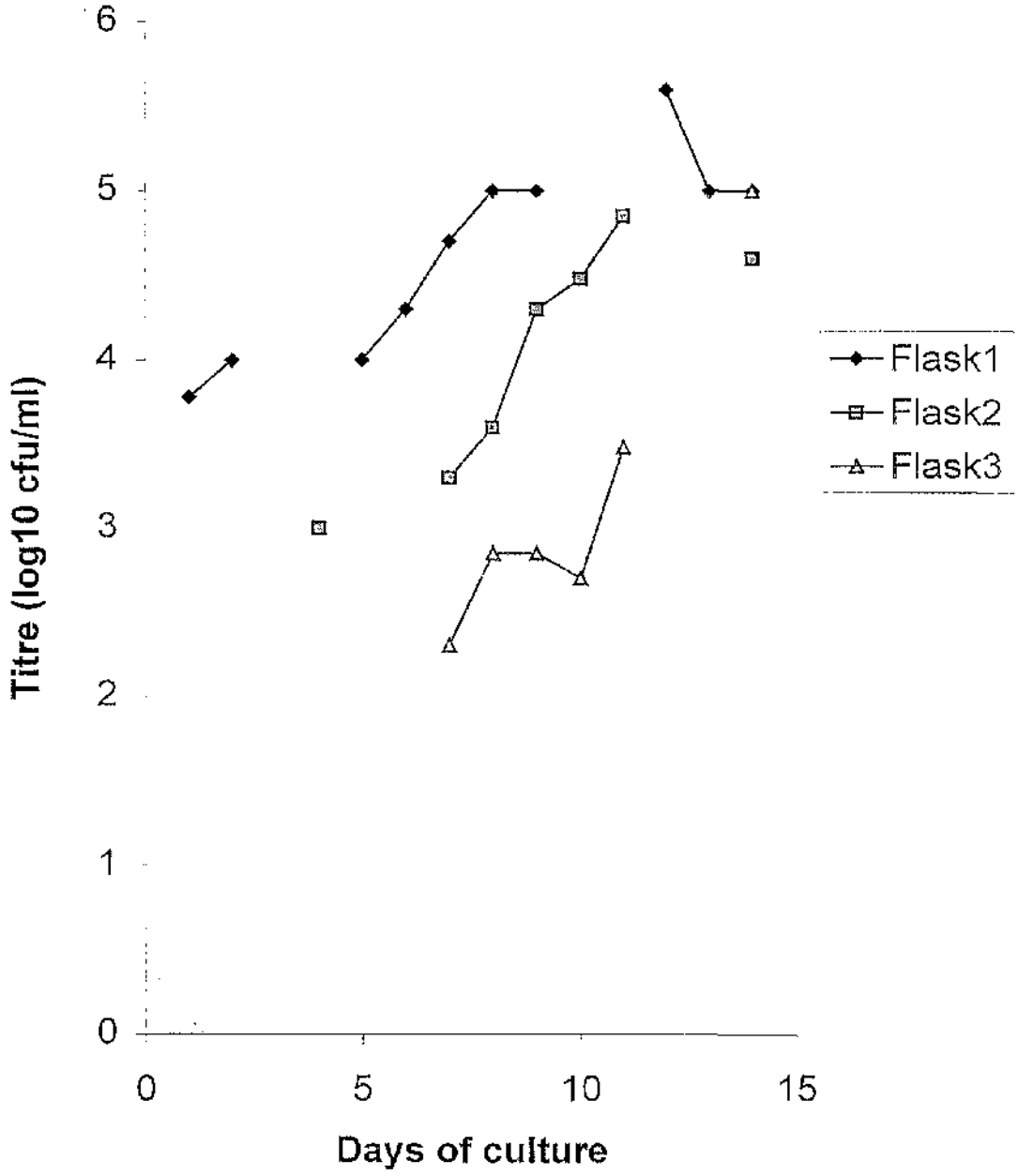

Figure 3.5 Retroviral vector titres from PALidSN cells cultured in roller bottles in ultraculture serum free media

3.3.4 Optimisation of viral vector production by PALidSN cells cultured in 850cm^2 roller bottles

3.3.4.1 Viral vector production from supernatants harvested over a 15 day period from PALidSN cells cultured in roller bottles in DMEM containing 10% FBS

PALidSN cells were cultured in roller bottles in high glucose DMEM containing 10% FBS, and the viral vector titre was established by assay of supernatant on NIH-3T3 cells. Table 3.10 presents vector titres from PALidSN cultures in high glucose DMEM with 10% FBS from three separate roller bottles from which supernatant was harvested over a fifteen day period, as described in section 3.2.6.

The results of the assays indicated that viral vector titres in roller bottles with high glucose DMEM supplemented with 10% FBS were of similar titre to virus harvested from tissue culture flasks (see Table 3.5). Titres in the region of 10^4 colony forming units per ml (cfu/ml) were obtained in the first three days of culture (roller bottle 2), rising to $2X10^5$ cfu/ml after four days. Thereafter, the titres of supernatants from roller bottles over the fifteen-day period ranged from $1X10^5$ to $8X10^5$ cfu/ml, with the exception of supernatant on days 7 and 10 from roller bottle 3, for which slightly lower titres were obtained.

Table 3.10 **Titres of retroviral vector in supernatants harvested over a 15-day period from PALidSN cells cultured in DMEM (4500mg/L glucose containing 10% FBS) in roller bottles.**

Day	Roller Bottle 1	Roller Bottle 2	Roller Bottle 3
1	N/A[1]	4×10^4 [2]	N/A
2	N/A	2×10^4	N/A
3	N/A	4×10^4	N/A
4	2×10^5	2×10^5	N/A
5	8×10^5	N/A	N/A
6	5×10^5	N/A	N/A
7	4×10^5	1×10^5	2×10^4
8	6×10^5	N/A	N/A
9	N/A	3×10^5	6×10^5
10	N/A	N/A	3×10^4
11	2×10^5	3×10^5	2×10^5
12	6×10^5	N/A	N/A
13	3×10^5	N/A	N/A
14	8×10^5	4×10^5	4×10^5
15	3×10^5	4×10^5	3×10^5

1. titres measured by NIH-3T3 titration (cfu/ml)
2. not applicable

3.3.4.2 Viral vector production from supernatants harvested over a 15 day period from PALidSN cells cultured in roller bottles in DMEM containing 1% FBS

PALidSN cells were cultured in a roller bottle in medium containing 1% FBS, to determine the effect of low serum concentration on titre. Cells were cultured for five days in high glucose DMEM containing 10% FBS, whereupon the serum concentration was reduced to 1% FBS. The resulting titres from various harvests taken between day 5 and day 15 are presented in Table 3.11. Titres were found to be slightly lower when PALidSN cells were cultured in media supplemented with 1% rather than 10% serum concentration. However, by days 13 and 14 a titre of $2X10^5$ cfu/ml was obtained, which was comparable with supernatant from cells grown in media containing the higher serum concentration.

Table 3.11 Viral titres measured over a 10-day period from roller bottle culture of PALidSN cells in DMEM (4500mg/L glucose containing 1% FBS)

Day	Titre
5	$7X10^{3(1)}$
6	$7X10^4$
7	$7X10^4$
8	N/A(2)
9	N/A
10	$5X10^4$
11	$8X10^4$
12	$2X10^5$
13	$2X10^5$

1. titre measured by NIH-3T3 titration (cfu/ml)
2. not applicable

3.3.5 Feasibility of PALidSN cells for scaled-up production; PALidSN cell culture on Fibra Cel®disks in a Techne spinner flask.

The results demonstrating feasibility of culturing PALidSN cells on Fibra-Cel® disks in a Techne spinner flask are presented in Table 3.12. The viral titre in the 120-hour supernatant was measured by NIH-3T3 titration at $1X10^6$cfu/ml. The cells adapted well to culture in the spinner flask, and were found both to adhere to the disks and remain free in the supernatant. Counts for both adherent and suspension cells are presented. The results of growth of PALidSN Cells on Fibra-Cel Disks are therefore presented in Figure 3.6.

Table 3.12 PALidSN cell growth and retroviral vector production on Fibra-Cel® disks in a Techne spinner flask.

Time (hours)	Nuclei/disk[1]	Nuclei/ml supernatant[1]	Viral titre[2]
24	1.6×10^5	2×10^5	N/A[3]
48	1.3×10^5	2.1×10^5	N/A
120	5.2×10^5	2.5×10^4	1×10^6
144	1.8×10^5	N/A	N/A
168	2.3×10^5	3.9×10^5	N/A

1. viable cell counts
2. titre measured by NIH-3T3 titration (cfu/ml)
3. not applicable

