Cationic liposome-mediated transfection of mammary epithelial cells with AAV-based plasmid DNA

by

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Abstract

Cationic liposome-mediated delivery of adeno-associated virus (AAV) based plasmid DNA has potential for efficient and safe delivery of DNA into a number of different cell types. The objective of this thesis was to develop techniques for transfecting adult somatic mammary epithelial cells with the aim of producing non-germline transgenic animals. Initially, chemical-mediated techniques were used to deliver plasmid DNA to mammary epithelial cells *in vitro*: this work was subsequently extended to *in vivo* studies. The research then focussed on cationic liposome-mediated delivery of AAV-based plasmid DNA to enhance transfection efficiency, and the molecular characteristics of AAV-based plasmid DNA as a vector for transfection and integration of DNA into the genome.

Chemical-mediated procedures were used to deliver plasmid DNA to the mouse mammary epithelial cell line, HC11, and to primary bovine mammary cells *in vitro*. Four delivery vehicles were used; calcium phosphate co-precipitation, DOTAP, DMRIE-C and LipofectAMINE. The highest transfection efficiency resulted from using LipofectAMINE, although this efficiency was relatively low, with only 2.1% of cells found to express β-galactosidase.

To establish the efficacy of chemical-mediated delivery of plasmid DNA *in vivo* and to investigate an intramammary route of delivery, lactating rats were transfected *in vivo* with plasmid DNA complexed with LipofectAMINE. These complexes were delivered by infusion through the mammary teat canal or by direct injection into the gland. The ensuing level of transfection was extremely low, with only a very small number of rat mammary epithelial cells found to express β-galactosidase. These results indicated that alternative transfection procedures were required.

Inverted terminal repeats (ITRs) of AAV have previously been shown to enhance transfection efficiency, and these were included in a plasmid construct flanking a β-galactosidase reporter gene. LipofectAMINE was used to deliver this DNA to

HC11 cells, however, the efficiency of transient transfection was only increased by a modest 1.4-fold.

To evaluate the effect of AAV ITRs on plasmid DNA integration, a DNA construct containing a neomycin resistance gene flanked by ITRs was transfected into HC11 cells. Additionally, this construct was co-transfected with an AAV Rep protein expression construct, which is required for site-specific integration in human cells. Neither the inclusion of ITRs, or expression of the *rep* gene affected the frequency of integration of the neomycin gene.

In humans a specific advantage of using AAV is site-specific integration of the virus into a locus on human chromosome 19, known as AAVS1. Southern blot analyses were used to ascertain whether a similar site is present in mouse and other species. No clear cut evidence was found for such a site.

Further Southern blot analysis of clonal lines derived from mouse HC11 cells that were co-transfected with a neomycin resistance gene, and an AAV *rep* gene construct, suggested that the site of integration was random. A novel PCR technique was utilised to amplify the site of integration. This enabled sequence data to be obtained for two HC11 clones. The site of integration for one of the clones was identified within exon 16 of *Mus musculus* DNA coding for the DNA dependent protein kinase catalytic subunit. The site of integration for the second clone was an unknown region of the mouse genome. An interesting characteristic of both clones was the presence of mouse B1 repeat sequences in close proximity to the sites of integration.

These studies demonstrate that AAV-based plasmid DNA can enhance the transfection efficiency in mouse mammary epithelial cells, and they contribute toward the overall knowledge of AAV-based plasmid DNA integration into the mouse genome.

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List of Abbreviations

°C	degrees Celsius
µg	micrograms
µl	microlitres
µM	micromolar
AAV	adeno-associated virus
ASPA	aspartoacylase
BLAST	basic local alignment search tool
bp	base pairs
cDNA	complementary DNA
cm	centimeters
CMV	cytomegalovirus
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEAE	diethylaminoethyl
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
dNTP	an equimolar mix of dATP, dCTP, dGTP and dTTP
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
FISH	fluorescent in situ hybridisation
FBS	foetal bovine serum
g	centrifugal force
hGH	human growth hormone
ITR	inverted terminal repeat
kg	kilograms
kb	kilobases of DNA
kD	kilodaltons
µm	micrometer
µM	micromolar
M	molar
mg	milligrams
ml	milliliters
mM	millimoles
MMLV	Moloney murine leukaemia virus
MMTV	mouse mammary tumour virus
MQ	milli-Q (low conductivity) deionised water
mRNA	messenger RNA
ng	nanograms
nm	nanometers
ONPG	*o*-nitrophenyl-β-D-galactopyranoside
P	p-value
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PEI	polyethylenimine
PolyA	polyadenylation
rAAV	recombinant adeno-associated virus
RIA	radioimmunoassay
RBE	Rep Binding Element
RNA	ribonucleic acid
rpm	revolutions per minute
sem	standard error of the mean
TAE	Tris-acetate-EDTA buffer
trs	terminal resolution site
U	units (of enzymatic activity)
X-gal	5-bromo-4-chloro-3-indoyl-B-D-galactoside

Chapter One

Introduction

1.1 General introduction

Traditionally, transgenic farm animals, such as sheep and cattle, have been produced by germ line gene transfer. The goal of genetic modifications of this kind is normally to alter a specific physiological process, which may in turn lead to enhancement of animal productive efficiency, to the generation of a new value added product, or they may be used for the evaluation of gene promoters. The mammary gland is a particularly attractive target tissue for this type of gene transfer. The introduction and expression of foreign genes in the mammary gland of large mammals such as cows can be used to modify milk composition, or to produce novel proteins in milk. Unfortunately, progress in the production of germ line transgenic farm animals has been hindered by high costs, mainly due to the associated long time frames required for gestation and maturation through puberty. An alternative approach to alleviate these inherent problems is to produce transgenic tissues by gene transfer to somatic cells.

Gene transfer to somatic cells is a technology that holds many promises for gene therapy and considerable emphasis has been placed on developing efficient techniques for the *in vivo* treatment of human genetic disorders. To date the majority of gene transfer technologies tested in phase one human gene therapy clinical trials have involved viral vectors. However, a multitude of techniques have been explored, all with the common goal of designing the ultimate vector for delivery of foreign DNA to somatic cells of the particular target tissue. These technologies can be directly transferred to the agricultural industry for the production of somatic transgenic farm animals.

This chapter discusses current methodologies used for gene transfer to somatic cells. In particular, focus has been placed on delivery of foreign DNA to mammary epithelial cells, as it is the objective of this thesis. Also, liposome-mediated delivery of adeno-associated virus based plasmid DNA is featured extensively, as this was the method that formed the basis of work in this thesis.

1.2 Gene transfer to somatic cells

Gene transfer can be defined as the transfer, and subsequent expression of foreign genes, into cells. Vectors are the vehicles used to carry this genetic material into the target cells. A challenge exists to develop the perfect vector for gene transfer. Several features are considered essential for such a vector, depending on the application for which they are designed. In general the following attributes are considered desirable: safety, efficiency, minimal toxicity, a high level of controlled expression, target specificity, and minimal immunogenicity. Other features considered include; cost and efficiency of production, environmental and ethical factors, and potential limitations on the amount of DNA that can be inserted into the vector.

Gene transfer vectors can be broadly categorised on the basis of the mode of DNA delivery, as either viral or non-viral in nature. The most extensively studied viral gene transfer vectors are those based on retrovirus, adenovirus, or adeno-associated virus. The non-viral vectors are classified according to the method by which they deliver DNA to the target cell. This is either by a physical or a chemical procedure.

1.2.1 Retroviral vectors

Retroviruses are single stranded RNA viruses. They have genomes of between 7 and 10 kilobases (kb) which contain three conserved structural genes (*gag*, *pol* and *env*) and are flanked by two direct repeats, the long terminal repeats (LTRs). All of these components are essential for the normal retroviral life cycle (Watson *et al.*, 1987). The retroviral life cycle can be summarised in five stages. Firstly, the viral particle attaches to the host cell surface via specific binding of the viral envelope surface protein to cell surface receptors. Following attachment, the virus enters the cell by receptor-mediated endocytosis. Once within the cell cytoplasm the virus sheds its outer envelope coat and the viral reverse transcriptase directs synthesis of a double stranded DNA from the single stranded RNA genomic template. This DNA intermediate is then transported to the nucleus, in a process

that requires cell division, and integrates into a random site in transcriptionally active chromatin domains. Following integration the proviral genome can utilise host cell machinery to express viral proteins which can be used to produce new viral particles (Coffin, 1990).

Retroviruses have been associated with causing oncogenic activity within cells, often eliciting tumorigenic results. This induction of malignancy often results from the random insertion of the wild type viral DNA into the host genome, which may subsequently function as an oncogene or as a promoter to oncogenes. For example, the Moloney murine leukaemia virus (MMLV) can induce leukaemias and lymphomas in mice after a long latent period. Other members of the retrovirus family include Rous sarcoma virus and Human immunodeficiency virus (Coffin, 1990).

A critical factor for insertional mutagenesis to potentially occur, is the presence of large quantities of wild type viral DNA. Therefore, to make retroviruses safer for use as gene transfer vectors, they must be made replication defective. To achieve this, the viral structural genes (*gag*, *pol* and *env*) are removed, consequently creating space for up to approximately 8 kb of exogenous DNA to be inserted into the viral backbone. Recombinant, replication defective viral particles can then be made with the help of packaging cell lines containing the necessary structural proteins required to produce an active virion.

The primary feature of retroviral vectors that make them attractive for gene transfer is their potential to integrate into the genome of target cells and hence to potentially provide stable, long-term expression of the transgene. On the other hand, the fact that retroviruses can only integrate into actively dividing cells represents a disadvantage to their use. Recent development of lentiviral vectors, which are able to transduce post-mitotic cells, has assisted in expanding the potential applications of retroviral vectors. Lentiviruses are a subfamily of retroviruses and include the human immunodeficiency virus. A further drawback, and a potential risk of retroviral vector use, is the possibility of homologous recombination with a replication competent virus *in vivo* to produce a recombinant replication competent virus. Another major disadvantage of retroviral vectors is

the low efficiency of gene transfer *in vivo*. This is due to the low titre achieved in recombinant viral production and also to a lack of target cell specificity (Anderson, 1998). As a consequence, *ex vivo* application of retroviral vectors, where target cells are infected *in vitro* and are then transferred back to the target tissue *in vivo*, is a common method of delivery. This aids in avoiding problems associated with low titre and target specificity, as viral particles are not diluted by non-specific infection, and also allows integration as cells undergo mitosis while *in vitro*. Recently, attempts have been made to improve both the titre and specificity of retroviral vectors, in order to target specific cells. For an excellent summary of these methods, refer to W.F. Anderson's review (1998).

Despite these disadvantages, and the fact that they are difficult and expensive to produce, retroviral vectors are currently used in nearly 40% of all approved clinical human gene therapy trials (www.wiley.co.uk). Retroviral vectors based on the Moloney murine leukaemia virus (MMLV) are currently the vectors being used in several *ex vivo* human clinical gene therapy trials (Orkin and Motulsky, 1995).

Attempts have been made to transduce primary mouse mammary epithelial cells *in vitro*, with a replication defective retroviral vector containing a β-galactosidase reporter gene construct. Up to 30% of cells demonstrated stable β-galactosidase expression (Smith *et al.*, 1991). Also, there have been *in vivo* attempts to infuse retroviral vectors directly to rat mammary epithelial cells via the central mammary duct (Wang *et al.*, 1991), and to goat mammary epithelia via the teat canal (Archer *et al.*, 1994). The earlier experiments utilised a MMLV vector to drive a β-galactosidase reporter gene construct and achieved β-galactosidase expression in 0.3% of cells. In the later study, delivery of a replication defective Gibbon ape leukaemia virus (GaLV) pseudotype retroviral vector expressing human growth hormone (hGH) resulted in secretion of hGH into the milk during lactation. The level of hGH detected in the milk however was low, only in the ng/ml range. It was suggested that the low level of expression was a result of low viral titre, with the number of mammary epithelial cells in the lactating gland exceeding the number of viral particles infused by at least three orders of magnitude.

1.2.2 Adenoviral vectors

Adenoviruses have a double stranded linear DNA genome of 36 kb (Ginsberg, 1984). They are non-enveloped viruses, which are commonly associated with mild human infections such as respiratory tract infections, conjunctivitis and gastroenteritis. The adenoviral life cycle begins with receptor-mediated internalisation, nuclear translocation and episomal maintenance of the viral genome. Host cell machinery is subsequently utilised for viral gene expression which is followed by viral assembly, packaging and lytic release from the cell.

For use as gene transfer vectors the viral E1 replication genes are removed which allows up to approximately 8 kb of foreign DNA to be accommodated within the viral genome, and also renders them replication-defective. Recombinant viral particles are assembled by complementing missing replication functions *in trans* using engineered cell lines (Fallaux *et al.*, 1998).

There are several features of adenoviruses that make them attractive vectors for gene transfer. They can be produced at high titres, they have the ability to infect a wide variety of cell types, including post-mitotic cells, and the viral genome remains episomal which minimises the possibility of insertional mutagenesis (Benihoud *et al.*, 1999). Unfortunately there are also several limitations of adenoviral vectors that restrict their use, including vector induced inflammation, transient expression of transgenes and the development of adenovirus specific neutralising antibodies that hinder repeat administration of the vector. Several attempts have been made to alleviate the cellular and humoral responses to recombinant adenoviral infection. Deletion of additional viral genes such as E2 has assisted in the reduction of the cellular response to infection. Humoral responses can also be decreased by the administration of adenoviral specific antibodies (Chen *et al.*, 2000). Further disadvantages of adenoviral vectors include episomal maintenance of the introduced recombinant genome, which limits the stability of expression. Also, the potential for homologous recombination with wild type virus to produce replication competent adenoviral particles still exists. Deletion of viral genes reduces the chance of the later occurring.

Adenoviral vectors have been widely used for gene transfer *in vivo* with 26% of all current human gene therapy trials using this method of delivery (www.wiley.co.uk).

Replication-defective adenoviral vectors have been used to deliver a β-galactosidase reporter gene to primary human mammary epithelial cells *in vitro* and to mouse mammary epithelial cells *in vitro* and *in vivo* (Yang *et al.*, 1995). A high level of expression was detected in all cases. More recently, Jeng *et al.* (1998) have successfully expressed a β-galactosidase reporter construct in the rat mammary gland using a replication-defective adenoviral vector that was delivered by the cannulation of the main mammary milk ducts. Up to 70% of epithelial cells demonstrated positive staining for β-galactosidase four days after delivery, but β-galactosidase expression dramatically decreased after this time and was not detectable after nine days.

1.2.3 Adeno-associated viral vectors

Adeno-associated virus (AAV) is a member of the *Dependovirus* genus of the *Parvoviridae* family. It has a linear, single stranded DNA genome of 4680 nucleotides (*Figure 1.1*) (Srivastava *et al.*, 1983). At either end are GC rich (over 80%) inverted terminal repeats (ITRs) of 145 nucleotides. The terminal 125 nucleotides of these repeats are palindromic and form a T-shaped secondary structure when folded. This hairpin structure is thought to be critical for viral DNA replication and integration (During, 1997). There are two open reading frames between the ITRs, one encoding *rep* (for replication) genes, and the other the *cap* (for capsid) genes. The DNA molecule possesses three promoters, p5, p19 (for the *rep* genes) and p40 (for the *cap* genes). The transcripts derived from the p5 promoter are Rep78 and Rep68, named from their molecular weight in kiloDaltons. Rep68 is a spliced version of Rep78. The p19 promoter directs expression of Rep52 and Rep 40 (Green *et al.*, 1980). The four Rep proteins are non-structural regulatory proteins.

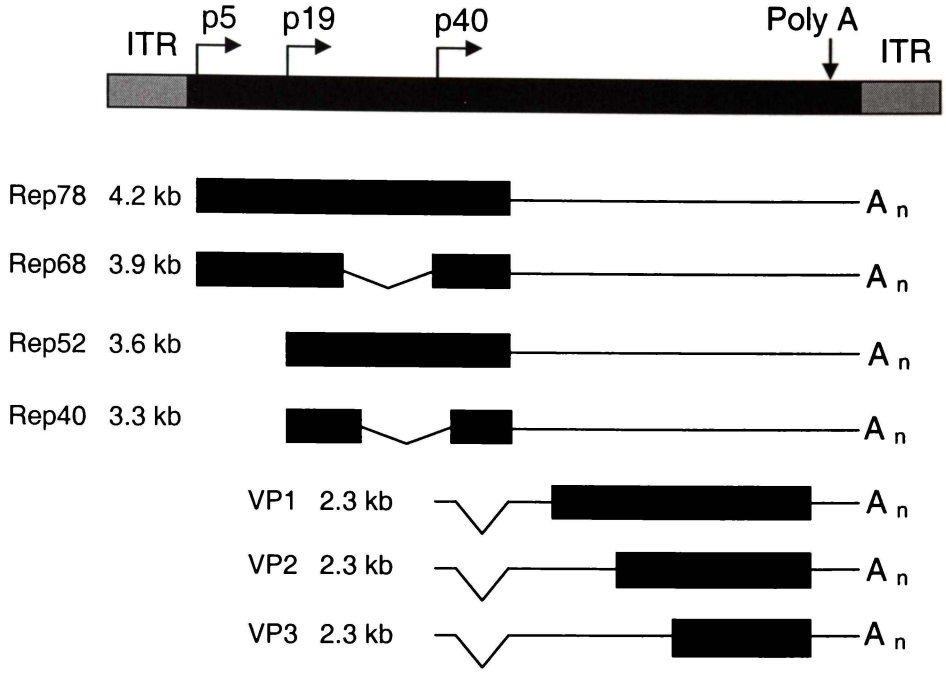


Figure 1.1 *Schematic representation of the AAV genome.* The relative positions of the inverted terminal repeats (ITR), common polyadenylation signal (PolyA) and the three transcription start sites of the viral promoters, p5, p19 and p40 are depicted. Transcripts derived from each of the viral promoters are also shown. Both p5 and p19 direct *rep* gene expression (Rep78, Rep68, Rep52 and Rep40), whereas p40 controls *cap* gene expression (VP1, VP2 and VP3). Coding regions are represented by solid boxes.

The two larger Rep proteins, Rep78 and Rep68 have been shown to be important for DNA replication (Labow and Berns, 1988), targeted integration upon latent infection (Weitzman *et al.*, 1994), and the regulation of AAV gene expression (Beaton *et al.*, 1989). The smaller Rep proteins, Rep52 and Rep40 have been implicated to have a role in facilitating packaging of the viral genome (Chejanovsky and Carter, 1989). The p40 promoter directs expression of the capsid genes, encoding the three structural proteins, VP1, VP2 and VP3. These proteins combine to form a non-enveloped icosahedral virion of 20-30 nm in diameter (Melnick *et al.*, 1965; Tsao *et al.*, 1991).

The name AAV reflects the viruses' dependence on a helper virus to co-infect a cell for lytic infection to proceed. The helper viruses most frequently identified and studied are adenoviruses, although herpesvirus is known to also act in this capacity (Pattison, 1994). The main role of adenovirus is mediated by the protein E1A, which assists in facilitating AAV gene expression (Bueler, 1999). In the absence of a helper virus, AAV will demonstrate the converse state of its biphasic life cycle and undergo latent infection by integrating into the genome of the host cell (*Figure 1.2*). This integrated provirus may be rescued and stimulated to proceed with lytic infection upon infection with a helper virus. Interestingly, external genotoxic stimuli such as UV irradiation, chemical carcinogens or heat shock may also stimulate lytic infection (Yakinoglu *et al.*, 1988).

A distinguishing feature of AAV latent infection in human cells is the site-specific nature of viral DNA integration. The locus into which AAV preferentially integrates, is chromosome 19q13.3qter and is known as AAVS1 (Kotin *et al.*, 1990; Kotin *et al.*, 1991; Samulski *et al.*, 1991; Kotin *et al.*, 1992). AAVS1 contains two DNA sequence features which are also found in the viral ITRs; a Rep Binding Element (RBE), and terminal resolution site (trs) (Linden *et al.*, 1996a). Recent work by Young *et al.* (2000) suggests that not just these two elements, but the entire AAVS1 locus is essential for site-specific integration *in vivo*. The mechanism of integration is not well understood, but it is clear that the larger Rep proteins (Rep78 and/or Rep68) are essential for site-specific integration into AAVS1 (Surosky *et al.*, 1997). In the absence of Rep78 and/or Rep68, DNA flanked by ITRs will still integrate, but at random sites within the

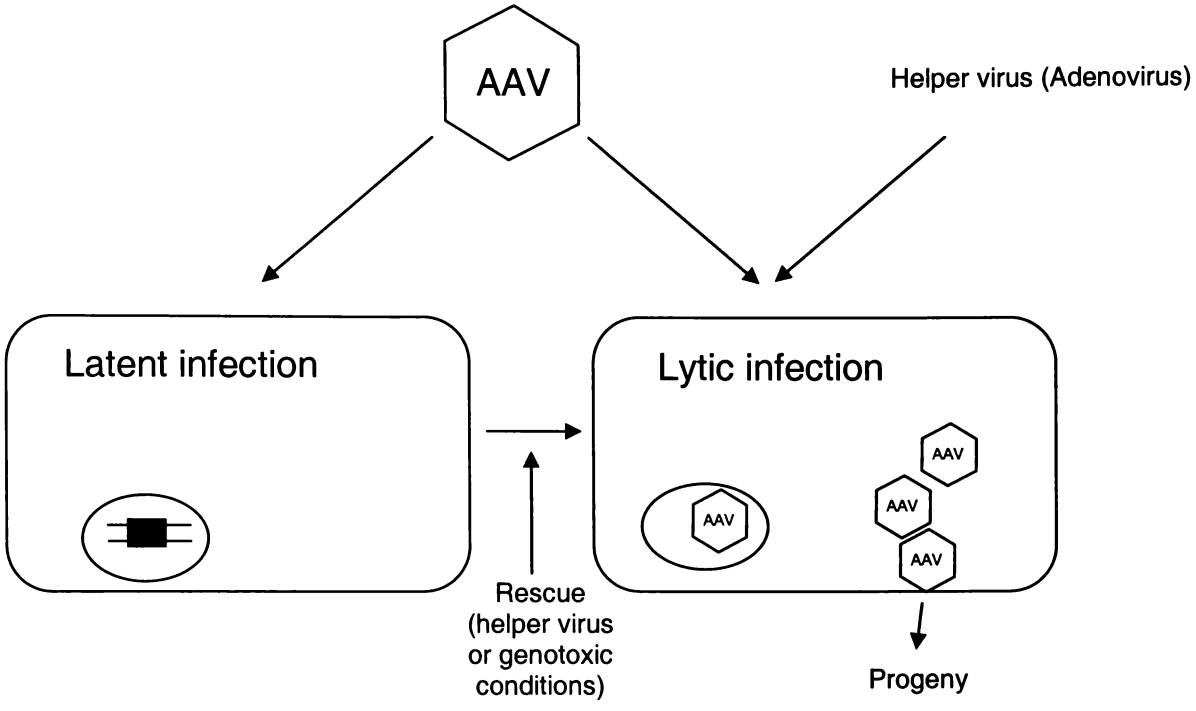


Figure 1.2 *Schematic representation of the AAV life cycle.* In latent infection, AAV integrates into the host cell genome. In lytic infection, which requires the presence of a helper virus or rescue from latent infection, viral replication and subsequent progeny release occurs.

human genome (Xiao *et al.*, 1997). A model for the integration process was proposed by Linden *et al.* (1996a) which involved binding of the Rep78 and/or Rep68 protein to the RBE of AAVS1 and also to the RBE of the viral ITRs, followed by nicking of AAVS1 and/or ITR sequence at the trs. Subsequent integration is likely to occur via a non-homologous recombination process involving replication of the viral DNA.

Production of recombinant AAV (rAAV) virus from packaging cell lines is possible because the ITRs are the only *cis* elements required for viral replication and packaging. Thus, all the viral genes may be deleted from the genome and replaced with a gene of interest. The *rep* and *cap* genes are supplied in *trans* by a helper plasmid (Samulski *et al.*, 1989). Earlier difficulties associated with the production of high titre virus, devoid of helper virus contamination, have recently been overcome with the development of more efficient packaging cell lines (Ferrari *et al.*, 1997; Xiao *et al.*, 1998; Liu *et al.*, 1999b). A potential disadvantage of rAAV vectors is that the size of the insert DNA that can be successfully packaged is limited to less than 5 kb, although this may be overcome by dividing the expression cassette between more than one vector (Nakai *et al.*, 2000).

AAV is considered to be the safest of all the viral vectors. Since no viral genes are present in rAAV, production of active virus would require infection with both wild type AAV as well as a helper virus, and their subsequent recombination. The human specific isolate, AAV-2 has never been associated with disease and is therefore believed to be non-pathogenic. Approximately 85% of the adult USA population are seropositive for this virus (Berns, 1990). To date no adverse symptoms or disease have been reported following rAAV infection. Also, the absence of a cellular immune response following infection suggests that rAAV vectors are not cytotoxic (Chirmule *et al.*, 1999; Hernandez *et al.*, 1999).

A wide variety of tissues in various animal models have been successfully transduced by rAAV *in vivo*. These include mouse skeletal muscle (Xiao *et al.*, 1996), rat and monkey brain (Kaplitt *et al.*, 1994; During *et al.*, 1998), mouse liver (Koeberl *et al.*, 1997; Snyder *et al.*, 1997), rabbit lung (Flotte *et al.*, 1993),

and rat retina (Rolling *et al.*, 1999). Expression from these rAAV vectors *in vivo* was not transient, therefore they are suitable for long term delivery of therapeutic genes. Nakai *et al.* (1999) provided the first direct evidence of integration into a host genome *in vivo* by a rAAV vector. They isolated junctions between vector and genomic DNA from mouse liver cells transduced *in vivo,* confirming the cellular integration of the virus. The site of rAAV integration was random, as expected since these vectors do not express Rep78 and/or Rep68. Also, a locus homologous to human AAVS1 that directs site-specific integration, has not been identified in any other species apart from green monkeys (Samulski *et al.*, 1991). The efficiency of rAAV integration *in vivo* is not clearly defined and varies depending on the cell type transduced (Carter and Samulski, 2000).

1.2.4 Naked DNA and physical methods of gene transfer

Microinjection of DNA directly into the cell nucleus is a very efficient method of gene transfer, with normally close to 100% of all the DNA that is delivered being successfully expressed (Capecchi, 1980). Unfortunately, as only one cell at a time can be injected, this approach is laborious, slow and not practical for *in vivo* gene transfer. Hence its use in animals has been limited to the production of transgenic animals by gene transfer to germ line cells *in vitro*.

Direct injection of naked plasmid DNA into mouse skeletal muscle to express a reporter gene was first demonstrated by Wolff and colleagues (1990). This is the simplest approach to *in vivo* gene transfer, as no other complexes are required, however the efficiency is poor. The uptake of the DNA through the cell membrane is a limiting factor.

Several modifications to the basic technique have been developed to enhance the efficiency of naked DNA delivery. Biolistic particle bombardment delivery of the plasmid DNA with a gene gun has been used. In this method the plasmid DNA is complexed with metal particles such as gold or tungsten and blasted into tissues. Yang *et al.* (1990) achieved transient expression of reporter genes in liver, skin and muscle tissue of rats and mice *in vivo* using particle bombardment. Similar

results were obtained with rat and human mammary explants and primary cell cultures. They found that approximately 3% of mammary tissue stained positive for β-galactosidase expression 48 hours after delivery of a β-galactosidase reporter construct driven by the mouse mammary tumour virus (MMTV) promoter. However, 25% of mammary tissue stained positive following delivery of a different construct, which had the immediate early cytomegalovirus (CMV) promoter driving the β-galactosidase reporter gene. This suggests that the lower percentage may under-represent the transfection efficiency, due to the relative weakness of the MMTV promoter. Furth *et al.* (1992) used a jet propulsion injection system to deliver naked plasmid DNA to skin, muscle, fat and mammary tissue of mice and sheep *in vitro* and *in vivo*, achieving low levels of localised β-galactosidase reporter gene expression. This gene gun approach has also been utilised to successfully transfect sheep mammary glands *in vivo*. Expression of a hGH transgene was detected by Northern blot analysis 48 hours following delivery (Kerr *et al.*, 1996). More recently, pressure-mediated delivery has been developed, where controlled non-distending pressure was used to deliver naked fluorescently (FITC) labelled oligonucleotides to rat cardiovascular tissue with greater than 50% of the label localised in the nucleus (Mann *et al.*, 1999). Similarly, hydrodynamic force by rapid injection via the tail vein successfully delivered naked plasmid DNA to numerous tissues of mice *in vivo*, and achieved expression in up to 40% of hepatocytes (Liu *et al.*, 1999a; Zhang *et al.*, 1999).

An alternative approach for gene transfer is electroporation. This method is very efficient and widely used *in vitro,* however, because of the difficulty of administration its use to deliver DNA to somatic cells *in vivo* is limited. Some success has been achieved, with skin (Banga and Prausnitz, 1998), corneal endothelium (Oshima *et al.*, 1998), and muscle (Aihara and Miyazaki, 1998). A disadvantage of electroporation for gene transfer is the resultant high mortality of cells. Methods using low voltage, high frequency electric pulses have recently improved this, with only transient tissue damage resulting from the procedure (Rizzuto *et al.*, 1999b).

These physical approaches to improve efficiencies of naked DNA gene transfer all assist penetration of the plasmid DNA through the cell membrane, hence reducing the extent of DNA degradation as a result of endocytosis. However, they all increase cell death as a result of physical damage to the cells.

1.2.5 Chemical methods of gene transfer

The general principle of chemical-mediated gene transfer is based on the formation of complexes between positively charged chemicals and negatively charged DNA molecules, which in turn can be taken up by the cell through endocytosis. Several chemicals have been utilised to form these complexes which assist in uptake of the DNA into cells.

Chemical introduction of foreign DNA into cells was first reported in 1965 using DEAE-dextran (Vaheri and Pagano, 1965). Since then various chemicals have been used. The most common and perhaps the cheapest method is calcium phosphate co-precipitation (Graham and van der Eb, 1973). The major drawback with calcium phosphate co-precipitation is that the variations from experiment to experiment are significant, due to inconsistency in the size of the calcium phosphate-DNA complexes. Its use is also limited due to its cytotoxic effect on cells and its impracticability for *in vivo* situations because of gravity dependence (Keown *et al.*, 1990). Both the DEAE-dextran and calcium phosphate co-precipitation methods rely on the formation of a complex between the DNA and the chemical, and subsequent internalisation by endocytosis. Various other polymers such as poly-L-lysine and polyethylenimine (PEI) have also been used to deliver DNA to cells *in vitro* and *in vivo* with mixed success (Luo and Saltzman, 2000).

In 1987, Felgner (1987) and co-workers described the first use of a synthetic cationic polymer, called lipofectin, to form complexes with plasmid DNA to be delivered to cells. They transfected a range of mammalian cells *in vitro*, yielding transfection efficiencies up to 100-fold higher than those obtained with other transfection methods such as calcium phosphate co-precipitation. Liposome-

mediated transfection is often more reproducible than other transfection methods because it appears to be less affected by contaminants, salt concentration, and pH of various DNA preparations. Subsequently, numerous developments have been made to improve these cationic lipids with the goal of enhancing transfection efficiency, and also to increase the versatility and simplicity of use for both *in vitro* and *in vivo* transfections. These improvements have reduced the toxicity of many reagents and broadened the range of cell types that are amenable to transfection. Numerous commercial liposomes are currently on the market, each with specific attributes for various situations. In general, they all have different efficiencies and toxicity's across a wide range of cell lines. What is apparent, is that no one particular liposome formulation will ubiquitously transfect all cell types, and that the requirements for *in vivo* transfection are very different to those *in vitro*.

The three basic parts of cationic lipids include; a hydrophobic lipid anchor group which helps in the formation of liposomes (or potential micellar structures) and can interact with cell membranes, a linker group, and a positively charged headgroup which interacts with plasmid DNA leading to its condensation (Mahato *et al.*, 1997).

The biochemical and cellular events required for successful cationic liposome-mediated delivery of plasmid DNA to the cell nucleus can be divided into five main stages; (1) formation of the lipid-DNA complex, (2) entry of this complex into the cell, (3) escape of the complex from endosomes, (4) dissociation of the DNA from the lipid and (5) transport from the cytoplasm and entry of the DNA into the nucleus (*Figure 1.3*).

The formation of the lipid-DNA complex involves a charge interaction between the multivalent cationic head groups of the lipid and the negative charge of the plasmid DNA molecule (Lee and Huang, 1997). Freeze-fracture electron microscopy has been utilised to identify the physical structure of the complex formed between lipid and DNA. Surprisingly, a heterogeneous population of complexes has been observed, ranging in morphology from condensed DNA

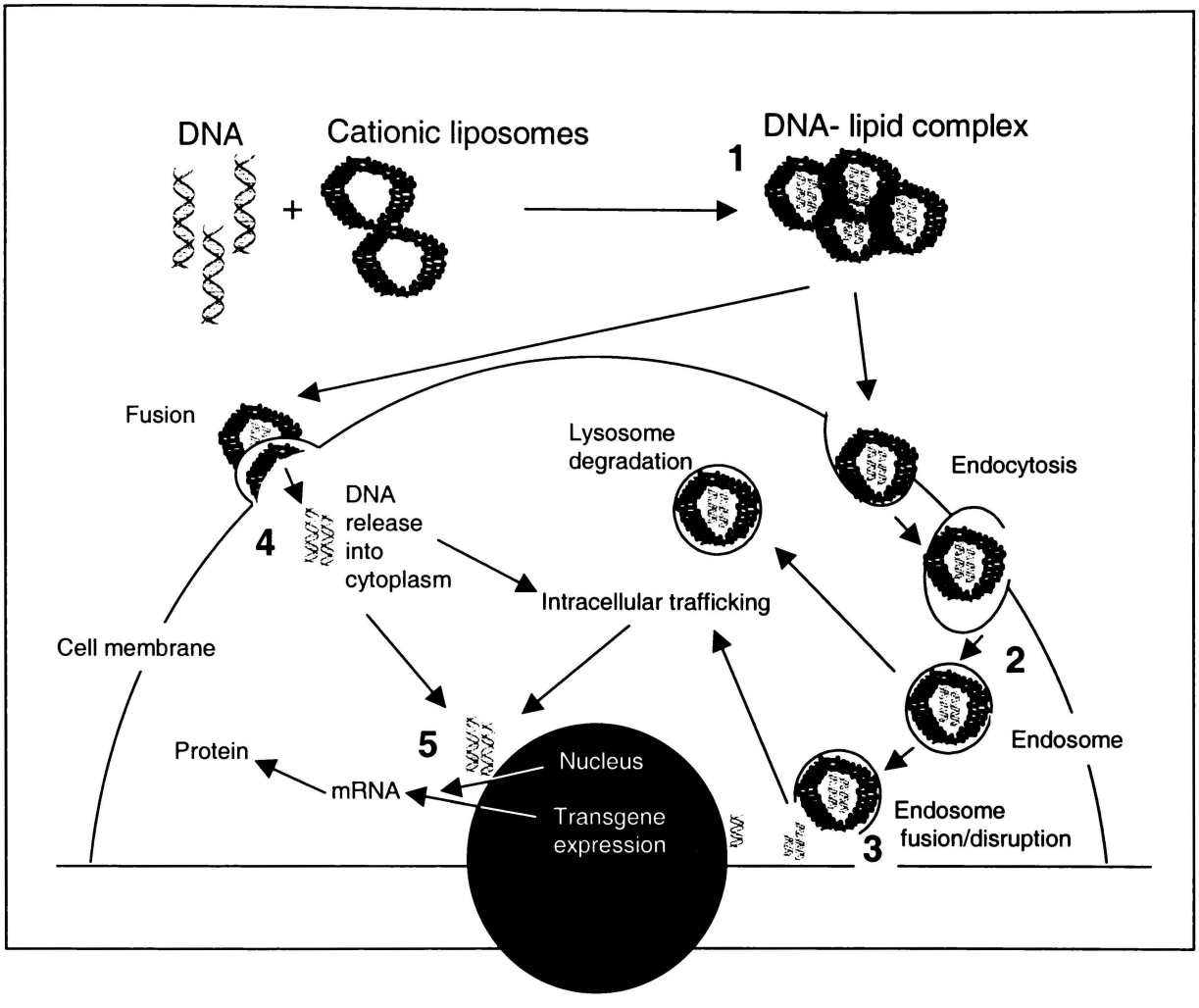


Figure 1.3 *Biochemical and cellular events involved in cationic liposome mediated delivery of plasmid DNA.* (1) formation of the lipid-DNA complex, (2) entry of this complex into the cell, (3) escape of the complex from endosomes, (4) dissociation of the DNA from the lipid and (5) transport from the cytoplasm and entry of the DNA into the nucleus.

surrounded by lipid, to very large aggregates of both DNA and lipid (Sternberg *et al.*, 1994; Zabner, 1997). The overall charge, stability and size of the complex is determined by the lipid-DNA ratio. Near neutral complexes usually form large aggregates whereas complexes with a net positive or negative charge have greater stability and are smaller in size (Lee and Huang, 1997). It remains to be determined which type of complex is best for successful transfection, as a homogeneous population of lipid complexes is not yet available. However, it is clear that the physical characteristics of the complex play an important role in determining the efficiency of transfection.

It is generally accepted that the entry of the lipid-DNA complex into a cell occurs mainly by endocytosis. In order to investigate this process, Zabner (1997) labelled plasmid DNA with gold particles, complexed them with a cationic lipid and transfected COS cells in culture. Electron microscopy at intervals of 5 and 30 minutes after delivery of the complex to cells showed that the uptake and internalisation occurred by endocytosis. An alternate path of entry may be by fusion of the complex to the plasma membrane and subsequent internalisation, however no conclusive evidence exists to support this hypothesis (Li and Huang, 2000).

Once within the cell, the lipid-DNA complex must escape from the endosome to avoid the normal endosomal pathway of degradation, so that it can be transported to the nucleus. El Ouahabi *et al.* (1997) observed that the efficiency of DNA delivery is correlated not only with the efficiency of uptake, but also with destabilisation and escape from endosomes. Using electron microscopy Zhou and Huang (1994) demonstrated that the neutral lipid DOPE (dioleoylphosphatidylethanolamine) enhances destabilisation of the endosome, allowing release of plasmid DNA into the cytoplasm. DOPE is a strong destabiliser of lipid bilayers (Litzinger and Huang, 1992). Consequently when DOPE is present the efficiency of transfection is enhanced (Farhood *et al.*, 1995). DOPE is a component of numerous commercially available cationic liposomes, including LipofectAMINE.

The adenoviral capsid is also capable of destabilising the endosomal complex. Capsid association with the endosomal membrane results in pore formation and lysis of the endosome (Greber *et al.*, 1993). Treatment of cells with replication deficient adenovirus after they had been transfected using a transferrin-polylysine/DNA complex was shown to enhance transfection efficiency (Wagner *et al.*, 1992). Currently, this method is not a practical option for *in vivo* gene therapy applications because of potential cellular and humoral responses to recombinant adenoviral proteins, however the concept has led to the development of other fusogenic substances aimed at achieving a similar result (Stayton *et al.*, 2000).

The major rate-limiting factor for successful transfection is the transport of the plasmid DNA from the cytoplasm to the nucleus. The precise mechanism of this nuclear delivery is unknown, however it is likely to involve passive diffusion, which is an inherently inefficient process. A recent *in vitro* study showed that only a small portion (0.3%) of radiolabelled DNA delivered to airway epithelial cells with the cationic liposome, LipofectAMINE, was detected in the nucleus 24 hours after delivery (Holmes *et al.*, 1999). This observation is supported by Schanbacher and Amstutz (1997), who delivered plasmid DNA complexed with a fluorescently labelled polyion to COMMA-1D mouse mammary epithelial cells or primary bovine mammary cells *in vitro*. They observed accumulation of the fluorescent label in the perinuclear region of most cells within 6-8 hours after delivery, however only a small percentage of cells also demonstrated fluorescent signal localised within the nucleus.

In an attempt to overcome this problem, it has recently been shown that inclusion of nuclear localisation signals in the delivery package can enhance the efficiency of nuclear targeting and hence increase transfection efficiency (Branden *et al.*, 1999; Ziemienowicz *et al.*, 1999). An alternative approach is to provide the necessary machinery for cytoplasmic expression and therefore avoid the need for nuclear delivery of the plasmid DNA. Gao and Huang (1993) used a T7 RNA polymerase cytoplasmic expression system to achieve this. More recently, a direct comparison was made between cytoplasmic and nuclear expression systems within a HEK-293 cell line expressing T7 RNA polymerase. It was shown that

once DNA is released into the cytoplasm, the cytoplasmic expression system shows immediate expression, proportional to the amount of DNA released. In contrast, DNA targeted for nuclear expression requires additional time for nuclear entry. The level of nuclear expression is also restricted by the limited amount of DNA that is imported into the nucleus (Brisson *et al.*, 1999).

Once within the nucleus, the plasmid DNA needs to be released from the lipid-DNA complex for expression to proceed. Naked plasmid DNA injected directly into the nucleus will be expressed, whereas plasmid DNA complexed to lipid injected into the nucleus will not (Capecchi, 1980; Zabner *et al.*, 1995). There must be a balance however, between "coated" plasmid DNA, where it is protected from degradation versus "uncoated" plasmid DNA, a state where transcription can proceed. The optimum state for maximal transfection efficiency will potentially be at an intermediate level of stability. Stable complexes restrict the rate of transcription whereas unstable complexes permit rapid plasmid DNA degradation (Schaffer *et al.*, 2000). The efficiency of transcription of the "uncoated" plasmid DNA may be improved by the presence of stronger promoter and enhancer elements. A popular strategy employed to maximise gene expression is to use a constitutive viral promoter such as the immediate early cytomegalovirus (CMV) promoter.

Overall, there are low efficiencies of gene transfer using physical or chemical methods of delivery compared to viral techniques, and the expression is transient. However, the safety aspect of their use makes them attractive and is hence the reason for perseverance with their use. Approximately 13% of all current human gene therapy clinical trials employ a liposome-based system of gene delivery. The first of these was for the treatment of skin cancer (Nabel *et al.*, 1993), and other trials include the treatment of cystic fibrosis (Caplen *et al.*, 1995).

A polyion complex was successful in delivering plasmid DNA containing the human growth hormone (hGH) gene to COMMA-1D mouse mammary epithelial cells and to primary bovine mammary cells *in vitro* (Schanbacher and Amstutz, 1997). A similar polyion complex has also achieved expression of this construct in guinea pig mammary glands *in vivo* (Hens *et al.*, 2000).

1.2.6 Hybrid vectors – a composite approach

The vectors developed for both viral and non-viral mediated gene transfer each have novel features which are beneficial for satisfying the wide ranging criteria that determine successful gene transfer. One of the more important of these criteria is high efficiency of DNA delivery and expression. However, this must be counterbalanced with consideration for the safety of the technique, with respect to both the microenvironment of the target organism and the macroenvironment within which it exists. Both requirements can be addressed somewhat by utilising expression features from viral vectors, which in general achieve higher levels and greater persistency of expression, and delivery techniques of chemical methods of gene transfer, which are generally safer.

One such composite technique is liposome-mediated delivery of AAV-based plasmid DNA. Inclusion of AAV ITRs within the plasmid to be transfected can enhance the efficiency and stability of transgene expression. Early studies demonstrated that a human interleukin 2 (IL-2) construct expressed up to 10-fold more IL-2 protein from AAV-based plasmids containing ITRs compared to control plasmid without ITRs, in primary human, and in cultured rat and human cell lines. Furthermore, long term expression up to 30 days post transfection was observed with AAV-based plasmids (Philip *et al.*, 1994; Vieweg *et al.*, 1995). These results suggested that the ITRs stabilise expression by either facilitating integration or by maintaining the foreign DNA in an episomal state. The AAV Rep78 and/or Rep68 proteins were not present in either of these studies.

These Rep78 and/or Rep68 proteins are essential for site-specific integration into AAVS1 (Surosky *et al.*, 1997). In their absence, DNA flanked by ITRs will still integrate, but at random sites within the human genome (Xiao *et al.*, 1997). Shelling and Smith (1994) initially showed by Southern analysis that transfection of AAV-based plasmids containing ITRs and *rep* genes can result in high frequency (75%) of site-specific integration at chromosome 19q13.3qter in human HEK-293 and HeLa cell lines. Recent experiments, where AAV-based plasmid DNA was delivered with liposomes to several human cell lines *in vitro*, has confirmed this earlier observation. Sequence analysis of AAVS1-plasmid DNA

junctions confirmed site-specific integration in the presence of Rep proteins (Surosky *et al.*, 1997; Lamartina *et al.*, 1998; Pieroni *et al.*, 1998; Rinaudo *et al.*, 2000; Tsunoda *et al.*, 2000). Also, Rizzuto and colleagues (1999a) created transgenic rats containing a 3.5 kb AAVS1 fragment from human chromosome 19. Subsequent transfection of AAV-based plasmid DNA containing *rep* genes into primary rat fibroblasts derived from these transgenic animals resulted in site-specific integration into the transgene locus, as observed by Southern blot and Fluorescent In Situ Hybridisation (FISH) analysis.

In vivo gene transfer, using transfection of AAV-based plasmid DNA has also been reported in mice (Baudard *et al.*, 1996). Furthermore, a construct expressing recombinant aspartoacylase (ASPA) was delivered to two children with Canavan disease (Leone *et al.*, 2000). This work is ongoing, but early reports suggest positive results for limited *in vivo* ASPA expression.

1.3 The mammary gland

The mammary gland is a structure that lies beneath the skin, exterior to the body cavity of mammals. The number, position and relative size of glands may vary between species, however the histological structure and resultant function, that of milk production, remains conserved. This structure accommodates two primary components, the parenchyma and the stroma.