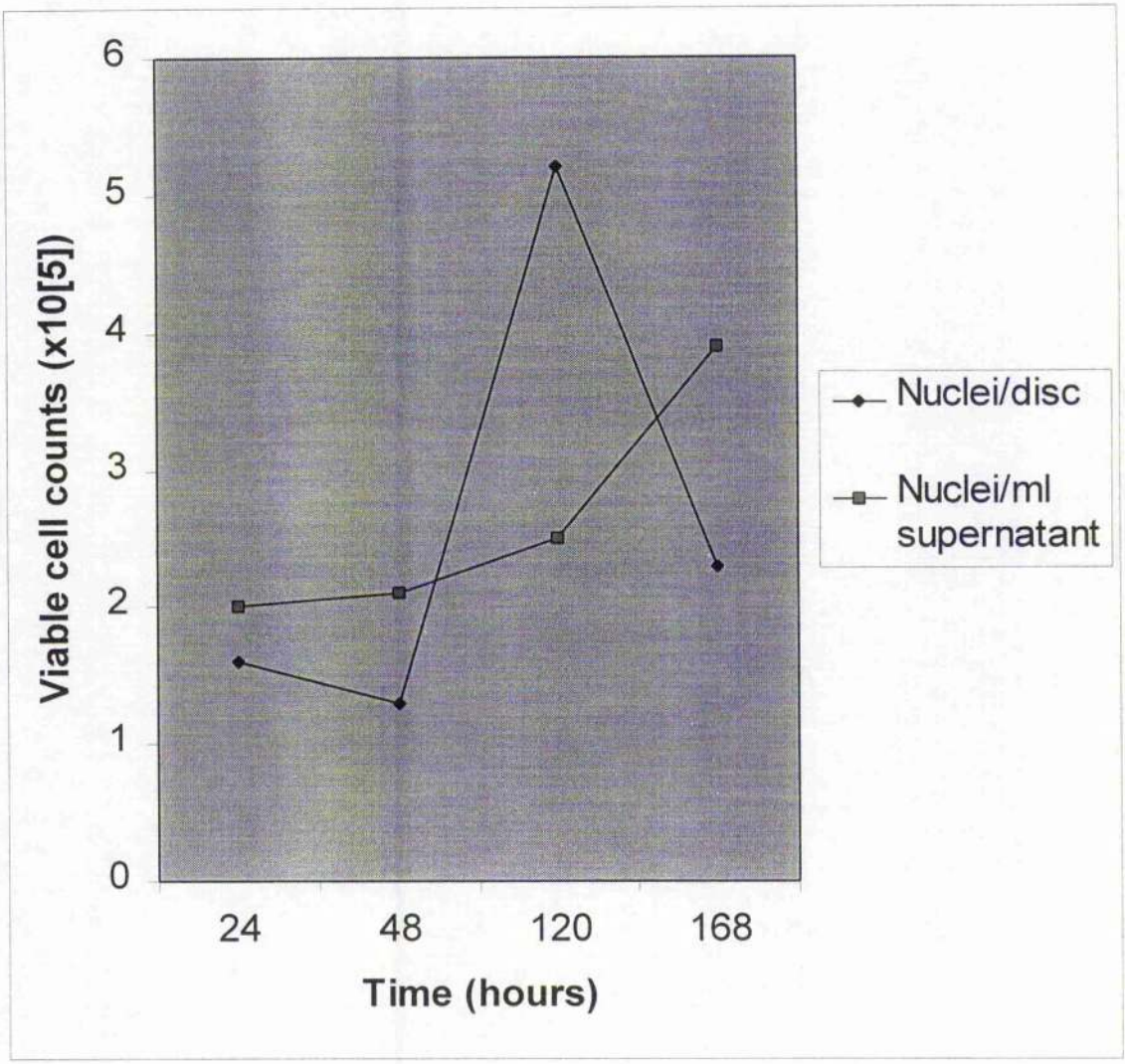

Figure 3.6 **PALidSN cell culture on Fibra Cel®disks in a Techne spinner flask**

3.3.6 Concentration of vector supernatant by filtration

Tables 3.13 and 3.14 present results obtained in two separate experiments performed to concentrate retroviral vector by ultrafiltration. The tables present retroviral titres of supernatant harvested from PALidSN cell cultures prior to concentrating by filtration, after concentration (the retentate), and for the filtrate which was drawn off as a control. In table 3.13, the initial volume of 0.45μM filtered retroviral vector supernatant was1140ml. The supernatant was concentrated over a 5-hour period, with the filtrate drawn off at a constant flow rate of 4.5 ml/minute. The flow rate was maintained by adjustment of restriction clamps in the cyclical system. The final volume of retentate (virus containing fraction) was 77ml, which represented 6.75% of the original stock volume. The supernatant was therefore 15 times more concentrated by volume, which would be expected to give an estimated 15-fold increase in viral titre. The data presented in Table 3.13 indicates that the concentration was successful. The titre of the concentrated retroviral vector increased from $2x10^5$ to $2x10^6$ cfu/ml, an increase of 1 log_{10}. The filtrate was shown to contain no vector, as expected.

Table 3.13 Concentration of vector supernatant by filtration (i)

Supernatant for titration	Supernatant volume (ml)	Titre[1]
Filtered starting material	1140	$2 \times 10^{5(1)}$
Retentate	77	2×10^6
Filtrate	1063	virus not detected

1. titre measured by NIH-3T3 titration (cfu/ml)

The data presented in Table 3.14 indicates that the second concentration was also successful. The initial volume of 0.45μM-filtered retroviral vector supernatant was

1300ml, and the final volume of retentate was 300ml, which was 23% of the original stock volume. The supernatant was therefore 4.3 times more concentrated by volume, which resulted in a 5-fold increase in viral titre from $6x10^5$ cfu/ml to $3x10^6$ cfu/ml.

Table 3.14 Concentration of vector supernatant by filtration (ii)

Supernatant for titration	Supernatant volume (ml)	Titre[1]
Filtered starting material	1300	6×10^5
Retentate	300	3×10^6
Filtrate	1000	virus not detected

1. titre measured by NIH-3T3 titration (cfu/ml)

3.4 Summary

3.4.1 Optimal cell culture conditions

The retroviral vector producer line, PALidSN, was initially examined for the effect of varying culture media serum and glucose content on cell growth and viral vector titre. Of the four media formulations examined (DMEM containing 4500mg/L or 1000mg/L glucose supplemented with either FBS or NBS), total viable cell counts were highest for PALidSN cells at the 96-hour timepoint. Optimal cell yield coupled with lowest doubling time was best achieved by culturing PALidSN cells in high glucose DMEM supplemented with 10% FBS (doubling time during exponential growth of 13.99 hours and a highest cell yield of 2.64×10^5 viable cells). Correlation between the results of the enzyme and infectivity assays was observed, in that the highest value of IDUA enzyme activity per milligram of protein (14,940 U/mg) and highest viral vector titre

measured by NIH/3T3 assay ($7X10^4$ cfu/ml) were obtained from supernatant harvested from cells after 96 hours culture in high glucose DMEM containing 10% FBS (Figure 3.1). Reduction of serum concentration from 10% to 5% FBS had negligible effect on viral titres. Reduction of serum concentration to 1% FBS in roller bottles (section 3.4.3) also produced a comparable viral titre ($2x10^5$ cfu/ml), although an extended culture period was required to reach this titre (12 days).

A study was performed to determine the potential for adaptation of PALidSN cells to various types of commercially available serum-free media; Ultraculture, CHO-S-SFMII, QBSF5 and QBSF56 (Table 3.7). Details of the media ingredients were unavailable from the suppliers as the information is patented and proprietary. Of the four types, PALidSN cells were found to be most suitable for adaptation to culture in Ultraculture serum-free medium, for which a lag phase duration of 48 hours and log phase duration of 24 hours were determined. The doubling time was determined to be 22.43 hours with a viable cell yield of $3.09x10^4$ viable cells/cm^2. Although the doubling time appears to be short, it must be noted that the log phase of growth was only 24 hours and the cell culture began to decline after this point. This indicates that feeding or harvesting may be required every 72 hours.

3.4.2 Effect of incubation temperature and inducing agents on viral vector titre

Incubation of producer cells for 3 days at 32°C rather than 37°C had no effect on PALidSN viral vector titre. The induction of cultures for 3 days by TPA or NaB also had no effect on viral vector titre.

3.4.3 Scale-up of cell culture

PALidSN cells adapted well to culture scale-up from tissue culture flasks to roller bottles and Fibra-Cel® disks in a Techne spinner flask. Culture was scaled-up in high glucose

DMEM containing 10% FBS, and the viral titre in the supernatant harvested after 120 hours was found to be $1X10^6$cfu/ml.

3.4.3.1 Cell culture in roller bottles

Cells adapted well to culture in the roller bottle system, producing viral vector titres comparable to those obtained by culture in tissue culture flasks. In the roller bottle system, when the culture was incubated for four days prior to harvesting, viral titres increased to $2x10^5$ cfu/ml on day 4, and the maximum viral titre obtained was $8x10^6$ cfu/ml on days 5 and 14 (Table 3.10). This titre was not achieved from cells where supernatants were harvested daily, or when cultures were incubated for 7 days prior to harvesting. One roller culture of PALidSN cells was incubated in DMEM containing 10% FBS until confluent (4-5 days) after which serum concentration was reduced to 1% FBS to determine the effect on production of vector. The results (Table 3.11) indicate that reducing the serum concentration to 1% FBS (in 150mls media) reduced production of viral vector from $4.7x10^5$ cfu/ml (roller bottle 1, mean viral vector titre) to $4.93x10^4$ cfu/ml (1% FBS, mean viral vector titre). Decreasing the volume of media to 75ml resulted in an increased titre of $1.45x10^6$cfu/ml (Table 3.11, roller bottle 2 in 1% serum, Day 11 to Day 14, mean viral vector titre). The resulting viral titre is comparable to titres obtained from cells cultured in serum containing media, however, required an extended culture period of 11 to 14 days to reach this titre.

3.4.3.2 Cell culture on Fibra-Cel disks in a Techne spinner flask

PALidSN cells also adapted well to growth on Fibra-Cel disks in a Techne spinner flask. Initial observations of the data generated in the study showed that at the 24-hour timepoint 43% of the PALidSN cells remained free in the supernatant. The large volume of circulating medium owing to the disks not being tightly packed may explain the large number of cells in suspension. At the 48-hour stage the medium was

replaced resulting in removal of cells in suspension and it was noted that logarithmic phase growth continued from this point to the 120-hour timepoint. A harvest of the supernatant generated at the 120 hour timepoint was titrated and the titre found to be $1x10^6$cfu/ml, which is approximately 0.5 log higher that the average titre which was obtained by roller bottle production ($4.7X10^5$ cfu/ml). Unfortunately, further samples from later time-points were unavailable.

3.4.4 Concentration of vector supernatant by filtration

Retroviral vector supernatant was successfully concentrated by filtration using SARTOCON MICRO 100KD filters. In one study, the volume of supernatant was decreased 15-fold by concentration, which resulted in an increase in viral vector titre of 1 $\log_{10}$ unit (from $2x10^5$ c.f.u/ml to $2x10^6$ cfu/ml), and in the other, the volume of supernatant was decreased 4.3-fold by concentration, which resulted in a 5-fold increase in viral vector titre (from $6x10^5$ c.f.u/ml to $3x10^6$ cfu/ml). As expected, the filtrate which was drawn from the system was found to contain no virus particles.

4.0 STABILITY OF RETROVIRUS SUPERNATANTS

4.1 Introduction

Retrovirus-containing supernatant stocks are obtained from producer cells by harvesting the culture medium in which the cells have been growing. The log phase of cell culture is when exponential growth occurs and the proportion of cells in cycle is high. Since infected cells release virus at a maximum rate when growing exponentially (Roe *et al.*, 1993, Morgan *et al.*, 1995), the highest titres should be obtained before an adherant cell line reaches confluence. In practice, maximum achievable recombinant viral titres have been reported to be approximately 10^6cfu/ml. Retroviral particles are considered to be fragile, with short half-lives even under optimal conditions (Sanes *et al.*, 1986, Paul *et al.*, 1993). They are also reported to be relatively labile in terms of the narrow pH range and ionic strength within which retrovirus infectivity can be maintained (Naussbaum *et al.*, 1993). Therefore, harvesting should be performed gently, and materials should be kept cold in order to maintain viral infectivity.

Retroviral vector stocks are stable for long periods of time when stored frozen at -70°C in harvested tissue culture medium containing 10% FBS. Stocks should be frozen in small aliquots as repeated freeze thawing can considerably reduce viral titre. However, storage of retroviral vectors at -70°C in medium containing 10% serum is impractical for many clinical facilities, therefore other methods of storage have been explored. These include lyophilization with storage at -20°C, for which a half-life of over 200 days has been reported, and storage at 4°C, for which a shelf-life of over 3 months can be expected (Jolly, 1994). In addition it has been reported that sugars can act as stabilisers and help to preserve unstable samples by replacing water, thus providing a stable structure to biological molecules (Aldridge, 1995).