The parenchyma is made up of secretory and ductal tissue. The secretory tissue consists of specialised mammary epithelial cells that synthesise milk. They are cuboidal in shape and are polarised with basal and luminal sides. A single layer of epithelial cells line pear-shapes structures called alveoli, which exist in clusters. Milk is secreted into the hollow lumina of alveoli, from which it drains into the ductal system (*Figure 1.4*). The ductal tissue forms a system of branching ducts that leads to a teat from which milk is removed. There are differences between species in the structure of the ductal system.

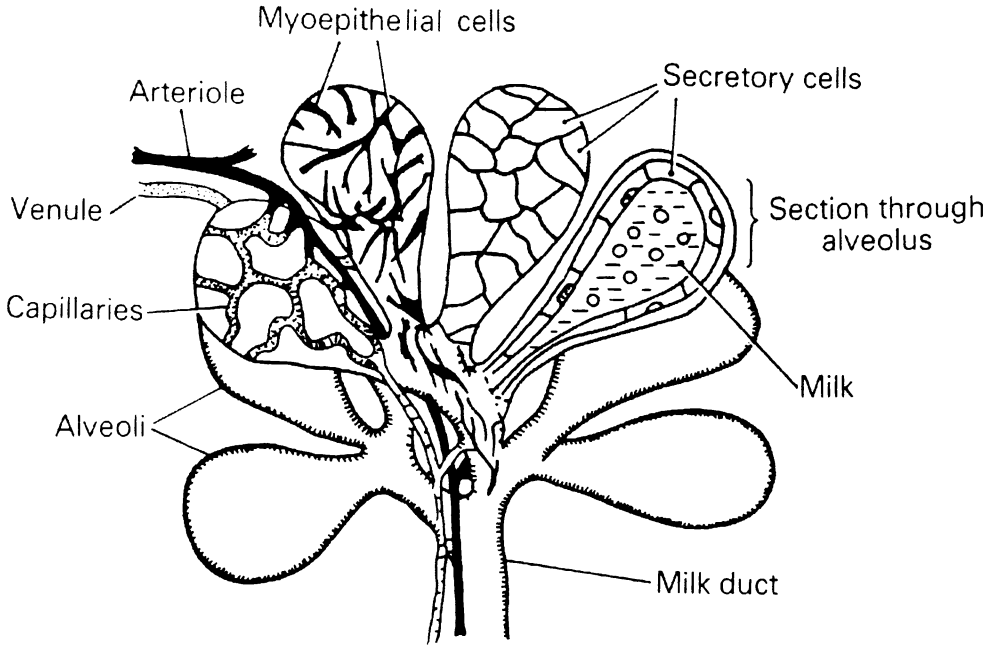


Figure 1.4 *Schematic representation of a cluster of mammary alveoli.* Reproduced from Mephan (1987) with permission.

The stroma consists of skin, connective tissue, adipose tissue, blood and lymph vessels and nerve tissue. It provides the substrate for the parenchyma to develop, and creates a network in which the essential supporting features necessary for milk production can function.

Several *in vitro* mammary epithelial cell models have been developed to study mammary function, including whole gland culture, primary explants, primary cell culture and cell lines. These models clearly demonstrate that no single *in vitro* system mimics the complex three-dimensional structure encountered *in vivo*, so the choice of model depends on the biological question being examined. For example, optimising delivery of plasmid DNA to the vicinity of target cells using an *in vitro* model is not a perfect substitute for the physiological environment encountered *in vivo*. Upon reaching the cell surface however, the cellular and molecular barriers to successful expression of foreign DNA are presumed to be similar *in vitro* and *in vivo*, therefore an *in vitro* model system is beneficial for examining the fate of DNA *in vivo*.

The potential use of the mammary gland as a bioreactor has been realised with the production of germ line transgenic animals that express proteins in the mammary gland (Wall *et al.*, 1997). An alternative to these germ line transgenic animals is somatic gene transfer. The mammary glands of domesticated farm animals such as goats, sheep and cattle are excellent targets for somatic cell gene transfer with the objective to either modify milk composition or express novel proteins.

The morphology of the mammary gland is such that it is essentially a closed system in which each epithelial cell is theoretically accessible from the luminal side. This allows for the prospect of direct intramammary infusion through the teat canal to deliver foreign DNA to the apical membrane of epithelial cells. Patton *et al.* (1984) demonstrated that such a pathway was successful in achieving uniform distribution of a dye marker throughout the mammary gland in a rat model.

Several different modes of delivery have been utilised for *in vivo* somatic gene transfer to the mammary gland, all of which have been discussed earlier in this

chapter. They include; recombinant retroviral (Wang *et al.*, 1991; Archer *et al.*, 1994) and adenoviral vectors (Yang *et al.*, 1995; Jeng *et al.*, 1998), physical delivery by direct needle injection (Kerr *et al.*, 1996) or jet injections by gene gun (Furth *et al.*, 1992), and chemical-mediated delivery of plasmid DNA (Schanbacher and Amstutz, 1997; Hens *et al.*, 2000).

1.4 Purpose and scope of this investigation

A diverse range of vectors are presently available for somatic cell gene transfer, each having merits for the specific application for which they were intended. The perfect vector for ubiquitous use however, has yet to be designed and remains an interesting challenge. Development of a vector for the delivery and expression of foreign DNA into mammary epithelial cells *in vivo* is an example of such a challenge. A unique set of criteria must be satisfied. These include the two essential prerequisites for all gene transfer vectors, which are efficiency and safety.

The focus of the work in this thesis is the somatic transfection of mammary epithelial cells. Previously several different approaches, that include retroviral, adenoviral, biolistic gene gun and polymer based vectors, have been utilised to deliver foreign DNA to mammary epithelial cells, each with varied success. In general no one technique has exhibited overwhelming advantages over others. An attractive method for somatic cell gene transfer is the use of liposome-mediated transfection of AAV-based plasmid DNA. This method encapsulates features of AAV to enhance the efficiency and longevity of transgene expression, yet still retains a non-viral mode of delivery. It has been used as a gene transfer vector for a wide range of target cells both *in vitro* and *in vivo*, but has not been used for mammary epithelial cells.

This work therefore investigates the efficacy of gene transfer to mammary epithelial cells using liposome-mediated transfection of AAV-based plasmid DNA. As yet, sparse knowledge exists about the site of integration resulting from

the use of these vectors, therefore characterisation of this event would improve knowledge in this area.

To achieve this goal, two main approaches will be employed. Firstly, several plasmid DNA constructs will be created and delivered to mammary epithelial cells *in vitro*. Secondly, the frequency of integration will be examined, and the site of integration will be investigated using Southern blots and sequencing.

Chapter Two

Materials and methods

2.1 Materials

Common laboratory chemicals and reagents were obtained from BDH Chemicals NZ, Ltd. and Sigma Chemical Co. Restriction enzymes were obtained from Roche. Mammalian cell culture medium components were obtained from Life Technologies GIBCOBRL. A Perkin-Elmer GeneAmp PCR System 9600 machine was used for PCR. Oligonucleotides were purchased from GIBCOBRL. *Milli Q* filtered water was used for all molecular biology and mammalian cell culture experiments. Diethylpyrocarbonate (DEPC) treated water and solutions were used for all experiments with RNA.

2.1.1 Solutions

Solutions used were prepared as described in Ausubel *et al.*(1987) or Sambrook *et al.* (1989).

Table 2.1 - *Common solutions*

Church and Gilbert hybridisation buffer	0.5 M Na_2HPO_4 (pH 7.2) 7% SDS 1 mM EDTA
Crystal Violet	0.04% crystal violet in 1 M citric acid
DNA loading dye	15% Ficoll (Type 400) 0.25% bromophenol blue 0.25% xylene cyanol
Easyprep lysis buffer	10mM Tris-HCl (pH 8.0) 1mM EDTA 15% sucrose 2 mg/ml lysozyme 0.2 mg/ml pancreatic Rnase 0.1 mg/ml bovine serum albumin
MOPS	41.8% MOPS 50 mM sodium acetate 10 mM EDTA
PBS	OXOID tablets

RNA loading dye	10% MOPS (10x) 20% deionised formaldehyde 50% deionised formamide 0.02% bromophenol blue 5% glycerol 1 mM EDTA (pH 8.0) 40 μg/ml ethidium bromide
TE	10 mM Tris-Cl, 1 mM EDTA (pH 8.0)
TAE	40 mM Tris-acetate 2 mM EDTA (pH 8.0)
Trypsin-EDTA solution	0.25% trypsin 1mM EDTA in PBS
2 × HEPES-buffered saline	0.28 M NaCl 0.05 M Hepes 1.5 M Na_2HPO_4 (pH 7.1)

2.2 Methods

2.2.1 General molecular biological methods

General molecular biology techniques were performed as described by Sambrook *et al.* (1989) and Ausubel *et al.* (1987). These included: bacterial transformation and culture; nucleic acid isolation from bacterial and mammalian cells; restriction endonuclease digestions; agarose gel electrophoresis; DNA fragment isolation; Northern and Southern blotting and hybridisation; and DNA modifications including dephosphorylation, end filling or blunting; and ligation. Modifications to these procedures and non-standard protocols are described in more detail where appropriate.

(i) DNA isolation

For small-scale plasmid DNA preparation, either the "Easyprep" (Berghammer and Auer, 1993), or small-scale alkaline lysis (Sambrook *et al.*, 1989) procedures were used. QIAGEN plasmid DNA preparation ion exchange columns (QIAGEN Pty Ltd) were used for all large-scale plasmid DNA preparation that were

intended for use in transfection experiments. Otherwise, large-scale alkaline lysis isolation was used (Sambrook *et al.*, 1989).

Plasmid DNA was quantified by linearising the plasmid, running an aliquot on an agarose gel and comparing the amount to known concentrations of lambda DNA molecular weight standards.

Extraction of genomic DNA from mammalian tissues, blood or cells in culture was performed either as described by Ausubel *et al.* (1987), or with DNAzol Reagent (GIBCO BRL) following the manufacturers protocol.

(ii) RNA isolation

Total RNA was extracted from cells in culture using TRIzol Reagent (GIBCO BRL) according to the manufacturers protocol.

(iii) Subcloning

For ligation reactions either T4 DNA ligase (GIBCO BRL) in 5x pre-mixed buffer, or the pGEM-T Easy Vector System I (Promega) were used. Ratios of insert to vector, of two-, five- and ten-fold the molecular mass, were commonly used to optimise the ligation efficiency.

(vi) Competent cells

XL1-Blue (MRF') (Stratagene) competent cells were prepared according to the method of Inoue *et al.* (1990).

(v) Labelling of DNA

Oligonucleotides probes were end-labelled with [α-^{32}P]dCTP using Terminal Transferase (Roche). cDNA probes were labelled with [α-^{32}P]dCTP using Rediprime II random prime labelling system (Amersham).

2.2.2 Mammalian cell culture

RPMI 1640 Medium, Dulbecco's Modified Eagle Medium (DMEM), OptiMEM I and Medium 199 were prepared as described by the manufacturers. All media was filter sterilised with 0.22 μm filters. Cells were cultured at 37°C, in 5% CO_2 in sterile plastic dishes. Foetal bovine serum was heat inactivated by incubation at 56°C for 30 minutes. To passage cells, growth medium was removed and the cells were washed with phosphate buffered saline (PBS). A minimal volume of trypsin-EDTA solution (0.25 % trypsin, 1 mM EDTA) was added to cover cells, which were then incubated for 5-8 minutes at 37°C or until the cells had lifted off the plastic. The trypsin-EDTA solution was then inactivated by the addition of growth medium containing 10% foetal bovine serum (FBS).

(i) HC11 cells

The mouse mammary epithelial cell line HC11 was a gift from Dr Jerome Demmer (formerly of AgResearch, Ruakura). All experiments were carried out with cultures that were between nine and eighteen passages from original isolation (Danielson *et al.*, 1984). Cells were cultured in RPMI 1640 Medium containing 100 U/ml penicillin G, 0.1 mg/ml streptomycin, 10% (v/v) foetal bovine serum, and 10 ng/ml epidermal growth factor.

(ii) HEK-293 cells

HEK-293 cells (Graham *et al.*, 1977) were a gift from Dr Mike Eccles (University of Otago). The passage number of the cells since their original isolation was unknown, however for experiments carried out in this thesis the cells were passaged less than six times. HEK-293 cells were grown in DMEM medium supplemented with 100 U/ml penicillin G, 0.1 mg/ml streptomycin and 10% (v/v) foetal bovine serum.

(iii) Neuro-2a cells

Neuro-2a (ATCC Number CCL-131) were a gift from Dr Ian Garthwaite (AgResearch, Ruakura). Neuro-2a cells were cultured in RPMI 1640 Medium

containing 100 U/ml penicillin G, 0.1 mg/ml streptomycin, 2 mM glutamine, 1 mM pyruvate, and 10% (v/v) foetal bovine serum.

(iv) Primary bovine mammary cells

A 10 cm^3 block of tissue was aseptically removed from the centre of the left rear gland of a mid-lactating Holstein-Friesian dairy cow, which had been slaughtered. This was cut into smaller pieces of approximately 1 cm^3 and washed twice in wash solution (Medium 199 media supplemented with 1% (v/v) foetal bovine serum). A collagenase solution (15 ml of Medium 199 containing 1000 U/ml collagenase) was infused into the tissue using a 25-gauge needle so that it was completely saturated. The block was then diced into smaller pieces and incubated at 37°C with shaking (180 rpm) for 2 hours or until the digestion was complete. The cell slurry was filtered through 150 μm sterile gauze mesh to remove any remaining clumps of tissue. The filtrate was washed in 40 ml of wash solution, centrifuged at 180 × *g* for 5 minutes, re-suspended in 25 ml of wash solution and centrifuged at 90 × *g* for 5 minutes. The resulting pellet was suspended in 25 ml of wash solution and filtered through 30 μm gauze mesh to enrich for clumps of epithelial cells. These cells were grown in Medium 199, which contained 100 U/ml penicillin G, 0.1 mg/ml streptomycin, 0.25 μg/ml amphotericin B and 10% (v/v) foetal bovine serum. The cultured cells were further enriched for epithelial cells by selective trypsinisation as follows. After two, four or six minutes of exposure to the trypsin-EDTA solution, the cells were washed with growth medium and the wash solution (containing fibroblast cells that were more sensitive to trypsinisation) was discarded. After rinsing with PBS, fresh trypsin-EDTA solution was added to dislodge the remaining cells, which were then seeded in a new plate.

2.2.3 Plasmid DNA

The following plasmids were either supplied as gifts or purchased commercially for use in this study: pBluescript II SK (Stratagene); pGEM-T Easy (Promega); pCH110 (Pharmacia); pBR322 (Sutcliffe, 1979); pEGFP-N1 (Clontech) (gift from

Professor Floyd Schanbacher, Ohio State University); pHIVrep (Antoni *et al.*, 1991) (gift from Dr Matt During, University of Auckland); pAAV/PLAN (gift from Dr Matt During, University of Auckland) was created by digestion of psub201(+) (Samulski *et al.*, 1987) with *Xba*I to remove the AAV viral genome, leaving the inverted terminal repeats (ITRs). This DNA sequence was replaced with a 366 bp *Hind*III/*Pvu*II fragment consisting of polylinker and bovine growth hormone polyadenylation sequence from pcDNA3 (Invitrogen); pCS2-CMVlacZ (gift from Dr Russell Snell, AgResearch, Ruakura) consists of a 3.1 kb lacZ gene fragment from pMC1871 (Shapira *et al.*, 1983) inserted immediately downstream of the CMV promoter in pCS2+ (www.sitemaker.umich.edu/dlturner.vectors); pTK(ES) (gift from Dr Garry Udy, AgResearch, Ruakura) consists of a 1746 bp *Pvu*II/*Bgl*II herpes simplex virus thymidine kinase (HSV-tk) gene fragment from pTK1 (Cordingley and Preston, 1981) inserted into pSP64 (Promega). Furthermore, a 274 bp *Xho*I/*Pst*I HSV-tk promoter fragment from pMC1neo (Stratagene) was inserted immediately upstream of the HSV-tk gene to create pTK(ES).

The plasmids that were made for the experiments described in this thesis are described in the following sections.

pBS-ITR

pBS-ITR was created by isolating a *Pvu*II/*Eco*RI fragment from pAAV/PLAN which consisted of 190 bp of ITR and 50 bp of pcDNA3 polylinker sequence. This was ligated into pBluescript II SK + backbone digested with *Sma*I/*Eco*RI.

pAAV-GFP

pEGFP-N1 was digested with *Ase*I/*Xho*I to release a 607 bp fragment containing the CMV promoter and a 4.1 kb vector backbone. pBS-ITR was digested with *Eco*RV/*Xho*I and the 607 bp fragment from pEGFP-N1 was inserted to create pBS-ITR-CMV. pBS-ITR-CMV was digested with *Xba*I and *Xho*I to release a 857 bp fragment containing the ITR and CMV promoter. This 857 bp fragment was ligated into the 4.1 kb pEGFP-N1 vector backbone from the first step to create pEGFP-ITR-CMV. pBS-ITR was digested with *Xba*I and *Hind*III to release a 260 bp fragment containing the ITR. This fragement was ligated into

pEGFP-ITR-CMV, which had been linearised by digestion with *Bfr*I, to create pAAV-GFP.

pCMVlacZ (7.5 kb)

pEGFP-N1 was digested with *Hind*III and *Bfr*I to remove the GFP gene, but leaving the CMV promoter in the vector backbone. The lacZ gene, isolated from pCH110 by digestion with *Hind*III and *Bam*HI, was ligated into this vector backbone to create pCMVlacZ.

pITR-CMVlacZ (7.9 kb)

pAAV-GFP was digested with *Hind*III and *Eco*RI to remove the GFP gene, but leaving the CMV promoter in the vector backbone. The lacZ gene, isolated from pCH110 by digestion with *Hind*III and *Bam*HI, was ligated into this vector backbone to create pITR-CMVlacZ.

pITR-neo-tk (5.8 kb)

A neomycin resistance gene cassette of 1986 bp was isolated from pEGFP-N1 by digestion with *Dra*III and *Dra*II. pITR-neo-tk was created by digesting pAAV-GFP with *Sac*I to remove the GFP cassette. The neomycin resistance gene cassette was then ligated into this vector backbone to create pITR-2neo. The existing copy of the neomycin resistance gene was then removed from the pITR-2neo vector backbone by digestion with *Dra*III and *Dra*II. This was replaced a 2.2 kb copy of the herpes simplex virus thymidine kinase (HSV-tk) gene isolated as a *Hind*III fragment from pTK(ES).

pneo-tk (5.3 kb)

pEGFP-N1 was digested with *Ase*I and *Bfr*I to remove the GFP cassette. This was replaced by neomycin resistance gene cassette of 1986 bp was isolated from pEGFP-N1 by digestion with *Dra*III and *Dra*II to create p2neo. The existing copy of the neomycin resistance gene was then removed from the p2neo vector backbone by digestion with *Dra*III and *Dra*II. This was replaced a 2.2 kb copy of the herpes simplex virus thymidine kinase (HSV-tk) gene isolated as a *Hind*III fragment from pTK(ES).

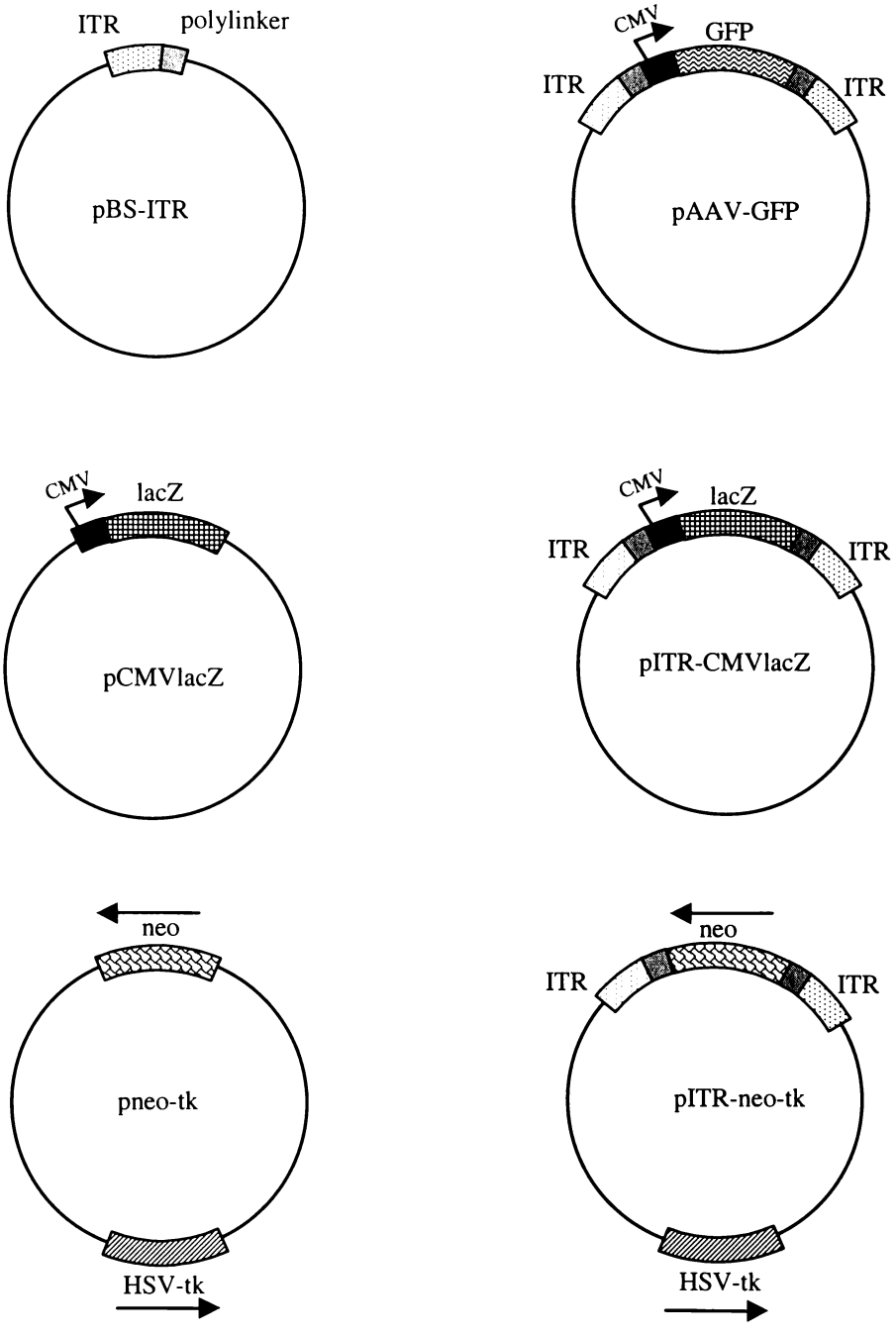


Figure 2.1 *Schematic representation of the plasmids made for experiments in this thesis.*

2.2.4 *In vitro transfection*

For the *in vitro* transfection experiments in this thesis, cells were seeded 16 hours prior to transfection. The number of cells that was seeded was determined from the surface area of the culture vessel, and calculated so that they would be 70-80% confluent at the time of DNA delivery. All DNA for transfection experiments was prepared using QIAGEN columns. For each experimental treatment, duplicate wells were transfected. Experiments were replicated between one and four times.

(i) Calcium phosphate co-precipitation

Calcium phosphate-mediated delivery of plasmid DNA was carried out as described in Ausubel *et al.* (1987). To transfect HC11 cells in a 6-well dish, the appropriate amount of plasmid DNA was diluted in 0.5 ml of 0.25 M calcium chloride. This solution was added dropwise to 0.5 ml 2 × HEPES-buffered saline (pH 7.1) while CO_2 was bubbled through the solution. The solution was incubated at room temperature for 30 minutes and then added to the cells. Growth media (1 ml) was added immediately and a further 1 ml was added 4 hours later. This solution was left on the cells overnight, then washed off the cells by rinsing them four times with PBS.

(ii) Cationic liposomes

Three cationic lipid formulations were utilised for transfection experiments in this thesis, DOTAP (Roche), DMRIEC (Life Technologies) and LipofectAMINE (Life Technologies). Unless otherwise stated, transfection was carried out using the manufacturer instructions. Control, mock-transfections were performed with lipid only, or DNA only, in the transfection mixture. DOTAP reagent (1 mg/ml) is N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethyl-ammonium methyl-sulfate in buffered saline. DMRIE-C reagent (2 mg/ml) is a 1:1 liposome formulation of the cationic lipid DMRIE (1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide) and cholesterol in membrane filtered water. LipofectAMINE reagent (2 mg/ml) is a 3:1 (w/w) liposome formulation of the polycationic lipid DOSPA (2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-

N,N-dimethyl-1-propanaminium trifluoroacetate) and the neutral lipid DOPE (dioleoyl phosphatidylethanolamine) in membrane filtered water.

To transfect cells in 6-well culture flasks with LipofectAMINE, a defined amount of plasmid DNA was diluted in 100 µl OptiMEM I medium. Likewise, the appropriate volume of LipofectAMINE was diluted in 100 µl OptiMEM I medium. These two solutions were then mixed and incubated at room temperature for 20 minutes. Following incubation, 800 µl OptiMEM I was added to this lipid-DNA mix and the resultant solution was added to cells, which were pre-washed in OptiMEM I. After 4 hours, the transfection solution was removed and replaced with 2 ml of growth medium. To transfect different sized culture vessels, the volume of each reagent was adjusted according to surface area of the vessel.

2.2.5 In vivo transfection

Lactating Sprague-Dawley laboratory rats were used for *in vivo* transfection experiments. On day seven to seventeen of lactation, the pups were removed one hour prior to the beginning of manipulations and sacrificed by CO_2 inhalation. The rats were anaesthetised by a single intra-peritoneal dose of Sagatal (60 mg/kg body weight) and the body temperature was maintained using a 37°C heating block. Transfection complexes were delivered to either the third or fourth mammary glands and milk was clearly present in these glands at this time. The delivery route was either by infusion into the mammary gland via the teat canal using a blunt 32-gauge needle, or by direct injection into the mammary gland through the wall of the gland. Two days after delivery of the transfection complex, the rats were sacrificed and mammary tissue was isolated and immediately frozen at -80°C. Controls glands were mock-transfected with OptiMEM I only. Cryosections of 20 µm, were subsequently prepared and mounted on poly-L-lysine coated glass slides. These sections were stained *in situ* for β-galactosidase expression, counter-stained with eosin and then mounted in DPX.

All animal procedures were approved by the University of Waikato (Protocol number 243), and Ruakura (Protocol number 2414) Animal Ethics Committees and the Ruakura Biosafety Supervisory Committee (Application number 23).

2.2.6 β-galactosidase reporter gene assays

(i) In situ histochemical staining

Cultured cells and tissue sections were assayed histochemically for β-galactosidase expression using a modified method of Sanes *et al.* (1986). They were washed once with PBS and then fixed *in situ* with fixative solution (2% formaldehyde, 0.05% glutaraldehyde in PBS: cells in the culture dishes and tissue sections on the glass slides) for five minutes at room temperature. They were washed twice with PBS then exposed to the stain solution (5 mM ferricyanide, 5 mM ferrocyanide, 2 mM $MgCl_2$, 1 mg/ml X-gal) and incubated at 37°C for between 2 and 16 hours.

(ii) ONPG β-galactosidase assay

The amount of β-galactosidase enzyme activity in the cultured cells was quantified using the substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (Jagota *et al.*, 1981). The cells were harvested 48 hours after liposome-DNA complex delivery, resuspended in 0.1 M Tris (pH 7.8) and lysed by repeated freeze-thaw cycling (five times alternating between dry ice and 37°C). The cell debris was pelleted and an aliquot of the cell lysate (4 µl) was assayed for β-galactosidase enzyme activity. Solution Z (150 µl, 36 µl β-mercaptoethanol/10 ml PBS) and the ONPG solution (30 µl) were added to the lysate, and the reaction was incubated for 30 minutes at 37°C. The colour change was measured at 405 nm. Units of β-galactosidase were calculated by comparison to known standards, where one unit of β-galactosidase is defined as the amount of enzyme that will hydrolyze 1 µmole of ONPG in one minute at 37°C. The total protein concentration was quantified using the method of Bradford (Bradford, 1976), and was used to normalise the samples.

2.2.7 *Selection for integration of plasmid DNA*

HC11 or HEK-293 cells were seeded into 60 mm culture plates, at a concentration of 1.5×10^6 cells/plate, 16 hours prior to transfection. Plasmid DNA was combined with LipofectAMINE and delivered to cells as described in Section 2.2.4. The cells were then passaged and seeded into plates containing growth media with either G418 (at the minimal lethal dose for the cell line which was determined as described in the following paragraph), or G418 and FIAU (0.2 μM), until discrete colonies were visible (14-24 days post-transfection).

The minimum lethal dose of G418 (GIBCO BRL), determined for both HC11 and HEK-293 cells, is the minimum concentration of G418 that gave zero percent survival of non-transfected cells. Each cell line was seeded at 30-40% confluence (8.0×10^4 cells/well for HC11 and 6.0×10^4 cells/well for HEK-293 in 12-well plates) in their respective growth medium. This was supplemented with G418 at concentrations ranging from 0 to 1000 μg/ml, initially with increments of 100 μg/ml. Once the broad range was determined, the experiment was repeated with smaller 25 μg/ml increments to more precisely define the minimum lethal dose of G418. The media was changed every day for days one to seven and then on days nine and twelve. On day fourteen, the media was removed and the plates were stained with crystal violet to stain any cells that remaining attached to the plate. The lowest G418 concentration where no cells were stained was determined to be the minimum lethal dose.

Following selection with G418, or G418 and FIAU, individual colonies were isolated by covering the colony with of 5 μl trypsin-EDTA solution, and scraping from the plate with a 200 μl pipette tip. Isolated colonies were placed in 96-well tissue culture plates containing growth medium and allowed to grow for 24 hours. The cells were then selected with G418. When the cells reached confluence, they were expanded by sequentially passaging them into larger culture vessels (*Table 2.2*). Control non-transfected cells, cultured in growth medium supplemented with G418, had all died before day fourteen. A small aliquot of cells from each transfection were seeded separately and stained *in situ* for β-galactosidase

expression. No significant differences in transfection efficiencies were observed between different vectors delivered on the same experimental day.

Table 2.2 *Summary of the selection regime for HC11 and HEK-293 cells.*

Day 0	Transfect cells in 60 mm culture plates.
Day 2	Supplement growth media with G418.
Day 3	Cells were passaged into 4 x 100 mm culture plates (A, B, C and D) at dilutions which allowed counting and selection of single colonies.
Days 4-15	Supplement growth media of all plates (A, B, C and D) with G418.
Day 15	Isolate and expand 12 colonies from plates A and B. Remaining colonies were stained with crystal violet and counted.
Day 15-24	Supplement growth media of plates C and D with G418 + FIAU.
Day 24	Isolate and expand 12 colonies from plates C and D. Remaining colonies were stained with crystal violet and counted.

2.2.8 PCR amplification of integration junctions

Genomic DNA-transgene junction sequences were identified essentially as described by the method of Siebert *et al.* (1995), with the following modifications (*Figure 2.2*). The 'Adaptor' was created by annealing the 'adapt' oligonucleotide (5' cta ata cga ctc act ata ggg ctc gag cgg ccg ccc ggg cag gt 3') to the 'link' oligonucleotide (5' acc tgc cc 3'), by combining equimolar amounts of each oligonucleotide, heating to 95°C for 2 minutes and cooling to room temperature. The templates for PCR were prepared by digesting 2.5 µg genomic DNA in 100 µl reaction volumes with 30 Units of *Sca*I restriction endonuclease and incubating overnight at 37°C. Digested DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), then with chloroform. The DNA was precipitated with ethanol, and resuspended in 20 µl TE. Ten µl of this *Sca*I digested DNA was ligated to 5 µM 'Adaptor' in a 20 µl reaction with 5 Units T4 DNA ligase (GIBCO BRL), incubated overnight at 16°C. Ligated DNA was diluted 10-fold in TE and stored at -20°C. Primary PCR reactions were conducted in 20 µl volumes containing 1 µl of template DNA, 0.4 µM AP1 primer (5' gga tcc taa tac gac tca cta tag ggc 3'), 0.4 µM neo primer (neo3341 (5' acc gct tcc tcg

tgc ttt ac 3') or neo3392 (5' cct tct atc gcc ttc ttg ac 3')), 0.25 mM dNTPs, 2 U Taq DNA polymerase (Roche) in 1 × Roche PCR buffer (10mM Tris-HCl, 1.5 mM $MgCl_2$, 50mM KCl, pH 8.3). The primary PCR amplification products were diluted 100-fold in TE buffer and used as template DNA for the secondary amplification. Secondary PCR reactions were conducted in similar conditions as for the primary reactions, except with the nested primers AP2 (5' aat agg gct cga gcg gc 3') and neo3608 (5' aac tga aac acg gaa gga ga 3'). The primary cycle parameters were an initial denaturation of 95°C for 2 minutes, followed by 34 cycles of 95°C for 30 seconds and 68°C for 6 minutes, and a final cycle of 95°C for 30 seconds and 68°C for 15 minutes. Secondary cycle parameters were as for primary except only 19 rather than 34 cycles were used.

The specificity of the amplification products was determined by Southern blotting then hybridisation with a neomycin gene specific oligonucleotide, neo3685 (5' gtg ttg ggt cgt ttg ttc at 3'). Specific products were re-amplified by removing a 'stab' with a 2 µl pipette tip, directly from within the DNA band on an agarose gel, and using this agarose plug as template DNA for a repeat of the secondary PCR amplification reactions. This enabled isolation of unique products, rather than mixed pools of products, which could then be subcloned and sequenced. Amplification products were subcloned into pGEM-T Easy (Promega) using the conditions described in the manufacturer instructions. Sequencing of clones was carried out at the University of Waikato DNA sequencing facility (ABI 377, Applied Biosystems).

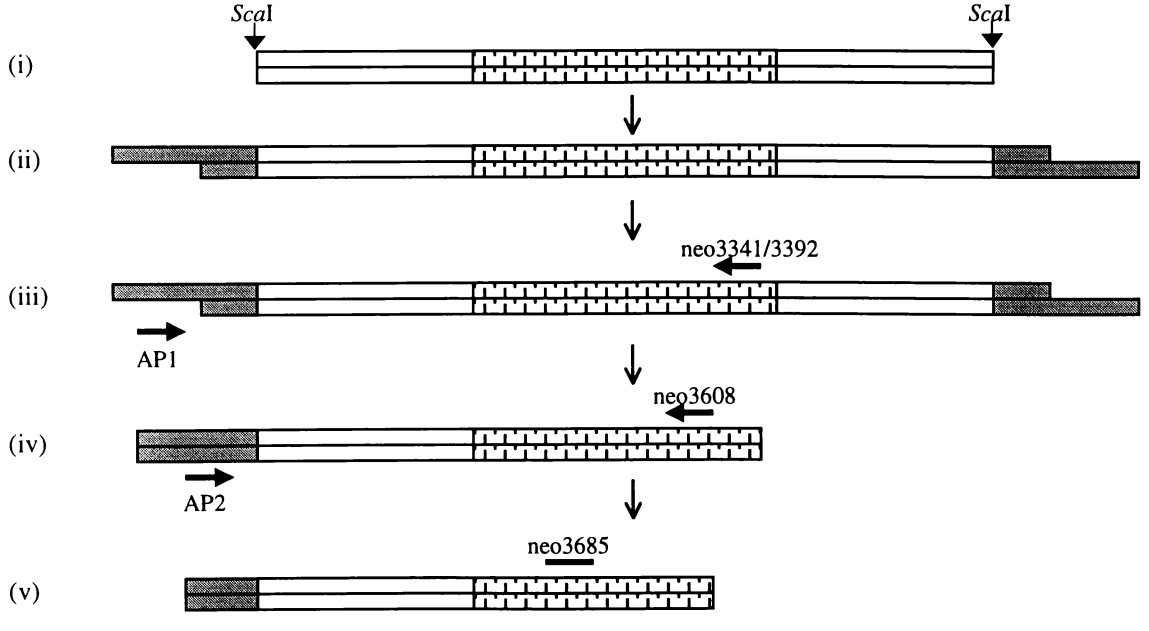
2.2.9 Probes

A 703 bp DNA probe specific to the neomycin resistance gene was isolated from the plasmid pneo-tk, by digestion with the restriction endonuclease *Nco*I. The digested DNA was fractionated by agarose gel electrophoresis, and the desired fragment was isolated from the gel using a scalpel blade to cut out a slice of agarose. The DNA was then purified from the agarose using a GFX DNA purification column (Amersham Pharmacia Biotech Inc.).

A 623 bp AAVS1 DNA probe was amplified by PCR from HEK-293 genomic DNA using primers, AAVS1-1 (cca ttt ccc agg ctc ccg cta cct) and AAVS1-4 (acg acg ggg cgg atc gag ac), that are specific to the human AAVS1 locus (GenBank Accession number S51329). The 623 bp product spans a region of human genomic DNA that contains the consensus sequence for the terminal resolution site and the Rep Binding Element (Linden *et al.*, 1996a).

A 508 bp DNA probe specific to the AAV Rep78/68 gene was isolated from the plasmid pHIVrep, by digestion with the restriction endonucleases *Bgl*I and *Bam*HI. The digested DNA was fractionated by agarose gel electrophoresis, and the desired fragment was isolated from the gel using a scalpel blade to cut out a slice of agarose. The DNA was then purified from the agarose using a GFX DNA purification column.

Figure 2.2 *Schematic representation of the Siebert PCR assay.* (i) digestion of genomic DNA with *Sca*I, (ii) ligation of the Adaptor (shaded box) to digested DNA, (iii) primary PCR reactions with AP1 and neo3341 or neo3392 primers, (iv) secondary PCR reactions with AP2 and neo3608 primers, (v) detection of specific products with neo3685 oligonucleotide probe. Genomic DNA (open boxes) and plasmid DNA (vertical hatching) are as indicated.



Chapter Three

Mammary epithelial cell transfection in vitro and in vivo

3.1 Introduction

Various techniques have been developed to deliver genes to somatic tissues *in vivo*, as outlined in Chapter One. The objective of this work was to develop an efficient system for the delivery of genes directly to the mammary gland. Somatic mammary epithelial cell modification has a wide range of potential applications. These include the ability to introduce and express novel genes in the mammary gland, which may change milk composition or alter mammary gland function.

The objective of this chapter was to firstly, assess and optimise the efficiency of chemical-mediated delivery of plasmid DNA to mammary epithelial cells *in vitro*. Two vehicles for delivery were utilised, calcium phosphate co-precipitation and cationic liposomes.

In order to obtain the highest transfection efficiency with cationic liposome-mediated delivery of plasmid DNA to any cell line, optimisation of two important parameters is required. It is essential to calibrate both the ratio of lipid to plasmid DNA, and the final concentration of these components, to achieve this goal of maximal efficiency. Other parameters which may affect transfection efficiency, but with much less significance, include the cell density at the time of delivery, time of exposure of the lipid-DNA complex to the cells, and DNA purity. These were kept constant in all *in vitro* experiments described in this work. For calcium phosphate co-precipitation, the amount of plasmid DNA delivered to cells can influence the transfection efficiency; therefore this was also optimised in these experiments.

The second objective of this chapter was to assess the efficacy of cationic liposome-mediated delivery of plasmid DNA to rat mammary epithelial cells *in vivo*.

3.2 In vitro transfection

In order to carry out these *in vitro* studies, three cell lines were used as models, Neuro-2a, HC11 and primary bovine mammary cells. The murine neuroblastoma cell line Neuro-2a was used as a positive control for transfection (Olmsted *et al.*, 1970). This cell line was chosen because it has previously been shown to efficiently take up DNA following transfection (Hitoshi *et al.*, 1996) and it was readily available at Ruakura. HC11 is a clonal cell line derived from COMMA-1D cells (Ball *et al.*, 1988), which are an epithelial cell line derived from the mammary tissue of mid-pregnant BALB/c mice (Danielson *et al.*, 1984). HC11 cells exhibit typical epithelial-like morphology and provide a cellular environment similar to that of *in vivo* mammary cells. Primary bovine mammary cells were isolated for this study from a mid-lactating Holstein-Friesian dairy cow (by methods described in Chapter Two). Morphological features, such as a cobblestone-like appearance with cells spreading out from organoid-like attachments, suggest that they were representative of typical mammary epithelial cells. The cell population was not completely homogeneous however, with up to 10% of cells exhibiting a fibroblast-like morphology.

Four different chemical reagents were used to deliver plasmid DNA to the HC11 cell line. These were calcium phosphate co-precipitation, and the commercially available cationic liposomes, DOTAP (Roche), DMRIE-C (Life Technologies) and LipofectAMINE (Life Technologies). LipofectAMINE was also used to deliver plasmid DNA to Neuro-2a and primary bovine mammary cells.

All *in vitro* transfection experiments outlined in this chapter were performed with the plasmid pCH110 (Pharmacia). This plasmid is a 7.1 kb eukaryotic expression vector, which contains the β-galactosidase gene driven by the SV40 immediate early promoter. A large-scale bulk preparation of pCH110 was purified using a QIAGEN plasmid purification kit (see Chapter Two) and plasmid DNA from the same preparation of pCH110 was used for all experiments.

Following chemical-mediated delivery of pCH110, the efficiency of transfection was assessed by calculating the percentage of cells that expressed β-galactosidase

48 hours after the plasmid DNA was delivered to cells. To calculate this, cells were fixed *in situ* and histochemically stained with a solution containing X-gal (Sanes *et al.*, 1986). Transfection efficiency presented here thus represented the number of cells that stained blue compared to the total cell number 48 hours after transfection. It does not represent the absolute percentage of cells that have taken up plasmid DNA. During the 48-hour period from delivery of the DNA to staining, the cells would have divided at least twice. Hence the total cell number may have increased by up to 4-fold, consequently diluting the percentage of cells staining blue compared to the total cell number by the same amount.

Furthermore, in experiments with LipofectAMINE-mediated delivery of pCH110 to HC11 cells, β-galactosidase enzyme activity was also quantified. Cells were harvested 48 hours after liposome-DNA complex delivery and lysates were assayed for β-galactosidase enzyme activity using the substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (Jagota *et al.*, 1981). Units of β-galactosidase were calculated from known standards where one unit of β-galactosidase is defined as the amount of enzyme that will hydrolyze 1 μmole of ONPG in one minute at 37°C. The total protein concentration was quantified using the method of Bradford (Bradford, 1976), and used to normalise between samples.

All the *in vitro* transfection experiments described here were performed in 6-well culture dishes with cells seeded at a consistent density. Chapter Two contains details on cell growth and maintenance, methods of DNA delivery, and β-galactosidase assays. Control mock-transfection of HC11, primary bovine mammary and Neuro-2a cells with pCH110 alone (naked DNA) resulted in no detection of reporter gene expression following *in situ* histochemical staining (data not shown). For each experimental treatment, duplicate wells were transfected. Experiments were replicated between one and three times.

3.2.1 Neuro-2a cell line transfection

The murine neuroblastoma cell line Neuro-2a was transfected with the plasmid pCH110 complexed to LipofectAMINE. Over a range of ratios of LipofectAMINE (2-10 μl): 1 μg of pCH110, a peak transfection efficiency of 75%

was achieved using an optimal ratio of 6:1 (μl LipofectAMINE: μg pCH110) (data not shown). These experiments also confirmed that the β-galactosidase gene was expressed by pCH110 and the transfection efficiencies were consistent with what has been observed previously (Hitoshi *et al.*, 1996).

3.2.2 HC11 cell line transfection

A range of DNA amounts (2 μg to 10 μg per well of a 6-well culture flask) were used for calcium phosphate co-precipitation delivery of pCH110 to HC11 cells. At best, 0.9% of HC11 cells stained positive for β-galactosidase activity where 10 μg of pCH110 was delivered per well (data not shown).

The cationic lipids DOTAP and DMRIE-C were each combined with plasmid DNA at a range of ratios of lipid (2-10 μl): 1 μg pCH110, and delivered to HC11 cells. At best, a transfection efficiency of 0.3% was obtained with DOTAP at a ratio of 6:1 (μl DOTAP: μg pCH110). Higher concentrations of DOTAP were toxic causing cell death after 24 hours. The transfection efficiencies achieved with DMRIE-C were similar, with the maximal transfection efficiency being 0.2% using a ratio of 4:1 (μl DMRIE-C: μg pCH110) (data not shown). As these delivery methods were very inefficient at transfecting HC11 cells in these initial observations, they were not persevered with further.

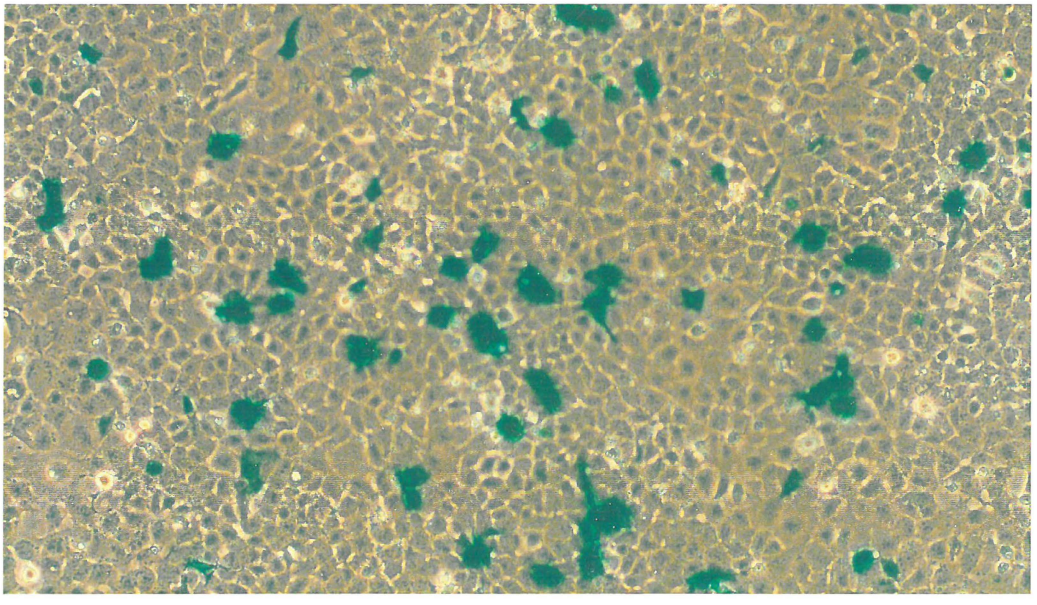
In addition, HC11 cells were transfected with the cationic lipid LipofectAMINE complexed to the plasmid pCH110. The transfection efficiency was optimised firstly by varying the ratio of LipofectAMINE: 1 μg pCH110 DNA. Transgene expression was quantified by the percentage of cells that stained positive for β-galactosidase *in situ* (*Figure 3.1A* and *Figure 3.2A*), or by the total amount of β-galactosidase enzyme activity (*Figure 3.2B*). Over a range of LipofectAMINE: DNA ratios, from 4 - 14 μl/μg, a maximal transfection efficiency of 0.8% was achieved with a ratio of 10:1 (μl LipofectAMINE: μg pCH110). At this ratio, β-galactosidase enzyme activity was also highest at 2.12 x 10^{-4} Units per microgram of protein (U/μg).