In this section of the study, the stability of retrovirus containing supernatants was

assessed by comparing titres of vector and wild-type AMLV stock after storage at varying temperatures and time. The effect on titre of storage in the presence of various sugars was also determined.

4.2 Materials and Methods

4.2.1 Virus and vector supernatants

The producer cells used in for these experiments were PALidSN cells, described in Section 3.1. These cells are based on the amphotropic retrovirus vector packaging cell line PA317. The wild-type A-MLV supernatant (strain 292A, ATCC VR-884) was also assessed for stability as a control. Supernatant from the *Mus dunni* cell line (ATCC CRL 2017) infected with A-MLV was used as control for comparison with vector supernatant.

4.2.2 Storage of virus stocks at varying temperatures

Retroviral vector and A-MLV containing supernatants were harvested, aliquoted, and the viral titres determined. In order to determine the stability of titre, aliquots of supernatant were held at -70°C, 4°C, 32°C, 37°C, and 56°C for varying periods of time and assayed for the presence of virus. The experimental procedure for titration of PALidSN supernatant in murine NIH-3T3 cells by *neo* resistance is detailed in section 2.3.1, and A-MLV titration on PG4 S^+L^- cells as described in 2.2.2.3.

4.2.3 Storage of supernatants at 4°C and -70°C in the presence and absence of sugar stabilisers

Supernatants were harvested and titrated as above (section 4.2.2.) then aliquots were placed at 4°C or -70°C in 50% v/v solutions of the sugars trehalose (Sigma), mannose (Sigma) and sorbitol (Sigma). The solutions were prepared in PBS and 0.22µm filtered.

The glucose solution was supplied as a 20% solution and was used as such (Gibco). An aliquot (1ml) of each of the sugar solutions was added to viral supernatant (19mls) derived from PALidSN producer cells or *Mus dunni* /A-MLV infected cells and aliquots stored at 4°C or -70°C. Stability of retrovirus containing supernatants was assessed by assay of virus stored at both temperatures at various time-points. Virus stored in the absence of various sugar stabilisers was tested at each time-point as a control.

4.3 Results

Stability of retrovirus containing supernatants was assessed by titration following storage at different temperatures for varying periods of time in the presence and absence of sugars. The resulting titres are displayed in $\log_{10}$ colony forming units of vector per ml ($\log_{10}$ cfu/ml) and $\log_{10}$ focus forming units of A-MLV per ml ($\log_{10}$ ffu/ml).

4.3.1 Inactivation of virus and vector supernatant at 56°C

Retroviral vector and A-MLV supernatants from three tissue culture flasks were harvested and held at 56°C for two hours. Over the two-hour period samples were removed for titration on NIH-3T3 and PG4 S^+L^- cells, respectively. The resulting titres converted to $\log_{10}$ ffu/ml virus and $\log_{10}$ cfu/ml vector are presented in Table 4.1. The results indicated that vector and A-MLV were rapidly inactivated at 56°C. However in three separate studies, both A-MLV and vector appeared to contain a fraction that was resistant to heat invactivation after two hours incubation at 56°C.

Table 4.1 **Titres of retroviral vector and wild type A-MLV after incubation at 56°C for varying periods**

	VECTOR[1]			A-MLV[2]		
Time	Expt. 1	Expt. 2	Expt. 3	Expt. 1	Expt. 2	Expt. 3
Initial titre	5.60	5.60	5.48	5.36	5.18	4.00
30-40 min	0	2.48	N/A[3]	3.43	0	N/A
1 hour	1.78	1.00	3.60	3.26	1.78	2.00
2 hours	N/A	2.48	2.70	N/A	1.30	0

1. titre measured by NIH-3T3 titration (log_{10} cfu/ml).
2. titre measured by PG4 S^+L^- assay (log_{10} ffu/ml).
3. not applicable

4.3.2 Stability of vector and A-MLV supernatants at various temperatures over 24 hours

Vector and A-MLV supernatants were stored at 4°C, 32°C and 37°C over a 24-hour period. During incubation, samples were removed for titration on NIH-3T3 and PG4 S^+L^- cells, respectively. The resulting titres displayed as log_{10} ffu/ml virus and log_{10} cfu/ml vector are presented numerically in Table 4.2, and graphically in Figure 4.1. No significant change in viral titre was observed after eight hours storage at each temperature for either virus or vector. However, after 24 hours, a decrease in titre was observed for vector stored at 32°C and 37°C, and A-MLV stored at 4°C and 32°C. A-MLV incubated at 37°C for 24 hours retained only 0.4% of the initial titre, a reduction of 2.36 log_{10} units. At 4°C, vector titre displayed greatest stability, decreasing by only 0.3 log_{10} units over 24 hours.

Table 4.2 **Stability of titres of vector and A-MLV after incubation at 4°C, 32°C or 37°C over a 24- hour period**

TIME	VECTOR[1]			A-MLV[2]		
	4°C	32°C	37°C	4°C	32°C	37°C
Initial titre	5.60	5.60	5.60	5.36	5.36	5.36
30 min	5.70	5.70	5.60	5.00	5.00	5.26
1 hour	5.70	5.60	5.70	5.34	5.34	5.32
2 hours	5.70	5.60	5.70	5.04	4.95	4.85
4 hours	5.60	5.48	5.60	5.20	5.04	5.23
8 hours	5.60	5.30	5.30	5.38	5.04	N/A[3]
24 hours	5.30	4.48	4.60	4.30	4.48	3.00

1. titre measured by NIH-3T3 titration (log_{10} cfu/ml).
2. titre measured by PG4 S$^+$L$^-$ assay (log_{10} ffu/ml).
3. not applicable

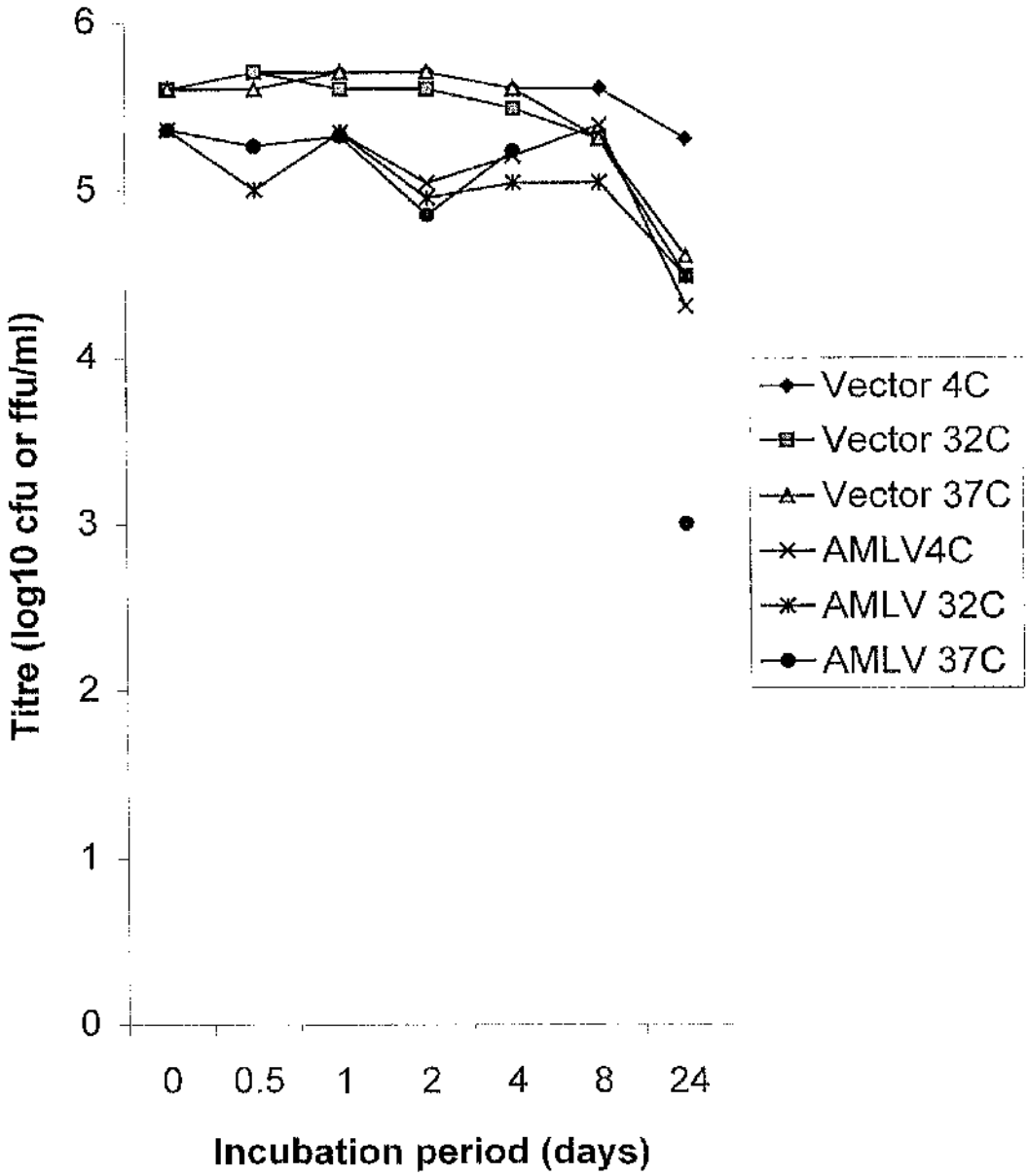

Figure 4.1 Titres of vector and A-MLV following incubation at varying temperature over a period of 24 hours.

4.3.3 Long-term stability of vector and A-MLV supernatants at 4°C, 32°C or 37°C

Vector and A-MLV supernatants were incubated at 4°C and 37°C over a longer period of time. Over a thirty-seven day period samples were removed for titration on NIH-3T3 and PG4 S^+L^- cells, respectively. The resulting titres converted to log_{10} ffu/ml virus, and log_{10} cfu/ml vector are presented in Table 4.3 and Figure 4.2. The decrease of 2.4 log_{10} units in titre for A-MLV over 24 hours at 37°C was similar to previous results (Table 4.2.). In this study, the vector titre decreased by 2.65 log_{10} over 24 hours at 37°C in comparison to a decrease of 1 log_{10} in the previous study. Both vector and A-MLV were completely inactivated by storage at 37°C for 3 days. However, differences were observed in that vector was apparently more stable than A-MLV over long-term incubation at 4°C. A-MLV titre declined throughout the study period and virus could not be detected after 37 days, whereas vector had decreased in titre by 0.6 log_{10} units after 37 days.