Using this fixed ratio of 10:1 (μl LipofectAMINE: μg pCH110), the amount of DNA and LipofectAMINE delivered per well (of a 6-well culture flask) was then altered. As shown in *Figure 3.3A*, 15 μl LipofectAMINE complexed with 1.5 μg pCH110 produced the greatest transfection efficiency of 2.1% and also the greatest enzyme activity of 3.83 x 10^{-4} Units β-galactosidase per microgram of protein (U/μg) (*Figure 3.3B*).

3.2.3 Primary bovine mammary cell transfection

Primary bovine mammary epithelial cells were difficult to establish and maintain in culture. A particular problem was contamination by fibroblast-like cells, which could not be avoided during isolation. Selective seeding and trypsinisation (described in Chapter Two) did initially provide a more homogeneous population of epithelial cells, but their maintenance proved difficult as fibroblasts characteristically grew faster than epithelial cells. Nevertheless, these primary bovine mammary cells were grown in 6-well culture flasks and then transfected with the plasmid pCH110 complexed to LipofectAMINE. The transfection efficiency was optimised by varying the ratio of LipofectAMINE: 1 μg pCH110 and transgene expression was quantified by the percentage of cells that stained positive for β-galactosidase expression *in situ*. It appears that both epithelial-like and fibroblast-like cells were transfected (*Figure 3.1B*). Over a range of LipofectAMINE: 1 μg pCH110 ratios from 2 - 12 μl/μg pCH110, maximal transfection efficiency of 0.3% was achieved with a ratio of 8:1 (μl LipofectAMINE: μg pCH110) (*Figure 3.4*). Concentrations of LipofectAMINE greater than 12 μl per well (of a 6-well culture flask) were toxic, causing cell death.

A



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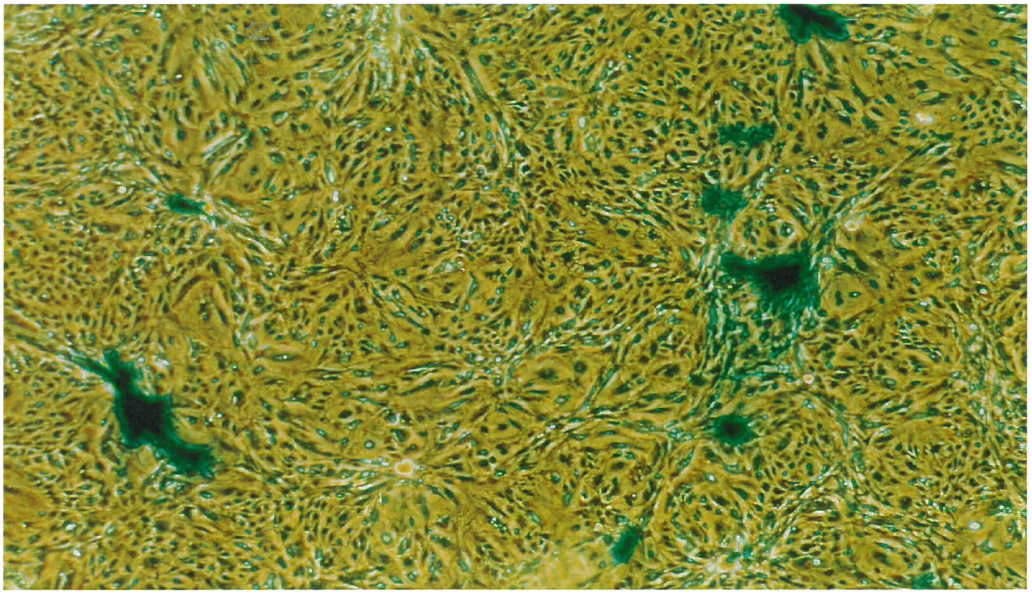
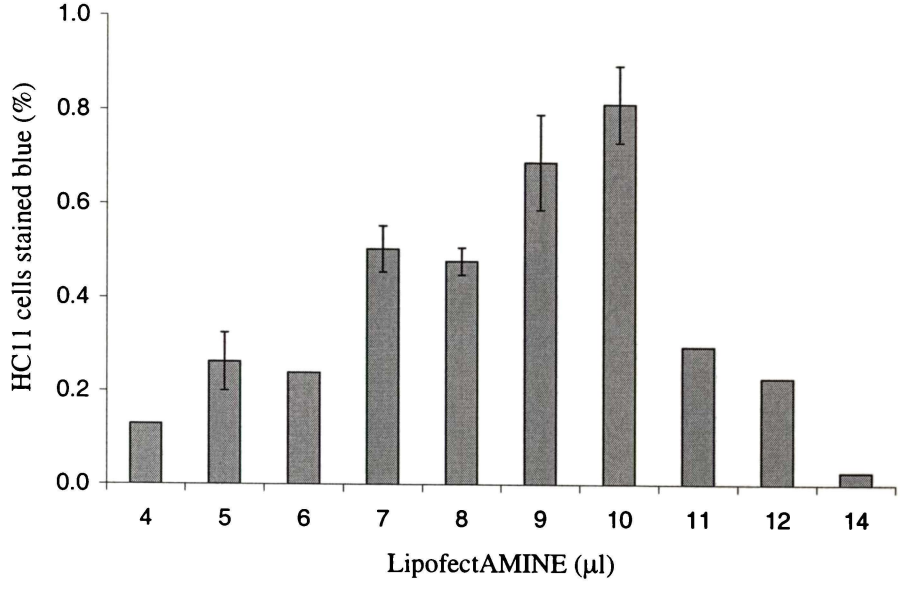


Figure 3.1 *In situ histochemical staining of cells for β-galactosidase expression.* (A) HC11 cells transfected with 15 µl of LipofectAMINE complexed to 1.5 µg pCH110. (B) Primary bovine mammary cells transfected with 8 µl of LipofectAMINE complexed to 1 µg pCH110. (40 × magnification).

A



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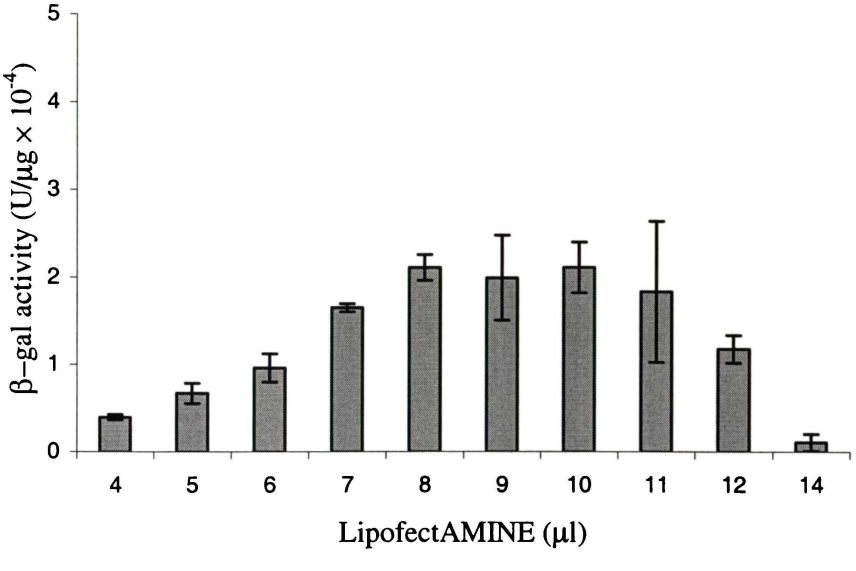
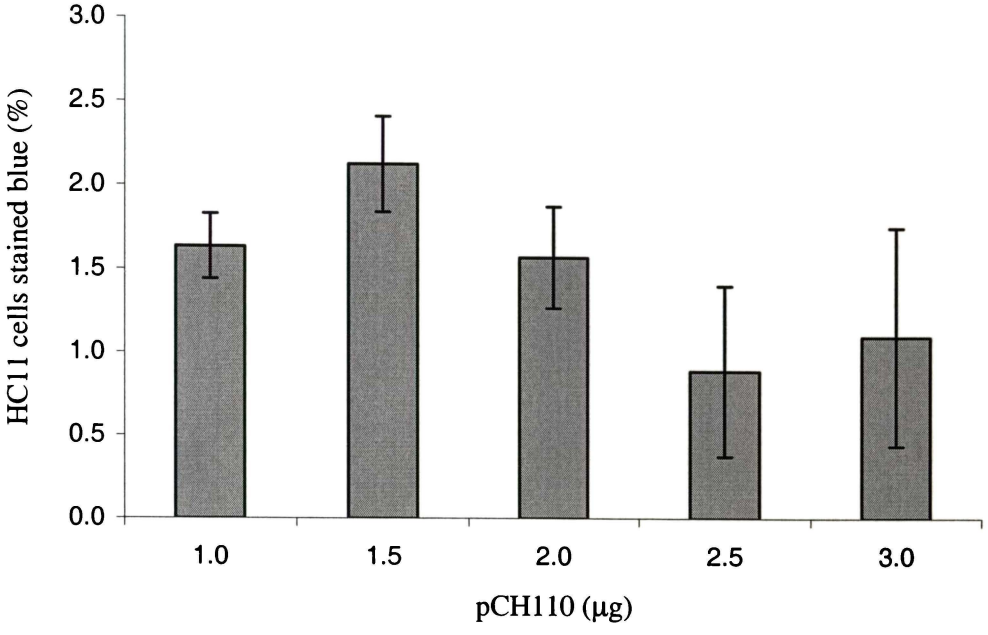


Figure 3.2 *Effect of cationic liposome amount on transfection of HC11 cells.* Cells were transfected with 1 µg pCH110 DNA and various amounts of LipofectAMINE then assayed 48 hours post-transfection. Cells expressing β-galactosidase were detected either by staining with X-gal to give the percentage of cells expressing the transgene (A), or by quantifying total β-galactosidase activity (U/µg) (B). (mean ± sem, n = 1-3 replicate experiments).

A



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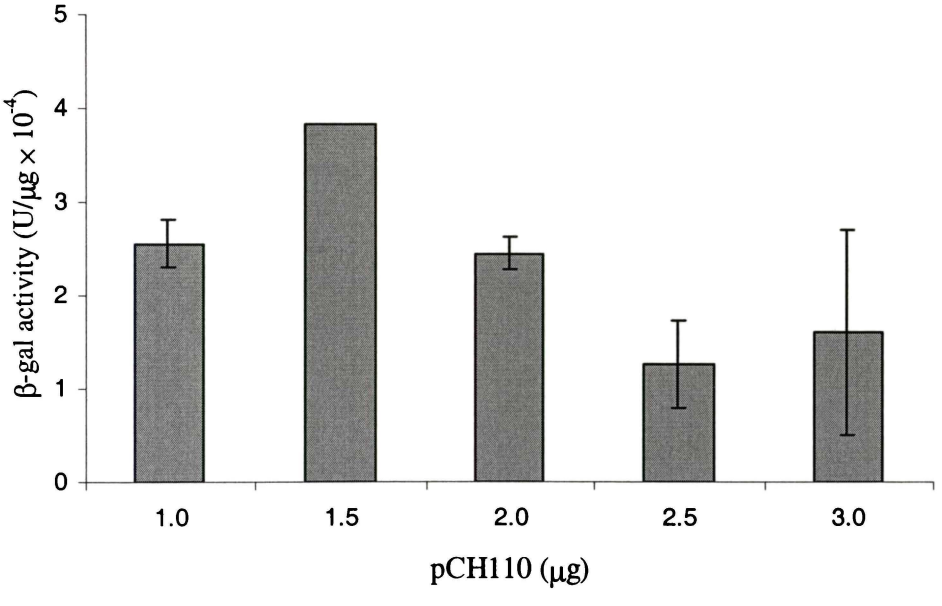


Figure 3.3 *Effect of the DNA quantity on transfection of HC11 cells.* Cells were transfected with various amounts of pCH110 DNA complexed to LipofectAMINE at a set ratio of 10:1, then assayed 48 hours post-transfection. Cells expressing β-galactosidase were detected either by staining with X-gal to give the percentage of cells expressing the transgene (A), or by quantifying total β-galactosidase activity (U/μg) (B). (mean ± sem, n = 1-3 replicate experiments).

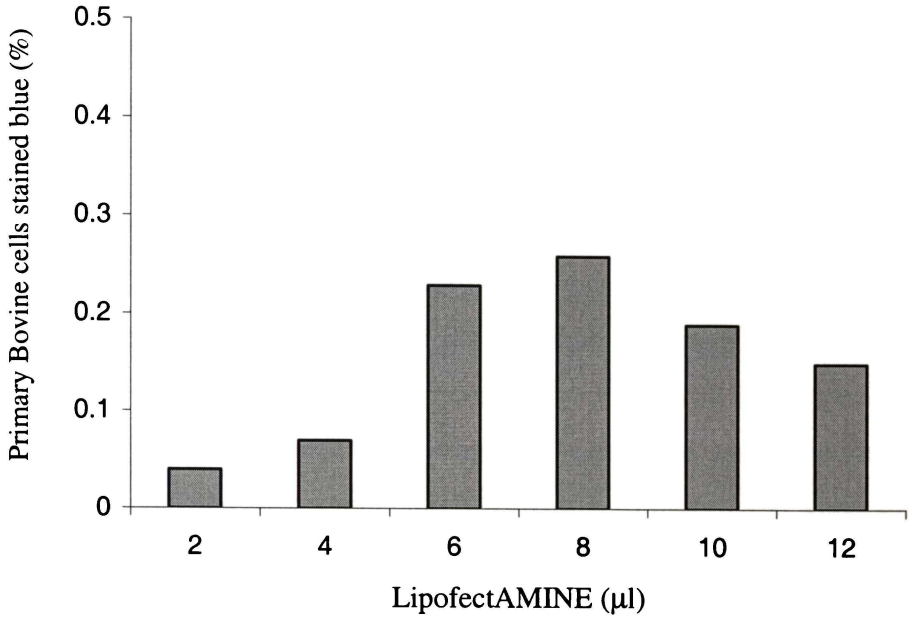


Figure 3.4 *Effect of cationic liposome amount on transfection of bovine primary mammary cells.* Cells were transfected with 1 µg pCH110 DNA and various amounts of LipofectAMINE. Cells were assayed 48 hours post-transfection to detect the percentage of cells expressing β-galactosidase by staining *in situ* with X-gal. (n = 1).

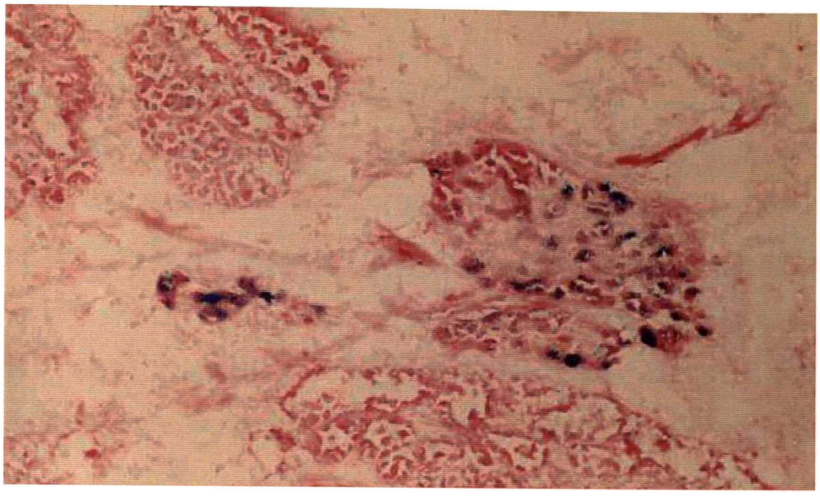
3.3 Rat mammary gland transfection in vivo

Lactating Sprague-Dawley laboratory rats were used in this work to assess the potential of liposome-mediated delivery of plasmid DNA to the mammary gland *in vivo*. LipofectAMINE was chosen because use of this reagent resulted in the highest transfection efficiency of HC11 cells *in vitro*. However, a different eukaryotic expression vector, pCS2-CMVlacZ, which contains the β-galactosidase gene driven by the immediate early cytomegalovirus (CMV) promoter, was used.

The optimal range of ratios and concentrations of LipofectAMINE and DNA applied *in vitro,* were utilised as an initial guide for *in vivo* delivery. Transfection complexes were prepared as for *in vitro* experiments with a range of ratios from 6:1 to 24:1 (μl LipofectAMINE: μg pCS2-CMVlacZ), where the amount of DNA was either 0.5 or 1 μg. These preparations were diluted into OptiMEM medium to a final volume of either 100 or 200 μl and delivered to the third or fourth mammary glands of a total of eleven lactating rats, ranging from day seven to seventeen of lactation. The delivery route was either specific infusion into the mammary gland via the teat canal using a blunt 32-gauge needle, or non-specific direct injection into the mammary gland through the wall of the gland. Two days after delivery of the transfection complex, rats were sacrificed and mammary tissue was isolated and immediately frozen. Cryosections were subsequently prepared, mounted on glass slides and stained *in situ* for β-galactosidase expression and subsequently counter-stained with eosin.

Positive staining for β-galactosidase was detected in a very low number of epithelial cells. This was observed in mammary gland tissue sections from only three out of the eleven animals utilised. These glands had 200 μl of the transfection complex containing 0.5 μg of pCS2-CMVlacZ complexed to 3, 6 or 12 μl LipofectAMINE, delivered either by teat infusion or direct injection. It was not possible to estimate the percentage of cells that had been successfully transfected, as positive β-galactosidase staining was very sparse. Therefore, transfection efficiency is best described as extremely low (*Figure 3.5*). Interestingly, all three of these animals, which gave positive β-galactosidase staining, were transfected on day 12 or earlier of lactation. Also, positive β-galactosidase expression from the same or adjacent cells was observed on serial cryosections, indicating that this positive signal was not a staining artefact (*Figure 3.5A and B*). Furthermore, no β-galactosidase expression was detected in tissue from mock-transfected glands.

A



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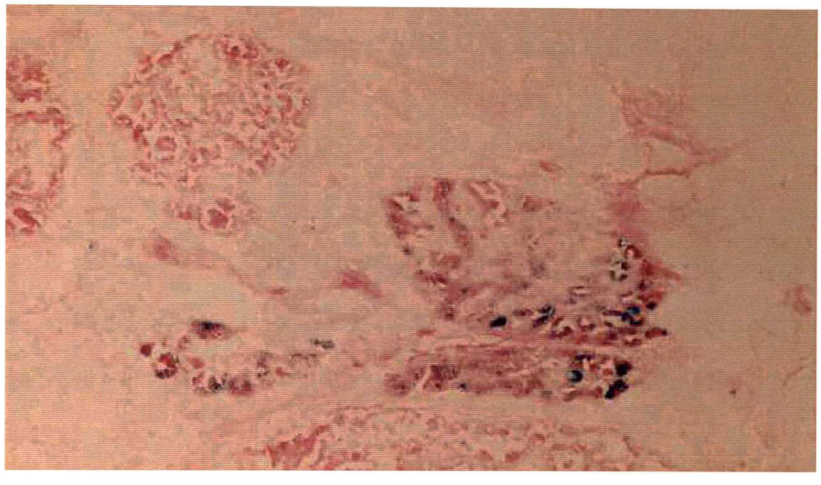

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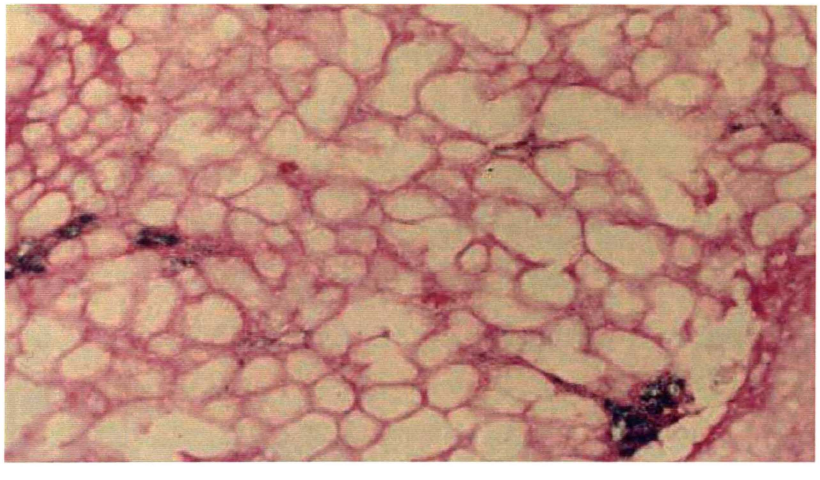


Figure 3.5 *In situ histochemical staining for β-galactosidase expression in rat mammary glands* transfected with 0.5 μg pCS2-CMVlacZ complexed to either 3 μl (A and B) or 12 μl (C) LipofectAMINE. The transfection complex was delivered either by infusion through the teat canal (A and B), or by direct injection into the gland (C). (250 × magnification).

3.4 Discussion

Of the chemical-mediated delivery techniques considered in this chapter, the cationic liposome, LipofectAMINE gave the highest transfection efficiency. Although the efficiency of transfection obtained with calcium phosphate co-precipitation was comparable to cationic liposome-mediated delivery, it was not subsequently used because, firstly, this method was not considered suitable for *in vivo* experiments involving direct delivery to the mammary gland because it is gravity-dependant (Keown *et al.*, 1990). Secondly, combined with its technical complexities, reproducible results often prove difficult to obtain, with variable results commonly due to slight changes in pH (Graham and van der Eb, 1973). Additionally, the amount of plasmid DNA required to achieve this level of transfection was high.

HC11 cells provided the best *in vitro* model to assess the potential of chemical-mediated delivery of plasmid DNA to mammary epithelial cells because they are a more homogeneous population of epithelial cells and are stable in culture, as opposed to primary bovine mammary cells which prove problematic to grow on a continuous basis.

Two conditions were optimised to attain maximal efficiency of transfection in HC11 cells: the ratio of LipofectAMINE to DNA and the overall concentrations of each respective component. The ratio of LipofectAMINE to DNA determines the overall charge and size of the complex. Interaction of the complex with the cell membrane, and subsequent uptake by endocytosis, depends on this charge normally being near neutral. However, different combinations of cationic liposomes and plasmid DNA have different charge ratios which are optimal for uptake by certain cell types (Tseng and Huang, 1998). The results in this chapter demonstrate that a broad range of both LipofectAMINE and plasmid DNA concentrations and ratios were effective at achieving transfection of HC11 cells. Furthermore, different ratios of LipofectAMINE to plasmid DNA were found to be optimal for transfecting the Neuro-2a and primary bovine mammary cell lines.

Several studies have shown that cellular entry is not the rate limiting molecular event of transfection (Zabner *et al.*, 1995; Tseng *et al.*, 1997). Other crucial events include, escape of the lipid-DNA complex from the endosome, dissociation of the DNA from the lipid, and entry of the DNA into the nucleus (Zabner, 1997). Differences in transfection efficiency between Neuro-2a (75%), HC11 (2.1%) primary bovine mammary cells (0.3%), and other cell lines reported elsewhere may reflect differences in the intracellular environment and also unique features of each cell line, which may subsequently impact on any one stage of the transfection process (Hawley-Nelson and Shih, 1993). It is unclear what stage of the transfection process results in the differences seen between cell lines in these experiments.

The level of transfection achieved with LipofectAMINE-mediated delivery of plasmid DNA to the rat mammary gland *in vivo* was extremely low. The reasons for this poor efficiency could be numerous. It may be an incompatibility problem with LipofectAMINE and the environment encountered in the mammary gland, leading to low transfection efficiency. Indeed, when a human growth hormone (hGH) reporter construct complexed to LipofectAMINE was delivered to the guinea pig mammary gland *in vivo* by infusion through the teat canal, no hGH expression was detected. However the same construct delivered with DEAE-dextran, did express hGH at detectable levels (Hens *et al.*, 2000).

Overall, the efficiency of transfection of mammary epithelial cells achieved both *in vitro* and particularly *in vivo*, in this chapter using cationic liposome-mediated delivery is low. This efficiency needs to be enhanced before this mode of delivery could be effectively used *in vivo* to modify somatic mammary epithelial cells for any chosen application.

Chapter Four

Transfection of mammary epithelial cells with AAV-based plasmid DNA

4.1 Introduction

The efficiency of transfection of HC11 cells *in vitro* achieved by delivery of plasmid DNA with the cationic liposome LipofectAMINE was poor. Furthermore, use of LipofectAMINE to deliver plasmid DNA to the rat mammary gland *in vivo* was also very inefficient. The aim of this chapter was to increase this transfection efficiency using HC11 cells in culture as a model.

One approach to potentially change cationic liposome-mediated transfection efficiency is to incorporate the inverted terminal repeats (ITRs) of adeno-associated virus (AAV) into the plasmid construct, so that the ITRs flank the expression cassette. This hybrid vector technique has previously been shown to enhance liposome-mediated transfection efficiency (Philip *et al.*, 1994; Vieweg *et al.*, 1995; Fan *et al.*, 1998).

To utilise this approach in these experiments, the ITRs of AAV were subcloned into a plasmid construct so that they flanked an expression cassette, which contained the β-galactosidase reporter gene driven by the immediate early cytomegalovirus (CMV) promoter. This construct (pITR-CMVlacZ) and the control construct (pCMVlacZ) which was identical except without ITRs, were then delivered to HC11 cells *in vitro* using LipofectAMINE. A detailed description of how the plasmids were constructed is outlined in Chapter Two.

Concurrently, an attempt was made to elucidate the mechanism by which the ITRs may enhance transfection efficiency. One hypothesis is that the ITRs may stabilise expression from the transfected plasmid by facilitating integration into the host genome. When wild type AAV infects human cells this integration occurs predominantly in a site-specific manner into the AAVS1 locus on chromosome 19 (Kotin *et al.*, 1990; Kotin *et al.*, 1991; Samulski *et al.*, 1991; Kotin *et al.*, 1992). A prerequisite for this site-specific integration is obviously the presence of the AAVS1 locus. Previously it has been shown by Southern analysis that the AAVS1 locus does not exist in any other species, apart from human and the green monkey (Samulski *et al.*, 1991). Mouse, rat, dog, cow, rabbit, chicken and yeast, along with human and green monkey genomic DNA

was screened with a 500 bp AAVS1 probe. However, more recently it was demonstrated using an *in vitro* model that a 33 nucleotide region of AAVS1 locus, which contains the terminal resolution site (trs) and Rep Binding Element (RBE), was the only requirement for site-specific integration to occur into this locus (Linden *et al.*, 1996a; Linden *et al.*, 1996b). Hence, in the present experiments an attempt was made to identify if any other species have sequence identity to this 33 nucleotide sequence.

4.2 Transient transfection of HC11 cells with AAV-based plasmid DNA

To assess the effect of ITRs on transfection efficiency, the two vectors created for this work (pCMVlacZ and pITR-CMVlacZ) were delivered to HC11 cells *in vitro* using the cationic liposome LipofectAMINE. The transfection conditions for HC11 cells as optimised in Chapter Three were used to deliver the plasmid DNA. HC11 cells, seeded in 6-well culture flasks, were transfected with 15 µl LipofectAMINE complexed to 1.5 µg plasmid DNA. Transfection efficiency was assessed 48 hours after plasmid DNA delivery to cells by fixing the cells *in situ* and histochemically staining with a solution containing X-gal to determine the percentage of cells which had expressed β-galactosidase. For each experimental treatment, duplicate wells were transfected. Data presented in *Table 4.1* represents the percentage of cells expressing β-galactosidase 48 hours post-transfection, in four separate experiments.

The average proportion of HC11 cells expressing β-galactosidase from pCMVlacZ 48 hours after delivery was 3.1%. This is a slight, but not significant, increase in transfection efficiency over that obtained with the pCH110 reporter construct, with the same parameters of DNA delivery, in which expression was detected in 2.1% of cells (Chapter Three). The pCH110 plasmid has the β-galactosidase reporter gene driven by the SV40 immediate early promoter, as opposed to the CMV promoter that drives the β-galactosidase reporter gene in pCMVlacZ.

Table 4.1 *Percentage of HC11 cells expressing β-galactosidase* 48 hours after LipofectAMINE-mediated transfection of an AAV-based plasmid with ITRs (pITR-CMVlacZ), compared to the control plasmid without ITRs (pCMVlacZ) (mean ± sem) (n = 4 replicate experiments).

Experiment	pCMVlacZ (%)	pITR-CMVlacZ (%)	Ratio
1	2.8	3.8	1.36
2	5.8	8.4	1.45
3	0.7	1.1	1.54
4	3.2	4.1	1.28
mean ± sem	3.1 ± 1.0	4.4 ± 1.5	1.41 ± 0.06

The CMV promoter drives constitutive expression in mammalian cells, and is regarded as a stronger promoter than the SV40 immediate early promoter, resulting in a greater level of expression. This may account for the observed increase in the number of cells in which expression was detected.

When HC11 cells were transfected with pITR-CMVlacZ, which has the β-galactosidase reporter cassette flanked by ITRs, the average number of cells expressing β-galactosidase 48 hours after delivery was 4.4%. This was a 1.4-fold increase over the control construct without flanking ITRs.

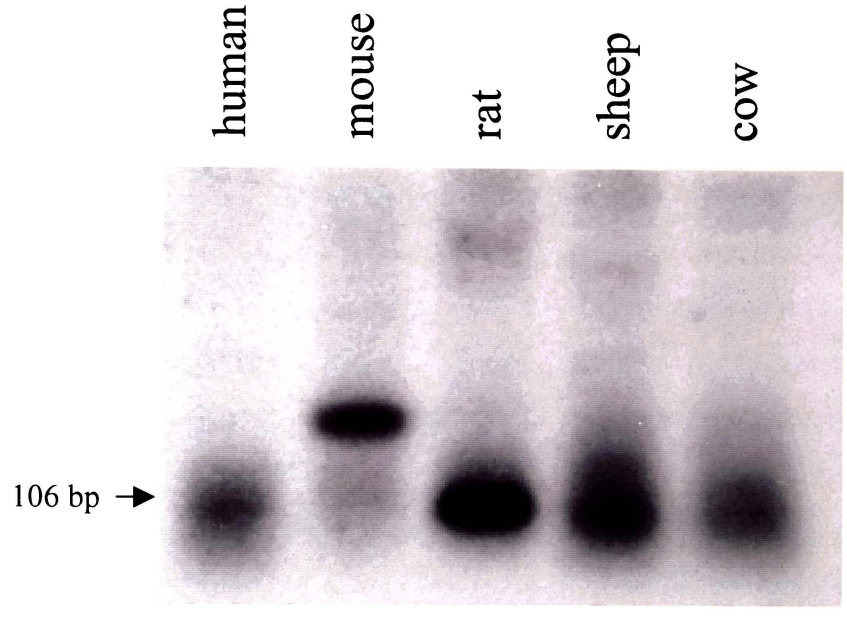
There was high variability in transfection efficiencies between the four replicates. There may be several reasons for this variability, which are discussed below. However, when the data from each replicate was converted to a ratio, representing the percentage of cells expressing β-galactosidase from the reporter plasmid with flanking ITRs (pITR-CMVlacZ) compared to the reporter plasmid without ITRs (pCMVlacZ), the increase was consistently 1.4-fold (1.41 ± 0.06).

4.3 AAVS1

Southern blot analysis was utilised to elucidate whether any other species had DNA sequence that is homologous to the minimal 33 nucleotide sequence of human AAVS1. Genomic DNA was isolated from mouse, rat, and sheep tissue, and human and cow blood. Ten micrograms of each genomic DNA was digested with *Hae*III, fractionated by electrophoresis on a 1% TAE agarose gel, transferred overnight to Hybond N+ membrane (Amersham) and hybridised to the AAVS1 oligonucleotide probe (*Figure 4.1A*). The sequence of the oligonucleotide probe was based on the region of human chromosome 19 that has been shown to be an essential requirement for site-specific integration to occur and which contains the consensus sequences for the trs and RBE (*Figure 4.1B*) (Linden *et al.*, 1996a). The AAVS1 oligonucleotide probe used in this chapter is 37 nucleotides in length and was based on the sequence of the human AAVS1 locus published in the GenBank database, accession number S51329 (nucleotides 384 to 420) (Kotin *et al.*, 1992). This is different to the oligonucleotide sequence published by Linden *et al.* (1996a), which is only 33 nucleotides. The difference exists in the region between the trs and the RBE. The GenBank sequence has seven extra nucleotides present between the trs and RBE recognition sites, whereas the 33-mer has three extra nucleotides 5' to the trs. The authors do not explain these differences in sequence. The probe was end-labelled with [α-^{32}P]dCTP and hybridised overnight at 60°C.

The expected fragment size for human genomic digested with *Hae*III was 106 bp. This band is identified in *Figure 4.1A* by an arrow. There was also fragments of similar size which hybridised to this probe present in rat, sheep and cow genomic DNA. Furthermore, there was a fragment of approximately 200 bp present in mouse genomic DNA. The resolution of the bands detected is poor because of the low percentage of agarose gel used for fractionation.

A



B

ggttggggctccgggcgcgtcgctcgctcgctcgctg

Figure 4.1 *(A) Southern blot analysis of 10 µg of genomic DNA from human, mouse, rat, sheep and cow, digested with Hae*III *and hybridised to the AAVS1 oligonucleotide probe. (B) Sequence of AAVS1 oligonucleotide probe.* The trs is underlined and the RBE is shaded.

4.4 Discussion

Inclusion of AAV ITRs within the plasmid DNA flanking the expression cassette enhanced the transfection efficiency into HC11 cells, albeit at a minimal level of 1.4-fold. In comparison, others have shown that the level of expression from constructs containing ITRs can be enhanced. Philip *et al.* (1994) transfected a rat prostate cell line using cationic liposomes complexed to plasmids containing the human interleukin-2 cDNA, either with or without AAV ITRs flanking the expression cassette. They recorded an increase of up to 10-fold more interleukin-2 protein secreted into the media from cells transfected with the construct containing ITRs as compared to the construct without ITRs. Likewise, Fan *et al.* (1998) observed increased levels of human apolipoprotein AI (apoA1) and lecithin-cholesterol acyltransferase (LCAT) protein expressed in mouse C2C12 muscle, or human embryonic kidney 293 cells, when transfected with a plasmid that had ITRs flanking the expression cassette, as opposed to a control construct without flanking ITRs. Up to 2.8- or 1.6-fold more apoA1, and up to 3.1- or 3.5-fold LCAT, was secreted into the culture media during the 48 hours following transfection of the C2C12 or 293 cells respectively. Neither of these studies quantified transfection efficiency as a percentage of cells that expressed detectable levels of the transgene for both the ITR containing plasmid and the control plasmid. Therefore, it is possible that the number of cells that had taken up and expressed the plasmid DNA was similar for both the ITR and control construct. This would suggest that the ITRs have enhanced gene expression from the plasmid and hence increased the amount of protein produced per cell. Alternatively, the amount of protein detected may have been enhanced by an increase in the total number of cells transfected. The authors suggest that this enhancement may be due to ITRs potentially facilitating nuclear entry and retention of the delivered plasmid DNA. A combination of both enhanced DNA delivery and expression is also possible. The mechanism by which the ITRs have caused an increase in transfection efficiency, as measured by the number of cells expressing detectable levels of the β-galactosidase reporter gene, in the experiments described in this chapter is unknown. The amount of β-galactosidase protein produced per cell was not quantified.

Variability in transfection efficiency can be the result of several factors. Excessive freeze/thaw cycling can lead to degradation of plasmid DNA, which may in turn reduce transfection efficiency. The results presented here, which were performed chronologically, are variable rather than just decreasing, suggesting that this is not the reason for variability in these experiments. Others have also observed large variations in transfection efficiencies. Boussif *et al.* (1995) transfected NIH3T3 or HepG2 cell lines with a luciferase reporter construct complexed with polyethylenimine. They noted that absolute values varied sometimes within an order of magnitude, depending on plasmid batch and the history of the cells, whereas relative values stayed within a factor of two. Additionally, NIH3T3 cells have been shown to have variable transfection efficiencies with different age of the culture. Cells that had either been freshly thawed, or in culture for 8 passages, before transfection with identical conditions resulted in a two-fold difference in efficiencies, with older cells being transfected less efficiently (Hawley-Nelson *et al.*, 1993). The same batch preparation of each plasmid was used for all experiments in this chapter. However, one possible reason for the observed variations may be the cell history. The passage number was maintained between 9 and 18 for all transfections, however the exact passage number for each experiment was not recorded.

Hybridisation products were identified in mouse, rat, sheep and cow genomic DNA as well as human DNA, when probed with the AAVS1 oligonucleotide. This suggested that sequence with identity to the minimal 33 nucleotide region of the human AAVS1 locus exist in these other species. However, the extent of this homology could not be determined from this result alone, and further analysis at the sequence level would be required to determine the exact identity of these possible homologous sites.

Chapter Five

Analysis of integration frequencies following AAV-based plasmid DNA transfection

5.1 Introduction

The results presented in Chapter Four indicated that the presence of AAV ITRs flanking a β-galactosidase expression cassette enhanced transient transfection efficiency of liposome-mediated plasmid DNA delivery to HC11 cells. It was also of interest to investigate whether the mechanism of transgene integration was altered due to the inclusion of ITRs in the construct. To assess whether the ITRs affect the stability of transgene expression, a selection regime was used whereby the neomycin resistance gene was inserted into an expression construct flanked by ITRs (pITR-neo-tk). The drug G418 was then used to identify the cells that expressed this selectable marker.

The frequency of integration was quantified by comparing the number of G418 resistant colonies that had formed from cells transfected with this construct (pITR-neo-tk) with the number of colonies resulting from cells transfected with a control neomycin construct without flanking ITRs (pneo-tk). Moreover, an AAV Rep78 and/or Rep68 protein expression construct (pHIVrep) (Antoni *et al.*, 1991) was co-transfected together with these neomycin constructs. The Rep78 and Rep68 AAV proteins have been extensively characterised and are essential for wild type AAV DNA replication (Labow and Berns, 1988). These Rep proteins are also essential for site-specific integration into the AAVS1 locus on human chromosome 19 (Surosky *et al.*, 1997). In the absence of Rep78 or Rep68 protein, DNA flanked by ITRs will still integrate into the host genome, but the integration is at random sites (Xiao *et al.*, 1997). Therefore, pHIVrep was included to assess whether Rep78 and/or Rep68 had any effect on integration and whether Rep-mediated site-specific integration occurred in these experiments.

The expression vector, pITR-neo-tk, was created for this work as was the control plasmid without ITRs, pneo-tk. A full description of their construction is given in Chapter Two. The vector pBR322 (which makes up the plasmid backbone of pHIVrep) was also co-transfected with the neomycin selection constructs as a negative control for pHIVrep (*Table 5.1*). Furthermore, the 0.5 μg of plasmid pCH110 was included in all transfections as a control for any variation in transfection efficiency.

Table 5.1 *Plasmid combinations transfected into cell lines for selection.*

	Plasmids		Summary
I	pneo-tk	pBR322	- ITR - *rep*
II	pITR-neo-tk	pBR322	+ ITR - *rep*
III	pITR-neo-tk	pHIVrep	+ ITR + *rep*
IV	pneo-tk	pHIVrep	- ITR + *rep*

Two cell lines were transfected with these plasmids; the mouse mammary epithelial cell line HC11 and the human embryonic kidney cell line HEK-293 (Graham *et al.*, 1977). HC11 cells were used as an *in vitro* model in this study for liposome-mediated transfection. HEK-293 cells are a human cell line in which site-specific integration into AAVS1 following AAV-mediated plasmid DNA transfection has previously been demonstrated and were included in these experiments as a positive control for such an event (Shelling and Smith, 1994; Pieroni *et al.*, 1998). LipofectAMINE was used to deliver the DNA using the optimised transfection conditions described in Chapter Three for HC11 cells, and as described by Life Technologies for HEK-293 cells (www.lifetechnologies.com). Transfection and selection procedures are outlined in Chapter Two.

The selection constructs created for this study with ITRs flanking the neomycin resistance gene also had the Herpes simplex virus thymidine kinase (HSV-*tk*) gene on the plasmid backbone. This allowed investigation into whether excision of the neomycin resistance gene from the transfected plasmid had occurred during integration. When selected with G418, colonies that express the neomycin resistance gene survive. When these colonies are subsequently selected with the drug FIAU, colonies that do not express the HSV-*tk* will survive. Hence this

positive-negative selection assay was aimed to identify colonies which have lost a functional copy of the HSV-*tk* gene from the plasmid backbone.

When selected with G418, each individual countable colony represents the successful transfection and subsequent stable integration and expression of the neomycin resistance gene in to a single cell that maintains its colony forming ability. If FIAU is included in the selective medium along with G418, colonies will only develop from single cells where the neomycin resistance gene and not the HSV-*tk* gene has been integrated. Raw colony numbers varied between replications due to different seeding dilutions and also because of variation in transfection efficiency. However, as these two parameters were constant within replicates, data was converted to ratios allowing comparison between experimental days.

For selection with G418, a value of 1.0 was used to represent the number of colonies resulting from transfection of the control plasmid combination pneo-tk + pBR322 (- ITR - *rep*). Ratios for the remaining three plasmid combinations were calculated by comparing the colony numbers for each, to the colony numbers for control plasmids. For selection with G418 and FIAU, ratios were calculated for each plasmid combination by comparing the number of colonies resulting from selection with G418 and FIAU to the number of colonies resulting from G418 selection only.

The minimum lethal dose of G418 was determined for both HC11 and HEK-293 cells. The G418 concentrations that resulted in zero percent cell survival are summarised in *Table 5.2*. At lower concentrations, cell survival was not visibly affected (data not shown). The G418 minimum lethal dose was 175 μg/ml and 250 μg/ml for HC11 and HEK-293 cells respectively.

Table 5.2 *G418 minimum lethal dose for HC11 and HEK-293 cells.* Survival of HC11 and HEK-293 cells after 14 days growth in the presence of G418 (μg/ml active concentration), expressed as a percentage of the total number of cells that survive (representative data from one of several experiments).

G418 (μg/ml)	HC11 (% survival)	HEK-293 (% survival)
125	30	95
150	5	50
175	0	25
200	-	10
225	-	5
250	-	0

A FIAU concentration of 0.2 μM was used to select HC11 and HEK-293 cells. This concentration was not toxic to wild type HC11 or HEK-293 cells, with no cell death observed over a period of 14 days growth. However, cells expressing thymidine kinase were killed at this concentration.

5.2 Transfection and selection of HC11 cells

HC11 cells were transfected with the four combinations of plasmid DNA as summarised in *Table 5.1*, using LipofectAMINE with conditions that had been optimised as described in Chapter Three. Cells were then cultured in selection media containing, either G418 (175 μg/ml) or G418 (175 μg/ml) and FIAU (0.2 μM), until discrete colonies were visible (14-20 days post-transfection). Colonies were counted and raw data was converted to ratios as described above. This transfection and selection regime was performed on four separate occasions.

The data presented in *Table 5.3* summarises colony number ratios following G418 selection. They suggest that when the neomycin resistance gene was flanked by ITRs (pITR-neo-tk + pBR322), the integration frequency was not significantly different from the control without ITRs (pneo-tk + pBR322) (P=0.87). Similarly, when the *rep* gene was co-transfected with the neomycin resistance gene, either flanked by ITRs (pITR-neo-tk + pHIVrep) or without flanking sequences (pneo-tk + pHIVrep), there was no significant difference in integration frequencies compared to the control (P=0.37 and 0.32 respectively).

The data presented in *Table 5.4* summarises colony number ratios following G418 and FIAU selection. All the colonies that grew when selected with G418 and FIAU have the neomycin resistance gene integrated into their genome, and only those colonies which do not have the HSV-*tk* gene integrated will grow. With the control plasmid combination (pneo-tk + pBR322) approximately 60% of integration events were of this kind. When the neomycin resistance gene flanked by ITRs (pITR-neo-tk) was co-transfected with the *rep* gene construct (pHIVrep) or its control (pBR322), the percentage of integration events of this type (where the HSV-*tk* gene is lost) was not significantly different from that of the control plasmid combination (P=0.57 and 0.70 respectively). Co-transfection of the neomycin resistance gene construct without flanking ITRs (pneo-tk), with the *rep* gene construct (pHIVrep) also resulted in no significant change in the percentage of integration events where the HSV-*tk* gene is lost, compared to the control plasmid combination (P=0.52).

Table 5.3 *Effect of ITRs and rep on the frequency of plasmid DNA integration into the HC11 genome.* Ratios represent the relative numbers of neomycin resistant HC11 colonies derived from transfection of plasmids containing various combinations of ITR and *rep*, compared to the control plasmid transfection (mean ± sem) (n = 4 replicate experiments).