Table 4.3 Titres of vector and A-MLV after long-term incubation at 4°C or 37°C

	VECTOR[1]		VIRUS[2]	
	4°C	37°C	4°C	37°C
Initial titre	5.60	5.60	5.18	5.18
D1	5.90	2.95	3.85	2.78
D3	4.00	0	3.90	0
D7	5.08	0	3.70	0
D16	5.30	N/A[3]	1.70	N/A
D37	5.00	N/A	0	N/A

1. titre measured by NIH-3T3 titration (log_{10} cfu/ml).
2. titre measured by PG4 S^+L^- assay (log_{10} ffu/ml).
3. not applicable

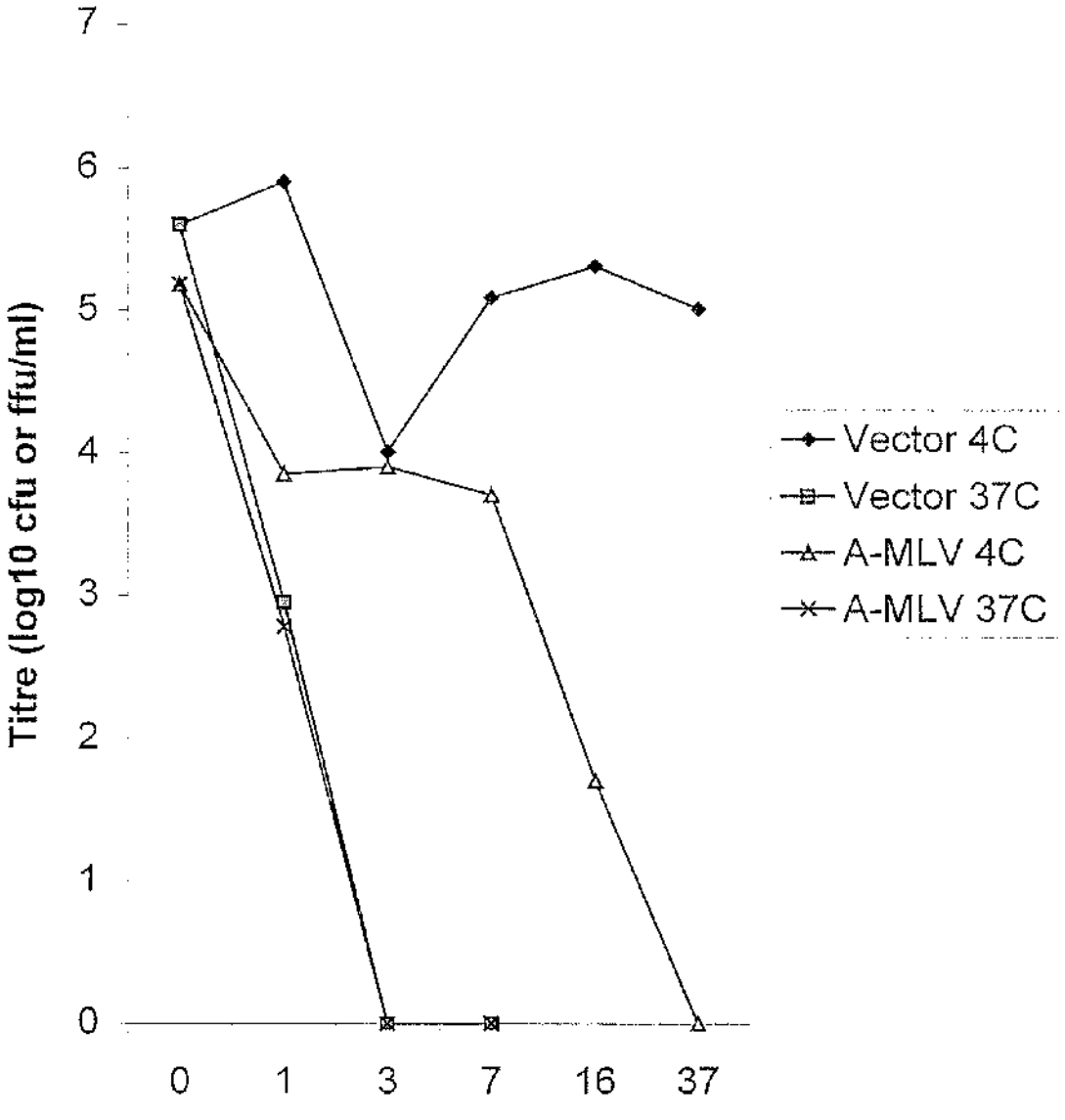

Figure 4.2 Titres of retroviral vector and A-MLV after incubation at varying temperature over a period of 37 days

4.3.4 Stability of vector and A-MLV supernatants at 4°C or -80°C with sugar stabilisers

4.3.4.1 Stability of vector and A-MLV supernatants at -80°C with sugar stabilisers

Vector and A-MLV supernatants were stored at -80°C in the presence of the sugar solutions mannose, sorbitol and trehalose as described in Section 4.2.3. Over the storage period samples were removed for titration on NIH-3T3 and PG4 S^+L^- cells, respectively. The resulting titres in log_{10} ffu/ml virus, and log_{10} cfu/ml vector are presented in Table 4.4. No significant change in titre (that is, greater than 0.5 log units) was effected by storage of vector supernatant at -80°C over 21 days, or A-MLV supernatant at -80°C over 65 days with or without sugar stabilisers present.

Table 4.4 Stability of vector and A-MLV at -80°C with sugar stabilisers

	VECTOR[1]					A-MLV[2]				
	-ve[3]	glc.[4]	mann.[5]	sorb.[6]	tre.[7]	-ve	gluc.	mann.	sorb.	tre
initial	5.48	5.48	5.48	5.48	5.48	4.00	4.00	4.00	4.00	4.00
D1	5.08	5.00	5.30	4.95	5.30	4.00	4.00	4.00	4.00	4.00
D21	5.00	4.78	5.48	4.78	5.00	4.30	4.00	4.00	4.00	4.04
D65	N/A[8]	N/A	N/A	N/A	N/A	4.60	4.48	4.48	4.30	4.48

1. titre measured by NIH-3T3 titration (log_{10} cfu/ml).
2. titre measured by PG4 S^+L^- assay (log_{10} ffu/ml).
3. negative control :
4. glucose
5. mannose
6. sorbitol
7. trehalose
8. not applicable

4.3.4.2 Stability of vector and A-MLV at 4°C with sugar stabilisers

Vector and A-MLV supernatants were stored at 4°C in the presence of the sugar solutions glucose, mannose, sorbitol and trehalose as described in Section 4.2.3. Over the storage period samples were removed for titration on NIH3T3 and PG4 S^+L^- cells, respectively. The resulting titres in log_{10} ffu/ml virus, and log_{10} cfu/ml vector are presented in Table 4.5. No significant change in titre (that is, greater than 0.5 log units) was observed in vector or viral supernatant stored at 4°C over 16 days with or without sugar stabilisers present. However, when viral vector supernatant in the absence of sugar was titrated following 70 days storage at 4°C, no colonies were observed. This contrasted with titres obtained for viral vector supernatant stored in the presence of each of the sugars studied, where the titre obtained was comparable to titres obtained following 16 days storage at 4°C. These titrations were repeated, and identical results obtained.

Table 4.5 Stability of vector and A-MLV at 4°C with sugar stabilisers

	VECTOR[1]					A-MLV[2]				
	-ve[3]	glc.[4]	mann.[5]	sorb.[6]	tre.[7]	-ve	gluc.	mann.	sorb.	tre
initial	5.48	5.48	5.48	5.48	5.48	4.00	4.00	4.00	4.00	4.00
D2	5.00	5.00	5.48	5.00	5.48	3.60	3.70	3.30	3.95	3.70
D8	4.85	4.78	4.60	4.95	5.00	N/A[8]	N/A	N/A	N/A	N/A
D16	5.48	5.00	4.85	5.30	5.00	4.60	4.48	3.70	4.00	4.00
D70	0	4.84	4.95	4.90	4.85	N/A	N/A	N/A	N/A	N/A

1. titre measured by NIH-3T3 titration (log_{10} cfu/ml).
2. titre measured by PG4 S^+L^- assay (log_{10} ffu/ml).
3. negative control
4. glucose
5. mannose
6. sorbitol
7. trehalose
8. not applicable

4.4 Summary

In these initial studies, the retroviral vector exhibited similar stability to wild type A-MLV. Both vector and virus contained a fraction that was resistant to inactivation following two hours incubation at a temperature of 56°C. In addition, the results were variable, with virus detected at later time-points, although apparently undetectable after 30-40 minutes of incubation. Virus validation studies previously performed at Q-One Biotech have produced similar results (unpublished data). This may be caused by variations in sampling in accordance with the Poisson distribution, however, with the limited data available in this study, these results should be treated with caution.

Following three days incubation at 37°C, both vector and A-MLV supernatants were completely inactivated. Supernatants were relatively stable at 4°C, vector supernatant slightly more so than virus supernatant; A-MLV was inactivated after 37 days storage at 4°C, whereas only 2 log units of vector titre were lost after storage at 4°C. In a limited study the presence of the sugars glucose, mannose, trehalose, or sorbitol, had no significant effect on virus or vector titre within three weeks storage at 4°C or -80°C. No decrease in titre was observed for vector or A-MLV stored at 4°C or -80°C in the presence or absence of sugar stabilisers. However, viral vector supernatant appeared to be inactivated following 70 days storage at 4°C. This effect was not observed in viral vector supernatant stored in the presence of sugar stabilisers, in that viral titres obtained following 70 days storage were comparable with those obtained within the initial three-week storage period. Further long-term studies on the effect of sugar stabilisers on MLV titre are necessary.

5.0 DISCUSSION

5.1 Occurrence of RCR

A potential problem in the use of retroviral vectors is the possibility of recombination events occurring which result in generation of replication competent retrovirus (RCR) (Miller *et al.*, 1993, Otto *et al.*, 1994, Vanin *et al.*, 1994). The principal factor influencing RCR formation is the level of homology between the vector genome and packaging cell line sequences, which may undergo genetic recombination to form RCR. The potential risk of RCR formation in clinical application was highlighted by the observation that murine RCR generated in this manner was associated with lymphomas in immunosuppressed rhesus macaques (Donahue *at al.*, 1992). Another factor influencing RCR generation is the method used to introduce the viral protein coding sequence into the producer cell line; if the *gag-pol* and *env* sequences are introduced as two separate plasmids, the possibility of RCR formation occurring during vector construction is decreased.

The rate of recombination that occurs in a vector-producing cell line is dependent on the particular characteristics of the cell line, and theoretically, therefore, would vary between producer cell lines. Third generation packaging cell lines were developed to minimise sequence homology beween the vector and packaging cell line sequences, where three separate recombination events are necessary for RCR formation. This has greatly improved confidence in the safety aspect of using retroviral vectors. However, the producer cell line GP+envAM12 (Markowitz *et al.*, 1988), which was developed by introducing vector sequences into a third generation packaging cell line on two separate plasmids, has been reported to generate RCR by a mechanism which is not yet fully understood (Chong and Vile, 1996).

Another potential hazard in vector safety is the contamination of retroviral vectors by endogenous murine proviral elements that also may recombine with packaging cell line sequences to generate RCR. Most stable packaging cell lines currently used are derived from rodent cell lines, such as NIH-3T3 cells, which have been engineered to express MLV *gag, pol* and *env* genes in order to generate recombinant retroviral vector particles (Miller and Buttimore, 1986, Markowitz *et al.*, 1988, Danos and Mulligan, 1988). Most rodent cell lines carry endogenous viral sequences, which have been reported to recombine with vector sequences to generate RCR (Otto *et al.* 1994, Purcell *et al.*, 1996). VL30 elements, which are common endogenous viral sequences in rodent cell lines, have been reported to be efficiently packaged into virions, and undergo reverse transcription, integration, and high level expression in the host cell (Hatzoglou *et al.*, 1993). Owing to the presence of such endogenous elements in addition to polytropic or xenotropic retroviruses that can infect human cells, murine cells are not ideal packaging lines for use in human gene therapy. Therefore it is beneficial to develop packaging lines and vectors which do not contain endogenous viral sequences. Feline cells have also been used as packaging cell lines. However, feline cells also contain endogenous viral sequences, including the RD114 virus, which could potentially be involved in RCR generation, and thereby compromise safety (Coffin, 1996). Packaging cell lines derived from human and canine cells, which lack endogenous retroviruses, are also potential candidates for vector development. The Madin-Derby canine kidney (MDCK) cell line has been found to be negative for endogenous type-C retrovirus production (Squires, 1991), and has potential for exploitation as a packaging cell line. Human cells can be readily infected with retroviruses from various animal species, and different receptor groups have been defined by interference assays (Somerfelt and Weiss, 1990). A packaging line derived from human 293 cells expressing an ecotropic MLV vector with amphotropic MLV *env* genes was recently described which contained minimal sequence homology between the vector genome and structural genes, and has not demonstrated RCR formation throughout extensive testing (Rigg *et al.*, 1996). Other limitations associated with murine packaging cell lines are the relatively low titres generated, and the complement sensitivity

of particles derived from them (Cosset *et al.*, 1995, Rigg *et al.*, 1996). The susceptibility of retrovirus vectors derived from MLV to inactivation in human serum has a negative impact on gene delivery *in vivo*, therefore, the production of vector in human cells may be more appropriate for *in vivo* therapy. A packaging cell line derived from 293 cells was reported to transduce target cells more efficiently than the NIH-3T3 CRIP packaging cell line (Danos and Mulligan, 1988) by a minimum of 10-fold (Davis *et al.*, 1997). This is advantageous both in terms of yielding producer cell lines with enhanced transduction efficiencies and theoretically in terms of safety.

Contamination of packaging cell lines with RCR may also be caused by introduction of virus from other cell lines within a facility. Such contamination should be preventable by adherence to Good Manufacturing Practice (GMP). However, failure in the management of the system can cause introduction of RCR from elsewhere in the facility as a contaminant of the system. RCR has also been mistakenly identified as an artefact of the detection system (Miller *et al.*, 1996).

5.2 Detection of RCR

The development of lymphomas in association with recombinant A-MLV infection in three of ten highly immunosuppressed macaques raised concern about the risks of MLV exposure in humans (Donahue *et al.*, 1992). All three animals died within 200 days, and high titre retroviraemia and murine RCR sequences were later detected within the lymphomas of these animals (Vanin *et al.*, 1994, Purcell *et al.*, 1996). Although RCR was not directly proven to be the causative agent of disease, it is reasonable to conclude from the results that exposure to high RCR levels under immunosuppressed conditions could result in lymphomas. Safety testing for the presence of RCR in production cells and retrovirus vectors is therefore of utmost importance. Current recommendations for the detection of RCR in retroviral vector products are set by the FDA (CBER, 1991,1996, 1998) in the U.S. and the CPMP (CPMP, 1995) in Europe. Specific recommendations for

testing retroviral gene therapy products from CBER indicate that RCR testing should be performed by infectivity or co-cultivation assays using an appropriate cell line such as the *Mus dunni* tail fibroblast cell for amplification followed by detection using the PG4 S^+L^- cell line . Other tests such as a marker rescue assay (Forestell *et al.*, 1996, Rigg *et al.*, 1996) are also suitable as an alternative to this testing.

Mus dunni cells were already in use at Q-One Biotech in tests for detection of xenotropic retrovirus in murine monoclonal antibody producing cell lines, and it seemed logical to adapt these assays for detection of RCR with A-MLV envelope. The assay format adapted for use at Q-One Biotech for RCR detection was infection or co-cultivation of samples with *Mus dunni* monolayers, followed by subculture over 5 passages in order to amplify infectious virus. Supernatants harvested after passage 5 of *Mus dunni* culture are assayed for infectious virus on feline PG4 S^+L^- cell monolayers.

The experiments performed in this study were undertaken in order to determine the sensitivity of the assay for detection of RCR and the optimal conditions for assay performance. Factors that may adversely affect assay sensitivity such as presence of interfering non-RCR were also investigated. *Mus dunni* cells were recommended by the FDA as a requirement for RCR testing as they are susceptible to infection by all known retrovirus classes, thereby allowing detection of anticipated infectious virus (Lander and Chattopadhyay, 1984). It has been reported that ecotropic Moloney MLV (E-MLV) infects *Mus dunni* cells poorly (Lander and Chattopadhyay, 1984). However, this is not of great concern with respect to human gene therapy protocols, as E-MLV infects cells of murine origin only. In the studies described in this report, comparison of relative sensitivities of mink S^+L^-, *Mus dunni* and feline PG4 S^+L^- cells by direct infectivity assay determined that *Mus dunni* and PG4 cells were of similar sensitivity. Mink S+L- cells were less sensitive than both *Mus dunni* and PG4 S^+L^- cells by approximately 1 log in titre (Table 2.1). Further comparison of extended infectivity assays for the detection of RCR evaluated that *Mus dunni* and PG4 S^+L^- assays were comparable in sensitivity to A-MLV infection over five

passages (Table 2.2). These results contrast with studies at Q-One Biotech (unpublished) which indicated that mink S^+L^- cells are more sensitive than feline PG4 S^+L^- cells for detection of xenotropic MLV. The Mn^{2+}-dependent RT activity and PG4 S^+L^- assay results at each passage of *Mus dunni* cells infected with A-MLV at low multiplicity suggested that 3 passages are adequate for viral detection, as enzyme activity and infectivity increased to reach a plateau at passage 3 (Figure 2.2). Infectivity results by PG4 S^+L^- assay of extended *Mus dunni* supernatants showed no change in virus titre from passage 2 onwards (a virus input of 10^{-1} infectious units resulted in confluent foci) indicating the high sensitivity of the assay. The data suggests that the current method for *Mus dunni* detection assay over five passages may be excessive, and that three passages may prove adequate for testing of supernatants by extended assay. This would be advantageous in reducing the time required for testing of retroviral vector prior to clinical use. However, it is possible that RCR generated from vector producing cell lines may be more fastiduous and slower growing than the wild type A-MLV used as a control in the present experiments.

A potential problem in testing supernatant for the presence of RCR is that the presence of high-titre retroviral vector may potentially affect the sensitivity of detection assays. In this study, wild type A-MLV was titrated through varying concentrations of PALidSN supernantant and tested by PG4 S^+L^- assay in order to determine if the presence of replication defective vector can mask the presence of low level RCRs in test supernatants. The sensitivity of the direct PG4 S^+L^- assay was not affected in the presence of vector supernatant with a titre of less that 10^5 cfu/ml, but there was a loss in sensitivity of the direct PG4 assay in the presence of retroviral vector in excess of 10^5 cfu/ml (Table 2.9). This observation is in agreement with the results of other reports which have indicated that the presence of high titre vector reduces the sensitivity of detection assays (Printz *et al* 1995, Cornetta *et al.*, 1993, Forestell *et al.*, 1996). This result was shown to be specific for vector supernatant, as viral titre was not reduced by titration of A-MLV in the presence of E-MLV at a titre of 10^5 pfu/ml. E-MLV uses a different viral

receptor to A-MLV, therefore the A-MLV receptor can be blocked by vector but not by E-MLV (Somerfelt and Weiss, 1990).

Validation of *Mus dunni* co-cultivation procedures demonstrated that the presence of 1 RCR in 10^7 cells can be detected by direct co-cultivation in tissue culture flasks or indirect co-cultivation using transwell dishes. The presence of vector producer cells in the co-cultivation assay was found not to inhibit detection of A-MLV. These results suggest co-cultivation and/or extended assays rather than direct assay should be performed for detection of RCR. The results suggest that although testing vector supernatants by infectivity assay provides a useful indication of safety, co-cultivation methods may be more reliable for detection of RCR. However, results of studies reviewed by CBER (Wilson *et al.,* 1997) indicated, suprisingly, that on occcasion positive results in RCR assays have been obtained by assay of supernatant while negative results were obtained in co-cultivation assays of the corresponding producer cells. Therefore, although the results of this and other reports indicate that co-cultivation assays for vector-producing cells are more sensitive than supernatant testing for RCR detection (Printz *at al.*,1995), CBER continues to recommend that both extended assay using vector supernatant, and co-cultivation assay using vector-producing cells are performed until further data is obtained (CBER, 1998).

A recent review of the CBER regulations for RCR testing during retroviral vector production and patient monitoring examined data which has been accumulated since the original guidelines were implemented in 1993 (Wilson, 1997). A number of important points are addressed in this paper. Firstly, the 'marker rescue assay' is recommended as an acceptable alternative assay system to the PG4 S^+L^- detection system (Printz *et al.*, 1995, Forestell *et al.* 1996, Miller *et al.*, 1996). The marker rescue assay is based on the presence of RCR in a sample resulting in the 'rescue' or mobilisation of a vector encoding a marker gene, which can be visualised following inoculation onto an indicator cell line. As recommended in previous guidelines (CBER, 1996), the marker rescue assay employed for RCR detection should be validated, and shown to be of similar sensitivity to the PG4

S^+L^- assay. Secondly, CBER has identified and deposited with the ATCC a producer cell line for production of a standardised RCR stock for use as a control in assays employed in the detection of RCR. The stock is based on the proviral clone Mo-MLV with *env* coding sequences replaced by the 4070A strain of A-MLV. This RCR standard will be characterised by electron microscopy to determine the particle count, PG4 S^+L^- assay to determine titre, and extended *Mus dunni* assay with PG4 S^+L end-point to determine end-point limiting dilution, in four independent laboratories (Wilson *et al.*, 1997). The use of this standard may have implications regarding the volume of supernatant required to be tested. The addendum to the CBER guidelines published in 1993 (CBER, 1996) recommends that a volume of 5% of the total vector-containing supernatant in each lot of manufactured vector product is amplified on *Mus dunni* cells, followed by PG4 S^+L^- assay to detect RCR. However, knowing the sensitivity of the assay in relation to the standard, the criteria limits for RCR, and the probability limits for detecting RCR, the required test volume can be calculated from the Poisson distribution (assuming the RCR is characterised by the Poisson distribution) (Wilson *et al.*, 1997). However, it is probable that a standard sensitivity of the infectivity assay would be difficult to validate given that different producer/cell-vector combinations may produce different levels of inteference in RCR detection systems. The results of the RCR testing presented in this report would require to be re-validated using the new vector and producer cell line controls that have been deposited with the ATCC.