	Experiment				
	1	2	3	4	mean ratio ± sem
pneo-tk + pBR322	1.0	1.0	1.0	1.0	1.0
pITR-neo-tk + pBR322	0.45	1.16	0.97	1.29	0.97 ± 0.19
pITR-neo-tk + pHIVrep	1.06	0.91	0.40	1.00	0.84 ± 0.15
pneo-tk + pHIVrep	1.16	0.77	0.46	0.92	0.83 ± 0.15

Table 5.4 *Effect of ITRs and rep on the nature of plasmid DNA integration into the HC11 genome.* Ratios represent the number of neomycin resistant/FIAU sensitive colonies relative to the total number of neomycin resistant colonies for HC11 cells transfected with each plasmid combination (mean ± sem) (n = 4 replicate experiments).

	Experiment				
	1	2	3	4	mean ratio ± sem
pneo-tk + pBR322	0.33	0.86	0.28	0.81	0.57 ± 0.15
pITR-neo-tk + pBR322	0.69	0.87	0.37	0.65	0.65 ± 0.10
pITR-neo-tk + pHIVrep	0.70	0.83	0.48	0.69	0.68 ± 0.07
pneo-tk + pHIVrep	0.45	1.22	0.44	0.83	0.74 ± 0.19

5.3 Transfection and selection of HEK-293 cells

HEK-293 cells were exposed to a similar experimental regime to that used for HC11 cells. They were transfected with the four combinations of plasmid DNA as summarised in *Table 5.1*, using LipofectAMINE with optimised conditions as described previously by Life Technologies GIBCOBRL. Cells were then selected with either G418 (250 μg/ml) alone, or G418 (250 μg/ml) plus FIAU (0.2 μM) until discrete colonies were visible (14-20 days post-transfection). Colonies were counted and raw data was converted to ratios as described above. This selection of HEK-293 cells was attempted five times but it was only possible to count colony numbers twice. Determining a dilution that allowed discrete colony formation was difficult. For the unsuccessful attempts, the cell densities reached confluence prior to completion of the selection regime hence it was not possible to differentiate individual resistant colonies for counting. In contrast, with sparse seeding, cell growth and subsequent colony formation was very slow.

The data presented in *Table 5.5* summarises colony number ratios following G418 selection and data in *Table 5.6* summarises colony number ratios following G418 and FIAU selection. There is high variation between the two replicates. Colony counts for the first set of recorded data were below 100 colonies per plate for G418 selection and less than 40 colonies per plate for G418 plus FIAU selection. Colony numbers were all greater than 200 per plate for the second replicate.

Table 5.5 *Effect of ITRs and rep on the frequency of plasmid DNA integration into the HEK-293 genome.* Ratios represent the relative numbers of neomycin resistant HEK-293 colonies derived from transfection of plasmids containing various combinations of ITR and *rep*, compared to the control plasmid transfection (mean ± sem) (n = 2 replicate experiments).

	Experiment		
	1	2	mean ratio ± sem
pneo-tk + pBR322	1.0	1.0	1.0
pITR-neo-tk + pBR322	1.00	1.18	1.09 ± 0.09
pITR-neo-tk + pHIVrep	2.50	0.76	1.63 ± 0.87
pneo-tk + pHIVrep	2.72	0.86	1.79 ± 0.93

Table 5.6 *Effect of ITRs and rep on the nature of plasmid DNA integration into the HEK-293 genome.* Ratios represent the number of neomycin resistant/FIAU sensitive colonies relative to the total number of neomycin resistant colonies for HEK-293 cells transfected with each plasmid combination (mean ± sem) (n = 2 replicate experiments).

	Experiment		
	1	2	mean ratio ± sem
pneo-tk + pBR322	0.31	0.60	0.46 ± 0.15
pITR-neo-tk + pBR322	0.50	0.51	0.51 ± 0.01
pITR-neo-tk + pHIVrep	0.19	0.73	0.46 ± 0.27
pneo-tk + pHIVrep	0.36	0.65	0.51 ± 0.15

5.4 Rep expression from transfected HC11 cells

Northern blot analysis was used to confirm that the pHIVrep construct was expressed in the transfected HC11 cells used in this study. Forty-eight hours following transfection with pHIVrep, total RNA was isolated and 15 μg was fractionated by formaldehyde/agarose gel electrophoresis, transferred to Hybond membrane and hybridised to a 508 bp probe specific to the *rep* coding sequence. The expected size of *rep* mRNA transcribed from pHIVrep plasmid is 3 kb. The arrow in *Figure 5.1* identifies this hybridisation band. There is extensive background hybridisation below 3 kb. The 28S and 18S rRNA bands were clearly visible on the ethidium bromide stained gel, which suggested that the RNA was not degraded. The background smear was attributed to contamination of the RNA with degraded plasmid DNA. The pHIVrep construct has been previously demonstrated by Northern blot analysis to be transcribed successfully in HEK-293 cells (Antoni *et al.*, 1991).

5.5 Discussion

Until the present study, the effect of ITRs and the Rep 78 and/or Rep68 protein on integration frequencies of AAV-based plasmid DNA into the mouse genome has not been thoroughly characterised. Analysis of the colony number ratios following transfection of HC11 cells with plasmids containing the neomycin resistance gene, with or without flanking ITRs, and selection with G418, indicated that the ITRs had no significant effect on the integration frequency of the neomycin resistance gene into mouse genomic DNA. This is in contrast with the findings of Vieweg *et al.* (1995). They transfected a rat prostate tumour cell line with constructs containing the neomycin resistance gene either without, or flanked by, ITRs and observed a "marked increase" increase in the number of G418 resistant colonies when the construct contained ITRs. However, it is difficult to make a direct comparison in their study between the ITR containing construct and controls without ITRs, as they were driven by different promoters, and exact numbers of colonies were not presented.

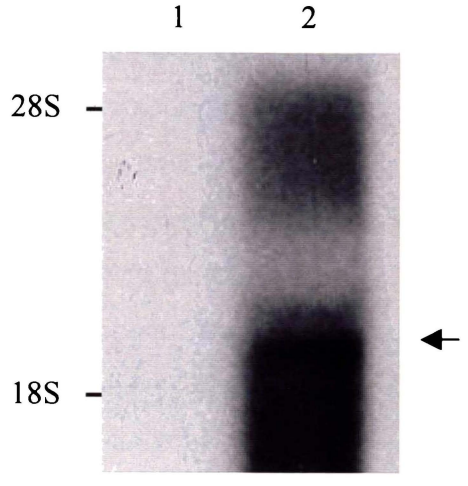


Figure 5.1 *Northern blot analysis of pHIVrep expression in HC11 cells.* Total RNA (15 μg), isolated from non-transfected (lane 1) or pHIVrep transfected (lane 2) HC11 cells, was fractionated by agarose gel electrophoresis, transferred to Hybond membrane and hybridised to a 508 bp *rep* specific probe. The position of the 28S and 18S rRNA bands are indicated. The arrow identifies the 3 kb *rep* mRNA.

Moreover, co-transfection in this study of a Rep78 and/or Rep68 protein expression plasmid with the plasmids containing the neomycin resistance gene, either with or without flanking ITRs, resulted in no significant change in the colony number ratios between those HC11 cells which received the *rep* plasmid and their respective controls. This suggests that Rep78 and/or Rep68 protein had no effect on the frequency of integration into HC11 cell genomic DNA. However, although expression from this *rep* construct was confirmed by Northern analysis, the presence of Rep78 and/or Rep68 protein was not confirmed. Therefore a role for the Rep78 and/or Rep68 protein for plasmid DNA integration into this mouse cell line cannot be excluded based on the data presented here. This could possibly be further elucidated by the addition of purified Rep78 and/or Rep68 protein at a range of concentrations directly to cells during transfection, followed by analysis of integration frequencies.

Unfortunately, the data presented in this study from transfection and selection of HEK-293 cells could not be analysed because of the high variation and low number of replicates. This is a limitation of this assay with HEK-293 cells because cell growth under selection with G418 is highly sensitive to seeding density.

Previous studies of liposome-mediated delivery of AAV-based plasmid DNA with HEK-293 cells have not attempted to investigate the effect of ITRs and *rep* on integration frequency as compared to a control. Rather, they have focussed on achieving long term expression and identifying the site of integration (Shelling and Smith, 1994; Pieroni *et al.*, 1998).

The second objective of the experiments described in this chapter was to assess whether ITRs flanking the neomycin resistance gene and/or the presence of *rep* leads to selective excision of this transgene from the plasmid backbone and integration into the HC11 cell genome. It was of interest to ascertain, firstly whether such an event may occur in this *in vitro* mouse model, and secondly, if so, whether there is specificity in the site of integration similar to what has been characterised in human cells (Surosky *et al.*, 1997).

In all examples of the different vector combinations co-transfected into HC11 cells (+/- ITR and +/- *rep*), approximately 60% of G418 resistant colonies were also FIAU resistant. Thus, in approximately one half of the integration events where the neomycin resistance gene is incorporated into the genome, a functional copy of the HSV-*tk* gene has also been integrated. This suggests that the integration event is random and not related to the presence of ITRs or *rep*. However, additional evidence, related to the molecular events involved in integration, is required before a conclusion can be drawn.

In a similar experiment, Tsunoda *et al.* (2000) reported a comparable proportion of colonies integrating both a selectable marker, which was flanked by ITRs, and a reporter gene cassette on the plasmid backbone. They transfected HeLa cells with a construct that contained the neomycin resistance and *rep* genes flanked by ITRs, and the β-galactosidase gene on the plasmid backbone. HeLa cells, which had integrated the neomycin resistance gene, were identified by selection with G418. From over four thousand G418 resistant colonies assayed, 51% also expressed β-galactosidase.

Tsunoda *et al.* (2000) also transfected HeLa cells with a plasmid containing the *rep* and neomycin resistance genes flanked by ITRs and a hygromycin resistance gene outside of the ITRs. They observed, by Southern analysis, that of the 22 neomycin resistant clones analysed where site-specific integration into AAVS1 was observed, 20 (91%) also had the plasmid backbone integrated, hence excision of DNA flanked by ITRs had not occurred. Furthermore, of these 22 neomycin resistant clones, 21 were also hygromycin resistant, confirming that a functional hygromycin resistance gene was present. These finding are also supported by the work of Surosky *et al.* (1997). They co-transfected a β-galactosidase reporter construct with flanking ITRs together with a *rep* expression construct into HEK-293 cells. Southern blot analysis determined that both the β-galactosidase gene and plasmid backbone sequences had integrated in six out of seven clones analysed where integration into AAVS1 was observed. In contrast, evidence also exists which suggests that excision of genes flanked by ITRs does occur following transfection of human cell lines. These other studies have demonstrated that the

plasmid backbone sequences were not integrated along with ITR flanked sequences site-specifically into AAVS1 (Shelling and Smith, 1994; Balague *et al.*, 1997; Pieroni *et al.*, 1998). These conflicting data suggests that ITR-mediated selective excision from plasmid DNA may be more complicated than initially suggested, and requires further investigation.

This study is the first to analyse the frequency of integration resulting from liposome-mediated delivery of AAV-based plasmid DNA versus non-AAV plasmid DNA. The results suggest that the ITRs or expression of the *rep* gene do not have any effect on the integration frequency or the nature of integration of the transgene into the HC11 (mouse) genome. There was high variation in the integration frequencies presented in this *in vitro* assay, with no clear differences or trends to indicate that the ITRs and/or *rep* were having any effect at the molecular level. This result may reflect a fundamental difference between the human and mouse genomes. Nevertheless, any enhancement of integration frequency would be beneficial to achieve longer-term expression from a transgene and further work can therefore be justified. Analysis with delivery of purified Rep78 and/or Rep68 protein could assist in understanding its effect on integration of AAV-based plasmid DNA into the genomic DNA of species other than human.

Since no significant differences were observed in the integration frequencies using this positive-negative selection assay, no assumptions about the site of integration could be made from this data. Southern blot analysis and sequencing of the integration junctions is required in order to determine if ITRs and/or *rep* have an effect on the site of transgene integration in the HC11 genome. These experiments are described in Chapter Six.

Chapter Six

Analysis of integration sites following AAV-based plasmid DNA transfection

6.1 Introduction

The results presented in Chapter Five indicated that the ITRs, or expression of the *rep* gene do not have a detectable effect on the frequency of integration into the HC11 (mouse) genome. The objective of this chapter was to analyse the effect ITRs and expression of the *rep* gene have on the site of integration into the mouse HC11 cell genome, and into the human HEK-293 cell genome.

To obtain information about the site of integration, two approaches were used. Firstly, Southern blot analyses were performed to confirm that the neomycin transgene was integrated into the genome and to identify any common hybridisation patterns amongst the different clones. Secondly, characterisation of the integration junctions was attempted by PCR amplification, subcloning and sequencing of these regions.

6.2 Southern blot analysis of plasmid integration into HC11 genomic DNA

HC11 cells were co-transfected with two different plasmid combinations, pneo-tk and pBR322 (-ITR - *rep*) or pITR-neo-tk and pHIVrep (+ITR + *rep*), and were then selected with G418 to identify the presence of the neomycin resistance gene. Several individual colonies were isolated and expanded as clonal cell lines. Genomic DNA was prepared from each clonal line as described in Chapter Two. This DNA was digested with *Hind*III, fractionated by agarose gel electrophoresis, transferred to Hybond N+ membrane and hybridised at 65°C to a 703 bp neomycin resistance gene probe. Digestion of genomic DNA with *Hind*III was chosen because no sites for this restriction endonuclease are present in either of the DNA constructs (pneo-tk or pITR-neo-tk). Thus, the neomycin specific probe will detect *Hind*III fragments encompassing integrated plasmid plus flanking genomic sequence.

Approximately 10 μg of genomic DNA from each of 12 individual clonal cell lines, transfected with pneo-tk and pBR322, was subjected to Southern analysis

(*Figure 6.1*). The neomycin specific probe hybridised to all clones (lanes 1-12). Hybridising bands ranged in size from 1.6 kb to greater than 12 kb (summarised in *Table 6.1*). Genomic DNA from non-transfected HC11 cells was also included (lane 14). No hybridisation to this control was detected.

Fifteen individual clonal lines transfected with pITR-neo-tk and pHIVrep were subjected to Southern analysis as described above (*Figure 6.2*). Neomycin probe hybridisation was detected with all clones, except 3a-15 (lane 15). Hybridisation bands ranged in size from 1.5 kb to greater than 12 kb (summarised in *Table 6.2*). No hybridisation to the HC11 genomic DNA control was detected (lane 16).

For bands greater than 12 kb, distinct bands could be resolved, such as in *Figure 6.2*, lanes 3 and 4, however accurate size estimation of these larger fragments was not possible on these gels. These bands probably ranged in size from 14 to 30 kb.

The unique pattern of hybridising bands shown by each individual clone in *Figures 6.1* and *6.2* can be interpreted to mean either that the site of integration is different for each clone, or that multiple copies (or part there of) have been integrated. The latter possibility would arise as a result of concatemers of head-to-head, head-to-tail, tail-to-tail or a combination of all three arrangements of all or part of the transfected plasmid. It cannot be determined from these results whether bands of similar size represent integration of the plasmid DNA into the same genomic site. While further Southern analysis with several different restriction endonuclease digestions might be expected to elucidate this, such experiments still tend to be inconclusive in practice.

Figure 6.1 *Southern blot analysis of G418 resistant clones derived from HC11 cells* co-transfected with the plasmids pneo-tk and pBR322 (lanes 1-12). Transfection and selection was carried out as described in Chapter Two. Ten μg of genomic DNA was digested with *Hind*III, fractionated on a 0.8% agarose gel, transferred to Hybond N+ membrane and hybridised at 65°C with a 703 bp neomycin gene specific probe. Lane 14 contains *Hind*III digested genomic DNA from non-transfected HC11 cells. Molecular size markers (M) are as shown (in kb).

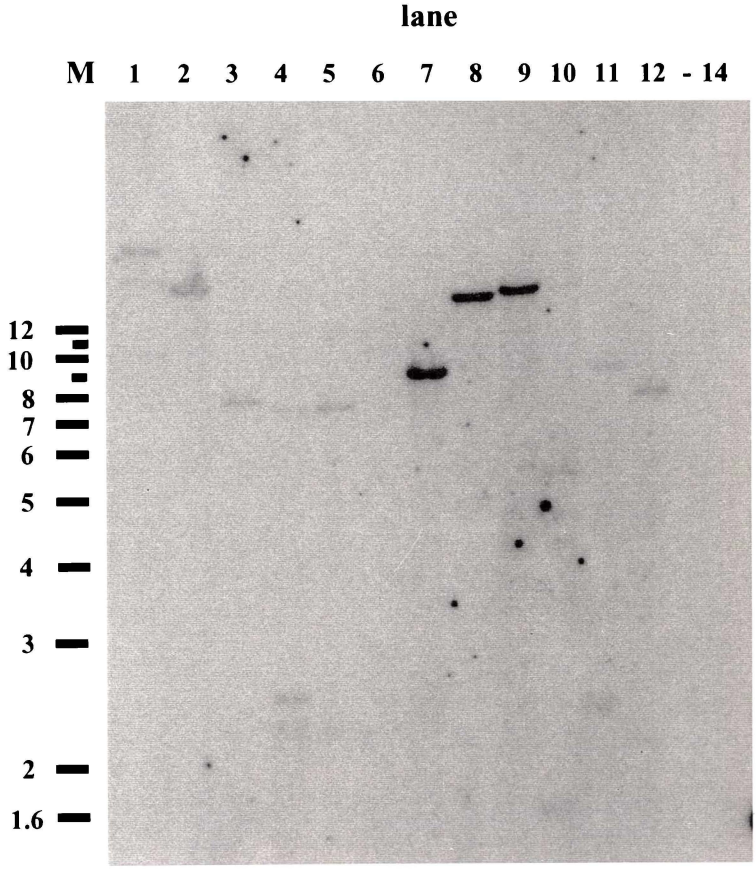


Figure 6.2 *Southern blot analysis of G418 resistant clones derived from HC11 cells* co-transfected with the plasmids pITR-neo-tk and pHIVrep (lanes 1-15). Transfection and selection was carried out as described in Chapter Two. Ten µg of genomic DNA was digested with *Hind*III, fractionated on a 0.8% agarose gel, transferred to Hybond N+ membrane and hybridised at 65°C with a 703 bp neomycin gene specific probe. Lane 16 contains *Hind*III digested genomic DNA from non-transfected HC11 cells. Molecular size markers (M) are as shown (in kb).

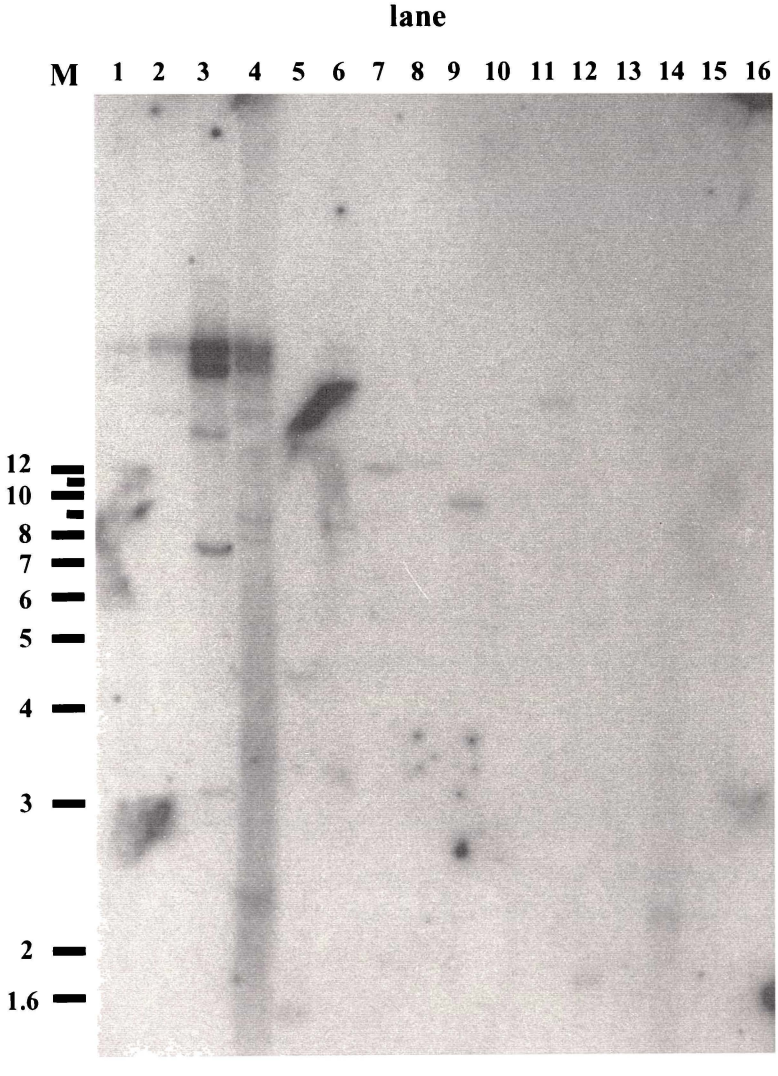


Table 6.1 *Size estimation of hybridisation products detected by Southern blot analysis of HC11 cells co-transfected with pneo-tk and pBR322.*

lane	Clone	Hybridisation band size (kb)
1	1-6	>12
2	1a-1	>12
3	1a-3	8.2
4	1a-5	7.8, 2.6, 2.4
5	1a-6	7.8
6	1a-7	11
7	1a-8	9.2
8	1a-10	>12
9	1a-11	>12
10	1a-12	5.7, 1.6
11	1a-14	9.5, 2.6
12	1a-15	8.5, 2.3
13	no DNA	-
14	HC11 (non-transfected)	-

Table 6.2 *Size estimation of hybridisation products detected by Southern blot analysis of HC11 cells co-transfected with pITR-neo-tk and pHIVrep.*

lane	Clone	Hybridisation band size (kb)
1	3-6	>12
2	3-10	>12 (2 bands)
3	3a-1	>12 (3 bands), 7.2, 3
4	3a-2	>12 (3 bands), 8, 2.4
5	3a-3	12, 4.5, 1.5
6	3a-4	8
7	3a-5	11
8	3a-7	12
9	3a-8	9.5
10	3a-10	>12, 10
11	3a-11	>12
12	3a-12	1.7
13	3a-13	>12
14	3a-14	2.3
15	3a-15	-
16	HC11 (non-transfected)	-