CBER is at present considering evaluation of a standard RCR-producing cell line for use in validating the sensitivity of co-cultivation assays. The guidelines (CBER, 1996) currently recommend co-cultivation of 1% or 10^8 (whichever is less), of the total pooled post-production cells or *ex vivo* transduced cells, with a cell line permissive for MLV replication eg. *Mus dunni* cells, and testing the resulting supernatants by PG4 S^+L^- assay. Should a standard be produced, the statistics used for determining the volume of supernatant to be tested above could also be applied to determine the number of cells to be used in the co-cultivation assay, again, depending on the sensitivity of the assay. The

results presented in this report for validation of the co-cultivation assays indicated the high sensitivity of the assay, suggesting that a detection limit of 1 infected cell in a total of 10^7 cells was attainable. Should the standard be produced, these results would also require re-validation to determine any difference in sensitivity of the co-cultivation assay when using the standard control. For *ex vivo* cells, there are a number of additional issues which should be taken into consideration, such as the number of cells in the total patient dose, and the length of time that the cells are in culture following transduction. Further data and discussions are required, in addition to production and characterisation of a co-cultivation standard and validation of assay detection systems, before such recommendations for co-cultivation procedures can be implemented.

Despite implementation of the above measures to minimise risk of RCR formation which have increased confidence in safety of the use of retroviral vectors, additional monitoring of risk is required by monitoring patients receiving treatment for evidence of RCR. Viral infection may be detected by assaying for either free virus, viral antigen, or viral antibodies which may be present in the patient's serum. Therefore, clinical trial patients treated with recombinant retroviral vector are monitored for evidence of RCR using immunological and/or polymerase chain reaction (PCR)-based methods. If productive infection with resultant viraemia occurs, viral DNA or RNA is detectable in peripheral blood lymphocytes (PBLs) by PCR, while viral antibody in patient serum can be detected by serological assays such as Western blot or ELISA. The ELISA assay is quicker and less expensive to perform, but cannot distinguish between an immune response induced by direct vector treatment or RCR infection and thus has the potential to generate false positives induced by vector treatment, or false negatives in immunocompromised subjects (Donahue *et al.*, 1992). Ideally, a PCR assay should be used for patients into whom vector has been directly administered, and ELISA for detection of RCR in the sera of patients receiving *ex vivo* treatment (Martineau *et al.*, 1997). According to CBER, European Regulatory Groups, and the Gene Therapy Advisory Committee (GTAC), the consensus decision on patient monitoring is to perform both PCR assay for viral *env* (or *gag*) sequences on PBL

samples, and Western blot or ELISA assay for detection of viral antibody to *env* proteins on serum samples. In the case of a positive result, where the possibility of a false positive has been eliminated, co-cultivation of PBLs with *Mus dunni* cells should be performed in order to detect infectious retrovirus. Patient monitoring should be performed 6 weeks, 3 months, 6 months, and 12 months after each treatment is complete, and annually after the first trial year (CBER, 1996). The frequency of testing performed, particularly in early stages of treatment is currently under review (Wilson *et al.*, 1997). In addition, Wilson *et al.* (1997) recommend that assays employed in patient monitoring should be chosen with specific consideration to the patient population and mode of vector administration. For example, serological methods may be misleading in an immunosuppressed patient population.

In the recent study in which RCR was erroneously detected as an artifact of the detection system, patient screening following administration of retroviral vector suggested the presence of RCR that originated from human haematopoietic cells (Miller *et al.*, 1996). Characterisation of the viruses, which were detected in all of the patients tested, showed all to be almost identical. Interference analysis suggested that this virus represented a new murine retrovirus group, which the researchers named *Mus dunni* endogenous virus (MDEV). It was reported that hydrocortisone or 5-iodo-2-deoxyuridine (IDU) can activate production of this virus. The presence of these agents in samples may therefore give rise to false-positive results in the RCR assay. However, a limited study at Q-One Biotech has failed to induce increased reverse transcriptase expression in *Mus dunni* cells following IDU treatment. The occurrence of such false positives may cause costly and time-consuming delay to clinical trials. An additional disadvantage with the use of *Mus dunni* cells is that they cannot be infected by some non-murine retroviruses, such as GaLV or FeLV (Q-One Biotech, unpublished data), on which some retrovirus packaging lines are based. There is a requirement for infectivity or marker rescue assays to be developed which detect retroviruses of different tropism and which are not prone to false-positive results. Marker rescue assays could also be employed as confirmatory assays to follow

co-cultivation of samples with MLV-permissive cell lines that resulted in suspected RCR infection.

5.3 Production of retroviral vector

The ideal retroviral vector should exhibit the following characteristics; a high titre to enhance transduction efficiency and reduce production costs, tissue-specific delivery of the therapeutic gene, optimal and sustained expression of the transgene (which can be regulated by physiological signals), and insertion into a specific site of the genome. Thus it is important to optimise methods for retroviral vector production and transduction. Cell banks used to produce retrovirus vector must be qualified, and the vector itself must be characterised and shown to be free of contaminating agents and substances. Evaluation of the vector includes constructing growth curves using appropriate cell culture medium and performing optimisation studies for production. Each producer cell line should be individually evaluated to assess optimal cell culture conditions and maximise and maintain production levels. Also, the growth and production characteristics of the producer cell line should be investigated by a variety of scale-up methods, such as roller bottles, microcarriers, and fermenters. In the case of the model vector used in these studies, the cell line was adherent, therefore small scale studies using tissue culture flasks, roller bottles and Fibra-cel disks were performed to optimise vector production and to set the parameters for scale-up. The parameters identified in the evaluation are used to scale-up production using cost-effective and appropriate technology.

In this study, initial titres resulting from the model producer cell line PALidSN were determined by NIH- 3T3 titration to be in the region of 10^5cfu/ml for both tissue culture flasks and roller bottle systems. PALidSN cells produced retroviral vector in the roller bottle culture system at an average of $4.7x10^5$cfu/ml (Table 3.10, roller bottle 1, mean viral vector titre). Typical cell culture harvest titres previously reported for producer cells constructed using the PA317 packaging cell line were reported to be in the region of 10^5-10^6 cfu/ml, but titres of up to $2x10^7$ cfu/ml have been achieved when cell culture

was scaled up using the Cellcube system (Kotani *et al.*, 1994). The feasibility of scaling-up culture of PALidSN cells in Fibra-Cel® disks in a Techne spinner flask resulted in a viral vector titre of $1x10^5$cfu/ml in a sample harvested after 120 hours cell culture. The large volume of circulating medium owing to the disks not being tightly packed may explain the large number of cells in suspension (Table 3.12). Although there were some problems with non-adherence of PALidSN cells to the disks, cell growth improved following feeding of the cells with fresh media. Different systems used to increase production have both advantages and disadvantages, and vary in their potential for manufacturing scale-up. Culture in roller bottles is straightforward, but relatively labour intensive in comparison to some recently manufactured systems. One of these is the microcarrier system (Pharmacia Cytodex 3™) which consists of a surface layer of denatured collagen, covalently bound to a matrix of cross-linked dextran spheres, 18.5g of which is equivalent to 100 roller bottles (surface area 4600cm^2/g). Another is the New Brunswick Celligen™ fermenter, which may provide an improved vector yield via controlled cell growth. The New Brunswick Celligen™ system is composed of fibrous polyester disks, of which 70g are the equivalent to 100 roller bottles. Both this and the New Brunswick Celligen™ system are disposable and use a smaller amount of medium more efficiently than roller bottle culture. These systems also allow culture conditions to be easily altered, and carry a lower risk of potential contamination than roller bottles. The Celligen™ system also allows easy monitoring of media circulation, including media input as and when required.

An improved production strategy for retroviral vector supernatants was recently described which is based on gene transfer potential rather than vector titre (Forestell *et al.*, 1995). A packed-bed bioreactor operated in perfusion mode was developed, which is easily scaled-up and produces large volumes of supernatant with high transduction efficiency. The supply of medium by continuous perfusion limits the time that virions are exposed to inactivating temperatures, and ensures a constant supply of nutrients, as well as continually removing inhibitors. This system could be applied to all

bioreactors that support high cell densities and can be operated in a perfusion mode.

Adaptation of cell growth to suspension culture is a goal of large-scale processes for viral vector production. For some cell lines, such as 293 cells, which are normally adherent, simply adapting the cells to serum-free media causes them to become lightly adherent or non-adherent. Suspension culture is more easily scaleable and adaptable to downstream processing, and also allows for higher density cell growth.

5.4 Use of serum and trypsin in culture media

Raw materials used in *in vitro* cell culture include components such as cell culture media, including synthetically manufactured supplements, such as amino acids, and materials of animal origin, such as bovine serum. In general, eukaryotic cells require the addition of serum to cell culture medium in order to grow *in vitro*. Serum is a complex mixture of a large number of constituents such as essential growth factors and nutrients. The use of serum in cell culture media poses a number of problems, including the addition of proteins to the culture medium, variability in the composition and quality of different batches, and risk of contamination with adventitious agents from the source animal (McLean *et al.*, 1997). Another raw material of concern in culturing anchorage-dependent cells such as the PALidSN cell line is trypsin, which is used to dissociate the cells. Trypsin is generally of porcine origin, in which the most common extraneous agent of concern is porcine parvovirus (PPV). Regulatory authorities such as the FDA and CPMP have ruled that sera must be sourced from selected countries such as Australia, New Zealand and the United States, where bovine spongiform encephalopathy (BSE) is not known to exist. A certificate of origin must be supplied, as well as information on the health status and age of the donors, and details on the manufacturing process. Internal quality control (QC) testing includes cell growth performance tests, testing for absence of bovine viruses, BSE, bacteria, fungus, mycoplasma and endotoxin contamination. The main viruses of concern in bovine

material are the pestivirus bovine viral diarrhoea virus (BVDV) and bovine polyomavirus (BpyV), which are widespread contaminants in bovine serum. Requirements for production include slaughterhouse licenses, adequate training of personnel, controlled transport of blood to the processing plant and extraction of serum using aseptic techniques, as well as sterile filtration and filling, and provision of full documentation.

In the studies presented in this report, the retroviral vector producer line, PALidSN, was initially examined for the effect of varying culture media serum and glucose content on cell growth and viral vector titre. Of the four media formulations examined (DMEM containing 4500mg/L or 1000mg/L glucose supplemented with either FBS or NBS), optimal cell yield coupled with lowest doubling time was best achieved by culturing PALidSN cells in high glucose DMEM supplemented with 10% FBS (doubling time during exponential growth of 13.99 hours and a highest cell yield of 2.64×10^5 viable cells). Correlation between the the results of the enzyme and infectivity assays was observed, in that the highest value of IDUA enzyme activity per milligram of protein (14,940 U/mg) and highest viral vector titre measured by NIH-3T3 assay ($7X10^4$ cfu/ml) were obtained from supernatant harvested from cells after 96 hours culture in high glucose DMEM containing 10% FBS (Figure 3.1). Results of a study comparing viral titres resulting from PALidSN cells cultured in tissue culture flasks in media containing either 5% or 10% serum content indicated that the effect of reducing serum concentration on viral vector titre was minimal (Table 3.5). The possibility of further reducing, or eliminating, serum during culture of PALidSN cells was indicated when reduction of serum concentration to 1% FBS in roller bottles (section 3.4.3) also produced a comparable viral titre ($2x10^6$ cfu/ml), although an extended culture period was required to reach this titre (12 days). This extended culture period may be explained by the lower availability of nutrients at the reduced serum concentration.

In studies reported here in which PALidSN cells were adapted to culture in serum-free media, cells adapted well to Ultraculture serum-free media, but not to CHO-S-SFMII,

QBSF51 or QBSF56 media. This suggests that a specific formulation is required for particular producer cell lines, indicating that evaluation studies for adapting cells to various media formulations would be necessary for individual producer cell lines. When the PALidSN cell line was cultured using Ultraculture serum-free media, the cells required daily feeding with fresh media in order to ensure a viral vector titre of at least $1x10^4$cfu/ml at an early stage of culture (Table 3.9). Leaving the culture for four days (Flask 2) resulted in a poor viral vector titre and deterioration of the cells. The culture did not recover until four days of daily feeding had occurred (Days 7 to 10) and the highest titre obtained was $7x10^4$cfu/ml. Flask 3 did not recover from the first seven days without feeding and viral vector titres were poor. Therefore, cells cultured in serum-free media appear to require a longer culture period, and more regular media replacement than cells cultured in serum-containing media. When the results of this study are compared to viral production in a serum-based system, there is on average a reduction in titre from $4.7x10^5$cfu/ml (Table 3.10, roller bottle 1) to $1.1x10^5$cfu/ml (Table 3.9, roller bottle 1) in the serum-free system. Although the titre is slightly lower in the serum-free system, the absence of serum facilitates the downsteam processing, and the material produced also has the potential to undergo concentration techniques. The feasibility of serum-free cell culture indicated in this study suggests that a completely serum-free production process is possible. However, it should be noted that some cell lines are more easily adapted to culture in serum-free media (SFM) than others. In addition, media termed SFM may contain serum fractions, and are therefore at risk of contamination by the same agents as in whole serum (Hodgson, 1995).

5.5 Production methods to increase viral titre

A limitation in using retroviral vectors is the relatively low titres of virus which have been obtained from existing packaging lines (10^6-10^7 cfu/ml) (Crystal, 1995). A complete discussion of vector design to increase titre is outwith the scope of this document. However, complementary packaging cells and vector constructs have been developed

which are reported to be of higher titre and safer than previously described producer cell lines (Morgenstern *et al.* 1990). Variations in the methods used in production of retroviral vectors have been employed in attempts to increase viral titre. Production at reduced temperature has been reported to improve vector titre (Kotani *et al.*, 1994). However, in the limited study on production temperature for the model vector PALidSN in this report, harvesting supernatant from cells incubated at for 3 days at 32°C rather than 37°C did not result in an increase in viral titre. This is in contrast with the results of Kotani *et al.* (1994), who reported a 5- to 15-fold increase in vector produced at 32°C in comparison to 37°C, and Olsen and Sechelski (1995) who reported an 8-fold increase in vector production at 32°C in comparison to 37°C. In their study, Kotani *et al.* (1994) reported that viral production rate was increased, and survival improved at 32°C. This was observed to be the case in this study, where viral inactivation was observed to be greater in supernatants incubated at 37°C rather than 32°C (Figure 4.1). However, cultivation of producer cells at 32°C rather than 37°C is more likely to lower the rate of viral thermal inactivation rather than increase virus production (Forestell *et al.*, 1995). Growth at 32°C results in retroviral vector supernatants with higher transduction efficiency by reducing the ratio of inactivated to transducing virus particles (Forestell *et al.*, 1995). Different clones of retroviral vector vary widely in the vector titre produced from them. Forestell *et al.* (1995) suggested that production at 32°C should be combined with harvesting supernatants at short time-intervals. They suggested that retroviral vector supernatants reach their maximum transduction efficiency between 3 to 5 hours following medium exchange. This contradicts the recommendations of Kotani *et al.* (1994), which are that supernatant should be harvested at 48-hour intervals. Forestell *et al.* (1995) also suggest that shorter incubation times would limit the accumulation of inhibitors of producer cell metabolism or non-viral factors that may reduce transduction efficiency.

Other potential methods that attempt to increase viral vector titre utilise agents that are known to stimulate virus production, such as sodium butrate (NaB) (Olsen and Sechelski, 1995) and TPA (Harada et al., 1986). In this study, a three-day induction period of

PALidSN cell cultures with either NaB or TPA did not have an effect on viral titre (Table 3.6). NaB was reported to increase production of a retroviral vector from between 20-fold to greater than 1000-fold, depending on the producer clone, resulting in vector titres of approximately 10^7 infectious units/ml (Olsen and Secheleski, 1995). Therefore, different producer lines would require assessment for susceptibility to viral inducing agents. NaB exerts its effect on virus production by affecting viral gene expression, although its inhibitory effects on the cell cycle may also be important in some cases (Shadan *at al.*, 1994). The increase observed by Olsen and Secheleski (1995) was partially accounted for by an increase in steady-state levels of full-length vector RNA within the producer cells. With some of the producer cell lines, combining NaB treatment with lowering the temperature of the virus harvest to 32°C resulted in an apparent synergistic increase in virus production. The effect of NaB treatment, however, is not consistent. When the producer cell line contained a different vector construct, NaB treatment resulted in only 2-fold to 10-fold increase in viral titre. In the limited experiment on PALlidSN producer cells reported in this study, no increase in viral titre was observed with NaB treatment. NaB treatment has been shown to induce production of HIV (Antoni *at al.*, 1994), but not Friend leukaemia virus (Zajac-Kaye *at al.*, 1986). Therefore the effects of NaB on gene expression may be virus specific or prompter/enhancer and cell-type dependent. The treatment may be useful to increase vector production from selected stable producer cell lines, but it will be necessary to validate the safety of producer cells and retroviral vector supernatants prepared in the presence of NaB.

The use of bioreactors to propagate retroviral producer lines has also been shown to increase viral production up to five-fold (Kotani *et al.*, 1994). Culture in bioreactors could be combined with other methods reported to increase viral titre such as incubation with deoxynucleotides to stimulate *de novo* reverse transcription (Zhang *et al.*,1995) or the addition of dexamethasone or NaB to stimulate viral production from retrovirus packaging cell lines (Pages *et al.*,1995). However, different producer lines would require individual assessment for susceptibility to viral inducing agents.

5.6 Concentration of retroviral vector

The problem of low titre virus may be partially addressed by retrovirus concentration methods (Paul *et al.*, 1993; Kotani *et al.*, 1994). It has been suggested that retroviral particles may be limited in their ability to be concentrated by ultracentrifugation owing to the possibility of damage to the envelope protein (Burns *et al.*, 1993). One approach to overcome this has been the use of pseudotyped retroviruses containing vesicular stomatis virus (VSV) G protein, but the toxicity of this protein may inhibit sustained virus production (Burns *et al.*, 1993). Bowles *et al.* (1996) reported the recovery of infectious amphotropic virus at approximately 95% following low speed centrifugation, whereas, after ultracentrifugation, only 50% of the virus was recovered. Virus particles which were initially concentrated by low speed centrifugation (6,000 x g for 16 hours at 4°C) were further purified by sucrose gradient centrifugation. The combination of low-speed sedimentation and sucrose banding steps resulted in titres up to 500-fold higher than in the original starting supernatant. Increasing viral titres from 10^6-1-2x10^7 cfu/ml was reported to result in a proportional increase in viral transduction activity *in vivo*, but a decrease in transduction efficiency was reported in virus particles concentrated beyond 10^7 ffu/ml. Jacomino *et al.* (1997) postulated that in both their study and that of Bowles *et al.* (1996), the decreased potency of highly concentrated retrovirus particles may have been caused by concentration of toxic materials during centrifugation. Le Doux *et al.* (1996) identified proteoglycans secreted by packaging cell lines as one of the interfering substances present in retroviral stocks. It is possible that these proteoglycans may have been concentrated by being bound to retroviral particles during the purification procedure. A producer cell line based on recombinant amphotropic retroviral vector was recently reported to produce a viral titre of up to 2x10^6 cfu/ml without inclusion of a concentration step (Kitten *et al.*, 1997). The producer cell line was constructed with particular attention to ensuring efficient *in vivo* gene transfer to the liver. No decrease

in transduction efficiency was observed with higher titre vectors in the study by Kitten *et al.* (1997), which is probably explained by the absence of a concentration step.

Another physical method used to increase viral titre examined in this study was ultrafiltration. Retroviral vector supernatant harvested from PALidSN cell cultures was successfully concentrated by filtration in two separate studies using SARTOCON MICRO 100KD filters (Table 3.13 and 3.14). In one study, the volume of supernatant was concentrated 15-fold, resulting in a 10-fold increase in viral vector titre (from $2x10^5$ c.f.u/ml to $2x10^6$ cfu/ml). This represents a level of 67% recovery of infective viral particles. In the other study, the volume of supernatant was concentrated 4.3-fold, resulting in a 5-fold increase in viral vector titre (from $6x10^5$ c.f.u/ml to $3x10^6$ cfu/ml). Therefore, no loss of vector activity was determined by NIH/3T3 assay following concentration by ultrafiltration in the second study. In both studies, virus was not detected in the filtrate that was drawn from the system. The samples were pre-filtered prior to titration, and the titration assay performed in the presence of antibiotics. Concentration of MLV by up to 24-fold was also demonstrated by Kotani *et al.* (1994) using 300kD membrane filtration, with over 90% recovery of infective viral particles. These results are encouraging for concentration of retroviral vectors by ultrafiltration, but individual producer cell lines would require stability testing to ensure that infectious viral particles can be recovered. However, as with concentration by centrifugation, a recent study reported that increasing end-point titre by ultrafiltration of amphotropic retroviral vector supernatants does not improve, and can even reduce, transduction efficiency (Forestell *et al.*, 1995). In this study, dilution of concentrated supernatant restored the level of transduction efficiency, suggesting that an inhibiting agent had been co-concentrated with the virus particles. This may have been caused by the presence of non-transducing virus particles causing a decrease in transduction efficiency without affecting the virus titre. As the study by Forestell *et al.* (1995) reported that lowered transduction efficiency is only observed in supernatants from amphotropic producer or packaging cell lines, the inhibition may be specific to the viral envelope protein.

5.7 Transduction of retroviral vector

As observed above, increased virus concentration does not always lead to increased transduction rate. Rather, there is an optimal titre, after which increased concentration does not lead to increased transduction rates (Chuck and Palsson, 1996). The number of cells that are transduced, however, is dependent on viral titre and polybrene levels. Recently reported data suggests that 10^6 c.f.u. recombinant retrovirus is the finite amount which can be safely and efficaciously administered through portal vein infusion in the rat (Bowles *et al.*, 1996).

Retrovirus mediated gene transfer is normally performed using static transduction methods, where retroviral supernatant is inoculated onto a bed of target cells. However, this system is limited by Brownian motion of the retrovirus, decay of the retrovirus, and absorption of retrovirus by the target cells. By relying on Brownian motion to deliver the retrovirus, the rate of virus delivery is largely determined by the proximity of the virus to the target cell, and the half-life of the virus. The limitations of Brownian motion can be overcome by directing the motion of the virus to the target cells using flow-through transduction methods (Chuck *et al.*, 1996). This method is achieved using a fluid flow of retroviral supernatant passing through a porous membrane that supports the target cells. This results in an increase in the number of successful gene transfer events which is much less sensitive to virus concentration than the static transduction method. Reproducibly high numbers of transduced cells can be obtained with a wide range of virus titres. Additional advantages in using flow-through transduction methods are that a smaller volume of viral supernatant is required for gene transfer and that the method can be carried out without the use of polybrene. (Chuck and Palsson, 1996).

5.8 Limitation of selectable markers

Selectable markers permit positive selection in that the selecting agent does not kill the cells of interest. One of the most frequently used selectable markers is *neo* (contained in the PALidSN cells in this study), which confers resistance to a group of antibiotics (including G418) which are toxic to cells. Two separate resistance genes found in certain bacterial strains can be functionally transferred into eukaryotic cells to permit growth in media containing G418. An issue of concern in the use of retroviral vectors containing *neo* as a marker gene is the variability of titration on NIH 3T3 cells and a number of factors have to be standardised in order to achieve reproducible results. Firstly, the NIH 3T3 cells must be in exactly the same state of growth before infection with vector, as G418 selection is dependent on cell density and non-resistant cells can survive in otherwise toxic concentrations if they are crowded on a tissue culture dish. It should be ensured that sufficient clones are counted for statistical accuracy and that the G418 antibiotic used to select *neo* resistant cells has been corrected for specific activity. The specific activity of the drug is batch dependent, therefore each lot should be independently titrated on NIH 3T3 cells to determine the minimum cytotoxic concentration for use in the assay. Its effects should be titrated on test cells over the range 200µg-2mg/ml. The problems encountered in the use of *neo* as a marker suggest that other markers may be more appropriate, for example lacZ, for colony counting or luciferase, which would enable automated counting.

Inclusion of a second gene as a dominant selectable marker may have unwanted effects in gene therapy studies. Firstly, the presence of a two promoters in a retrovirus may result in interference between the two transcriptional units, causing 'promoter suppression' (Emerman and Temin, 1984). Secondly, expression of selectable markers *in vivo* may induce host immune responses that could eliminate transgene-expressing cells. Some

retroviral vectors have no selectable marker with the result that titration of the vector is based on a measure of the gene product, for example enzyme activity or protein concentration. An example of this is included in this study where IDUA enzyme activity in PALidSN cells was determined by infection of human fibroblast MPSI cells, and enzyme activity in cell extracts measured by fluorimetry. Enzyme activity measured per milligram of protein was comparable with viral vector titres measured by NIH-3T3 titration (section 3.3.1). In such cases, measurement of titre by enzyme activity results in the characterisation of virus-producing clones being more laborious. A simple and reliable assay has recently been developed to identify clones producing recombinant retrovirus that lack dominant selectable markers (Onodera *et al.*, 1997). This is based on a validated two-day RNA dot-blot procedure, the titres obtained by which were demonstrated to correlate with titres obtained by conventional G418-resistant cfu/ml, and by Southern analysis of HeLa cells transduced with supernatant from each clone. In contrast with many conventional assay systems, the RNA dot-blot method allows comparison of titres from different packaging cell lines, therefore is useful as a method for directly comparing titres obtained from different producer clones.

5.9 Optimisation of stability of retroviral vectors

Extension of the shelf-life of pharmaceutical products, or storage at ambient temperatures would greatly benefit the pharmaceutical industry. Thus, it is important to optimise the stability of retrovirus vectors in order to maximise their clinical application. The European Agency for the Evaluation of Medicinal products (EMEA) recently implemeted ICH document Q5C, which provides notes for guidance on stability testing of biological products. Stability during the intended storage period must be confirmed; biological activity must be maintained and degradation avoided. Thus a minimum of six months stability study data should be provided for biological products to obtain market authorisation. As inactive virus particles can reduce transduction efficiency of retrovial vectors, storage conditions should be optimised to reduce the accumulation of these

competitor particles in retroviral vector supernatants (Forestell *et al.*, 1995). Forestell *et al* (1995) reported that supernatants incubated at 0°C for 4 days lost 50% of their gene transfer potential although the titre determined by infectivity remained stable. It was therefore recommended that retroviral vector supernatants should be rapidly frozen and stored at –80°C shortly after harvesting.

In this study, the stability of retroviral vector from PALid SN cells and wild-type A-MLV in culture supernatants was determined by measuring titre following short or long term storage at different temperatures. Retroviral vectors have been reported to be more stable at 32°C than at 37°C and this was found to be the case with PALidSN cells where viral titre decreased more rapidly in supernatants incubated at 32°C rather than at 37°C (section 5.5). The half-life of retroviral vectors has been reported to be 3.5 to 6.5 hours at 37°C, dependent on the vector construct (Paul *et al.*, 1993). The half-life of the amphotropic vector from the packaging cell line ψCRIP was measured to be between 5-8 hours at 37°C, while the half-life of another amphotropic retroviral vector at 37°C was recently determined to be 3.5 hours (Jacomino *et al.*, 1997). In another study a reduction of 92% in vector titre after 24 hours at 37°C was reported, whereas no significant reduction was observed after 48 hours at 32°C (Kotani *et al.*, 1994). In this study, vector titre was observed to decrease by 98% after 24 hours at 37°C, and by 70% after 24 hours at 32°C. After 3 days a low titre was detected in vector at 32°C whereas vector at 37°C was completely inactivated. In the present study the stability of the vector at 4°C was found to indicate that loss in viral titre over 3 days storage at 4°C is insignificant. These results are comparable to a report by Bowles *et al.* (1996), where the loss of viral titre by overnight storage at 4°C was found to be consistently less than 10%. The stability of the viral titre of virus and vector supernatants at 4°C or 32°C remained constant for at least the first eight hours of incubation, which is also similar to previously reported data (Forestell *et al.* 1995).

The mechanism by which MLV-derived vectors decay is not clear, although inactivation of

HIV has been proposed to occur by shedding of glycoproteins from the lipid envelope of virus particles (Layne *at al.*, 1992). Loss of infectivity would directly result from loss of ability to bind to cellular receptors. The short half-life of MLV-derived retroviral vectors significantly affects their ability to transduce genes successfully by limiting the distance that they travel before decaying (Chuck and Palsson, 1996). Therefore, a better understanding of the mechanisms that determine retroviral stabillity will be useful in increasing the efficiency of gene transfer. The presence of polybrene has been reported to significantly affect the kinetics of retroviral decay, owing to differences observed in the pattern of loss of retroviral activity in the presence and absence of polybrene (Andreadis and Palsson, 1997). In the presence of a high concentration of polybrene (13.2μg/ml), the kinetics of decay were exponential from the beginning of the decay period, whereas in the absence of polybrene, loss of retroviral activity did not follow simple exponential decay. The dynamics of viral inactivation showed a sigmoidal decrease, with an initial phase during which the transduction efficiency remained constant prior to exponential decay. The sigmoidal pattern of retroviral decay is in agreement with the pattern of decay reported for HIV-1 (Layne *et al.*, 1992). Two alternative mechanisms were proposed to address the data suggesting that the presence of polybrene affects the kinetics of retroviral decay. Polybrene may induce virus aggregation, which reduces the effective concentration of retroviral particles, or, neutralisation of polybrene (positively charged) by FBS components (negatively charged) could decrease the effective concentration of polybrene. These results outline the importance of considering the processes that may occur in solution during the steps of viral binding and entry into the target cell, and their effect on the stability of retroviral particles.

Freeze drying, or dehydration, is a process where solvent is physically removed from a sample such that the solids are maintained in an essentially dry (eg. water-free) condition. One of the benefits of freeze drying is to aid the long-term storage of pharmaceuticals, while retaining their biological activity. There are three steps involved in freeze drying a product. Firstly, the product is frozen solid, thus the water present in the material is

converted to ice. Next, the ice formed in freezing is removed by conversion from solid to vapour (sublimation). Thirdly, water which is strongly bound to the solid and therefore not frozen, is converted to vapour and removed from the product (desorption). The presence of sugars may aid freeze drying by protecting cell membranes and proteins during the desiccation process. Trehalose, a colourless and odour-free disaccharide constisting of two glucose molecules, is currently being applied to the improvement of stabilised vaccines, hormones, and blood components.

In this study the ability of trehalose, along with glucose, mannose and sorbitol to stabilise retroviral vector supernatant stored at -80C and 4C was examined. Viral vector titres obtained were found to be similar in the presence and absence of sugars. However, the study was not continued over a long enough period of time to draw any conclusions from the effect of the presence of sugar stabilisers.

5.10 Overview and future directions for retroviral gene transfer

As stated in a recent review, appropriate vector design and mode of delivery remain the main obstacles to successful human gene transfer (Crystal, 1995). The ultimate goal of gene therapy using retroviral vectors is to obtain tissue and cell specific targeting and regulated expression of the appropriate gene that is both safe and regulated. The first step in attempting to target retroviral vector gene delivery is in pseudotyping the packaging cell lines. The envelope of A-MLV can be used to deliver genes to human haemopoetic stem cells, however, not all cell types can be infected by A-MLV. The tropism of vectors can be increased by the use of other retroviral envelope genes or the glycoproteins of other viruses capable of forming pseudotypes.

In the present study extensive experiments were performed to validate co-cultivation and infectivity assays for RCR detection using *Mus dunni* cells and the PALIDSN retroviral vector-producing cell line. Further limited studies were performed on the

PALIDSN cell line that elucidated the characteristics of the cell line with respect to optimal conditions for vector production and on the vector with respect to comparative stability to temperature and concentration. The limited nature of the observations made here preclude general conclusions regarding optimal conditions for production and storage of retroviral vectors. It is probable that each producer cell-vector combination should be individually evaluated with respect to each of the parameters discussed above.

Further studies should be undertaken to validate the *Mus dunni* or a suitable marker rescue assay for RCR detection using the reference RCR producing cell line deposited by CBER with the ATCC.

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