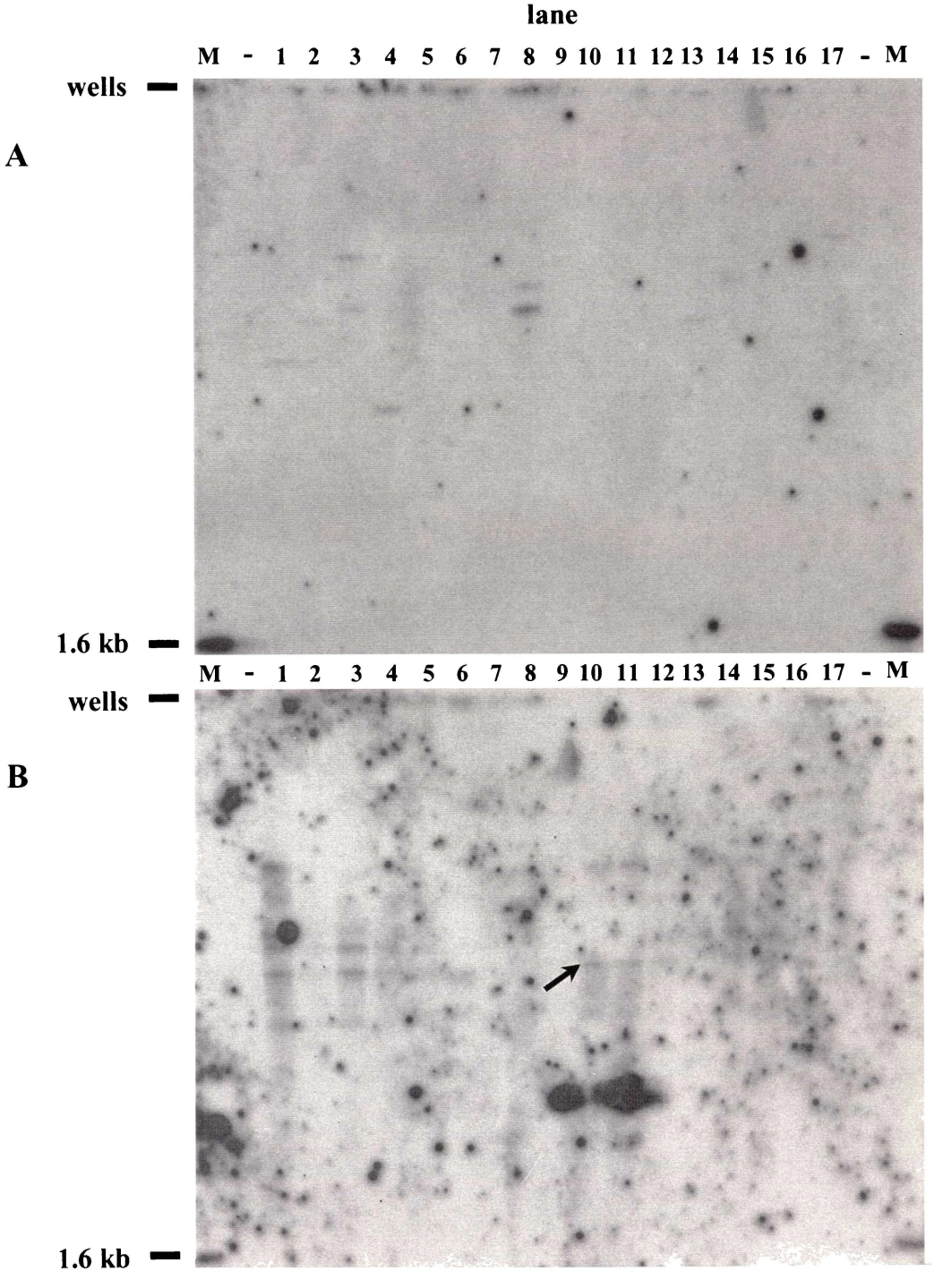
6.3 Southern blot analysis of plasmid integration into HEK-293 genomic DNA

HEK-293 cells were co-transfected with the same two plasmid combinations used to transfect HC11 cells; pneo-tk and pBR322 (-ITR - *rep*) or pITR-neo-tk and pHIVrep (+ITR + *rep*). Cells were then selected with G418 to identify the presence of the neomycin resistance gene. Several individual colonies were isolated and expanded as clonal cell lines. Genomic DNA was prepared from each clonal line as described in Chapter Two. Approximately 10 μg of genomic DNA was digested with *Hind*III, fractionated by agarose gel electrophoresis and transferred to Hybond N+ membrane for Southern hybridisation. Eight clonal lines transfected with pneo-tk and pBR322 (lanes 1-8), and seven clonal lines transfected with pITR-neo-tk and pHIVrep (lanes 11-17) were analysed. Genomic DNA from non-transfected HEK-293 cells was also analysed (lane 10) (*Figure 6.3*).

This membrane was hybridised at 65°C to a 703 bp neomycin resistance gene probe (*Figure 6.3A*). Hybridisation bands of a range of different sizes were detected in lanes 1, 2, 3, 4, 7, 8, 13, 14, 16 and 17. No hybridisation in lanes 5, 6, 11, 12 and 15, or the HEK-293 genomic DNA control in lane 10, was detected.

Subsequently, this membrane was stripped then hybridised at 65°C to a 623 bp human AAVS1 probe (*Figure 6.3B*). As *Hind*III does not have a restriction site within the 4067 bp published region of AAVS1 (GenBank Accession number S51329), the size of the expected hybridisation product was unknown. However, a specific band was present in the HEK-293 genomic DNA control (lane 10) (highlighted with an arrow in *Figure 6.3B*). This band was also detected in lanes 1, 2, 3, 4, 11, 12 and 14. Additionally, secondary hybridisation products, which were larger in size compared to the control band, were detected in lanes 1, 2, 3, 4 and 14.

Figure 6.3 *Southern blot analysis of G418 resistant clones derived from HEK-293 cells* co-transfected with the plasmids pneo-tk and pBR322 (lanes 1-8) or pITR-neo-tk and pHIVrep (lanes 11-17). Transfection and selection was carried out as described in Chapter Two. Ten µg of genomic DNA was digested with *Hind*III, fractionated on a 0.8% agarose gel, transferred to Hybond N+ membrane and hybridised at 65°C with either a 703 bp neomycin gene specific probe **(A)**, or a 623 bp human AAVS1 specific probe **(B)**. Lane 10 contains *Hind*III digested genomic DNA from non-transfected HEK-293 cells. Lane 9 had no DNA loaded.



6.4 *Amplification of integration junctions in HC11 clones by Siebert PCR*

The site of plasmid DNA integration into the HC11 genomic DNA was determined using a modified PCR technique based on the published method of Siebert *et al.* (1995). In this procedure flanking regions of integrated DNA are amplified, subcloned and sequenced. This method allows 'genome walking' into unknown regions adjacent to known sequence using a combination of 'vectorette' and 'suppression' PCR. It is based on 'adaptor' ligation and subsequent amplification using two rounds of PCR, firstly with adaptor specific and gene specific primers and, secondly, with nested primers to the same targets. This approach has the potential to reveal the specific site of integration.

Genomic DNA was digested with *Sca*I, and the 'adaptor' was ligated to the DNA ends. The *Sca*I restriction endonuclease was chosen because it is a six base cutter which generates blunt ends (important for blunt adaptor ligation) and no sites exist for it in either of the plasmids pneo-tk or pITR-neo-tk (confirmed by restriction endonuclease digestion and sequence analysis; data not shown).

Genomic DNA, isolated from clonal cell lines from each of the four different plasmid combinations transfected into HC11 cells described in *Table 5.1*, was used as template DNA for 'Siebert' PCR. In total, 12 clonal cell lines were used with three clones per plasmid combination. Two control reactions containing non-transfected HC11 genomic DNA were also included. (i) *Sca*I digested which had the adaptor ligated to it (H_A), and (ii) undigested HC11 genomic DNA (H_C).

PCR amplifications were performed as described in Chapter Two. Primary reactions were conducted with the adaptor primer AP1 and the gene specific primers neo3341 or neo3392. A secondary reaction was conducted with products from the primary PCR as template DNA using the adaptor primer AP2 and the nested gene specific primer neo3608. Products from primary and secondary PCR reactions were subjected to agarose gel electrophoresis, transferred to Hybond N+ membrane and hybridised to the neomycin specific oligonucleotide, neo3685.

6.4.1 Primary PCR amplification

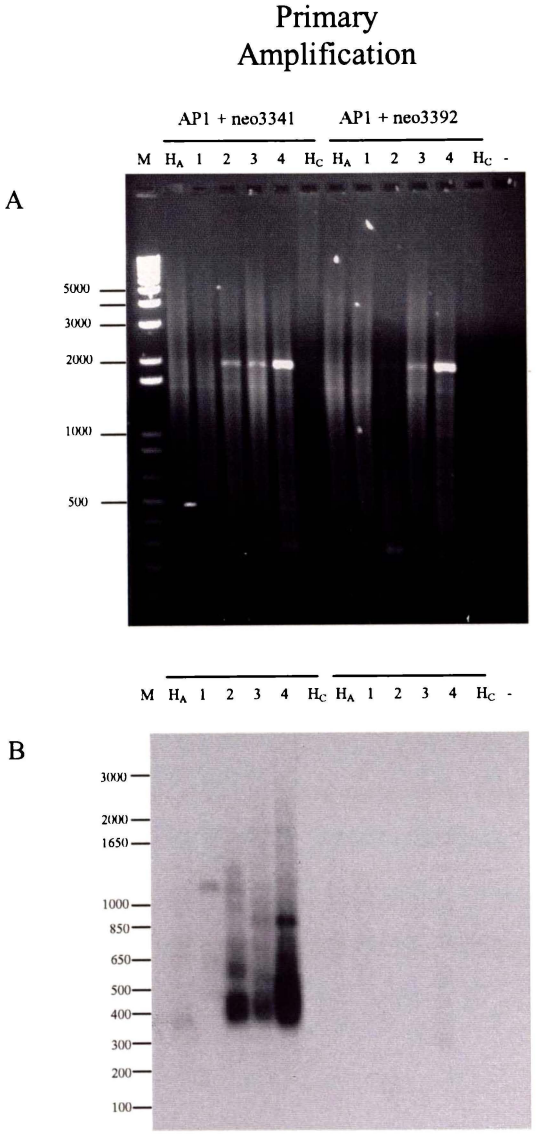
Four different templates were utilised for the first attempt at 'Siebert' PCR. They were HC11 clones 1b-1, 2b-10, 3b-3 and 4b-5 (designated the names *Sample 1, 2, 3 and 4* respectively). *Figure 6.4* shows the result from the primary round of amplification. As seen in panel A, a background smear from approximately 200 bp to greater than 5 kb was visible in all samples. This was produced by non-specific annealing of the primers to the template DNA. A distinct product of approximately 1.8 kb was clearly visible in *Samples 2, 3* and *4* amplified with AP1-neo3341 and in *Samples 3* and *4* amplified with AP1-neo3392 primer pairs respectively. This product was present in all four *Samples,* and HC11 DNA ligated to the adaptor (H_A), but not in the HC11 control DNA (H_C) (not visible in photograph). Another product of approximately 1.5 kb could also be seen in the same samples. As these bands were present in the control (H_A) sample, they were interpreted as non-specific artefacts (see discussion). No products were detected from amplification of the HC11 undigested control DNA (H_C) or the no DNA (-) control.

When transferred to Hybond N+ membrane and hybridised to the neomycin specific oligonucleotide neo3685, several hybridisation products were seen when the primary amplification was with AP1 and neo3341 primers. They ranged in size from approximately 400 bp to 1.2 kb in all four templates. Interestingly, the neo3685 oligonucleotide did not hybridise to any products from amplification with the AP1-neo3392 primer pair. Furthermore, no hybridisation products of 1.8 kb or 1.5 kb were seen, confirming that the bands seen in panel A were non-specific artefacts (*Figure 6.4B*).

6.4.2 Secondary PCR amplification

The primary PCR amplification products were diluted 100-fold in TE buffer and used as template DNA for the secondary amplification using the nested primers, AP2 and neo3608. All conditions were the same as for the primary reactions, except that the number of thermocycles was reduced from 35 to 20.

Figure 6.4 *Siebert primary PCR amplification products – Samples 1 - 4.*
(A) 1% agarose gel stained with ethidium bromide. (B) Agarose gel from panel A transferred to Hybond N+ membrane and hybridised to the neo3685 oligonucleotide. Genomic DNA from non-transfected HC11 cells (H_A), and G418 resistant HC11 clones 1b-1 (*Sample 1*), 2b-10 (*Sample 2*), 3b-3 (*Sample 3*), 4b-5 (*Sample 4*) were prepared for Siebert PCR by digestion with *Sca*I and ligation with the adaptor. These templates were then subjected to PCR using the methods described in Chapter Two. The primers AP1 and either neo3341, or neo3392 were used to amplify template DNA. Control reactions in lane "H_C" and "-" contain undigested HC11 genomic DNA and no DNA respectively. The DNA marker is the 1 kb plus ladder (Life Technologies).



Following agarose gel electrophoresis, a background smear was detected by ethidium bromide staining in all samples (except the negative controls "H_C" and "–"). Several PCR products specific to individual clones could also been seen (*Figure 6.5A*). Products of approximately 950 bp and 550 bp were distinctly visible in *Sample 1*, which was amplified initially with AP1 and neo3341. These two bands also hybridised to the neo3685 oligonucleotide, confirming that they contain plasmid sequences (*Figure 6.5B*). None of the other bands that were visible by ethidium bromide staining were detected by Southern hybridisation. The 950 bp and 550 bp products from *Sample 1* were subjected to further analysis.

A further eight templates were prepared for a second attempt at 'Siebert' PCR; HC11 clones 1a-1, 2a-1, 3a-3, 4a-1, 1b-2, 2b-8, 3b-4 and 4b-6 (designated the names *Sample 5* to *Sample 12* respectively). The AP1 and neo3341 primers were used for the primary amplification, and AP2 and neo3608 primers for the secondary amplification. A background smear was produced following primary amplification. Numerous specific products were produced however, following secondary amplification of the diluted primary products. They ranged in size from approximately 100 to 650 bp (*Figure 6.6A*). No products were detected from amplification of the HC11 undigested control DNA (H_C) or the no DNA (-) control. When transferred to Hybond N+ membrane and hybridised to the neomycin specific oligonucleotide neo3685, several of these secondary amplification products that were visible by ethidium bromide staining were also detected, indicating that they were neomycin specific (*Figure 6.6B*). Some non-specific hybridisation to the control HC11 DNA (lane H_A) was also seen.

Two specific hybridisation products were present in *Sample 11* of approximately 450 bp and 500 bp, however only the 450 bp product was clearly visible on the ethidium bromide stained agarose gel, and was subjected to further analysis.

Figure 6.5 *Siebert secondary PCR amplification products – Samples 1 - 4.* (A) 1% agarose gel stained with ethidium bromide. (B) Agarose gel from panel A transferred to Hybond N+ membrane and hybridised to the neo3685 oligonucleotide. Genomic DNA from non-transfected HC11 cells (H_A), and G418 resistant HC11 clones 1b-1 (*Sample 1*), 2b-10 (*Sample 2*), 3b-3 (*Sample 3*), 4b-5 (*Sample 4*) were prepared for Siebert PCR by digestion with *Sca*I and ligation with the adaptor. These templates were then subjected to PCR using the methods described in Chapter Two. Samples were initially amplified with the primers AP1 and neo3341 (lanes 3-6) or with the primers AP1 and neo3392 (lanes 9-12). Secondary amplification was with the primers AP2 and neo3608 (all lanes). Control reactions in lane "H_C" and "-" contain undigested HC11 genomic DNA and no DNA respectively. The DNA marker is the 100bp and 1kb ladder combined (New England Biolabs).

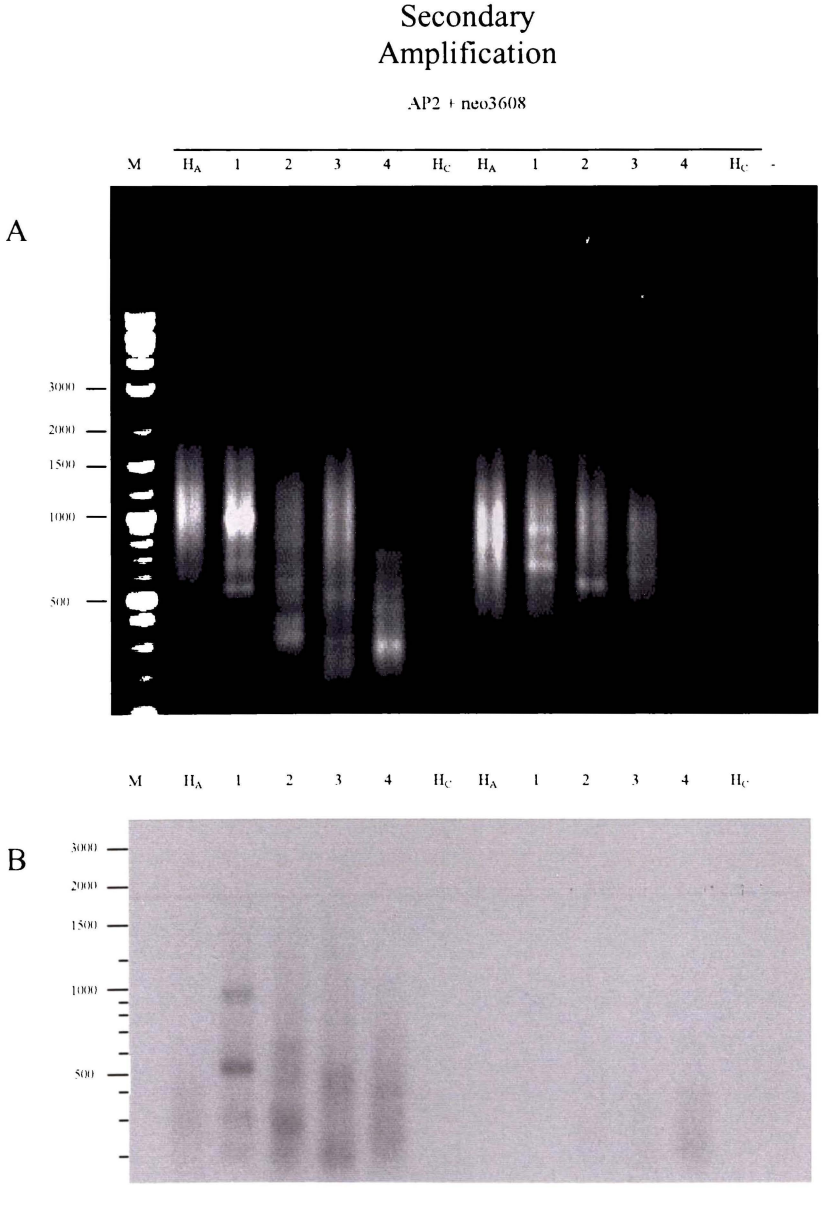
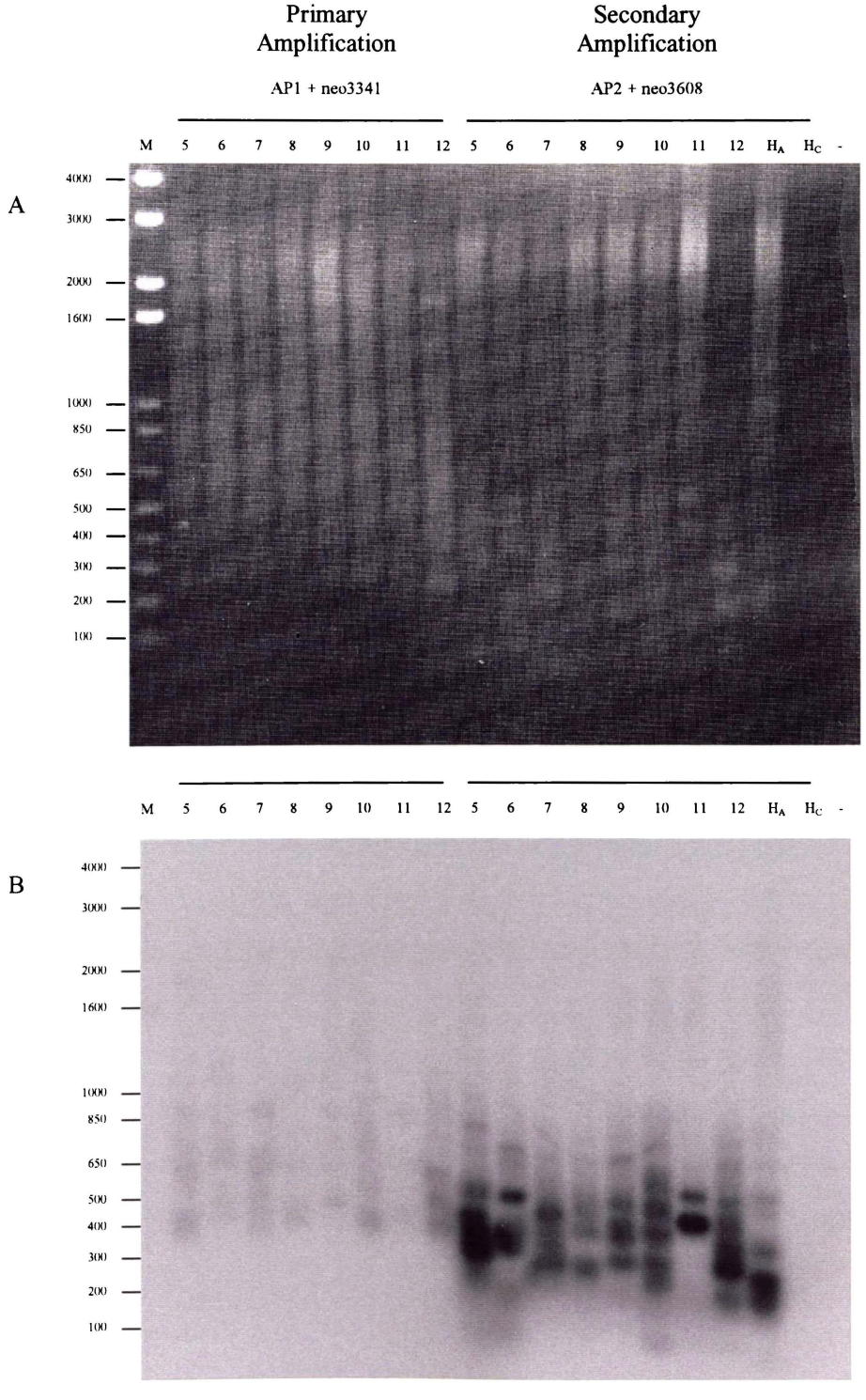


Figure 6.6 *Siebert primary and secondary PCR amplification products – Samples 5-12.* (A) 1% agarose gel stained with ethidium bromide. (B) Agarose gel from panel A transferred to Hybond N+ membrane and hybridised to the neo3685 oligonucleotide. Genomic DNA from non-transfected HC11 cells (H_A), and G418 resistant HC11 clones 1a-1 (*Sample 5*), 2a-1 (*Sample 6*), 3a-3 (*Sample 7*), 4a-1 (*Sample 8*), 1b-2 (*Sample 9*), 2b-8 (*Sample 10*), 3b-4 (*Sample 11*), 4b-6 (*Sample 12*) were prepared for Siebert PCR by digestion with *Sca*I and ligation with the adaptor. These templates were then subjected to PCR using the methods described in Chapter Two. The primers AP1 and neo3341 were used to amplify template DA for primary reactions. The primers AP2 and neo3608 were used for secondary amplification to amplify template DNA from PCR products from the primary amplification. Control reactions in lane "H_C" and "-" contain undigested HC11 genomic DNA and no DNA respectively. The DNA marker is the 1kb plus ladder (Life Technologies).



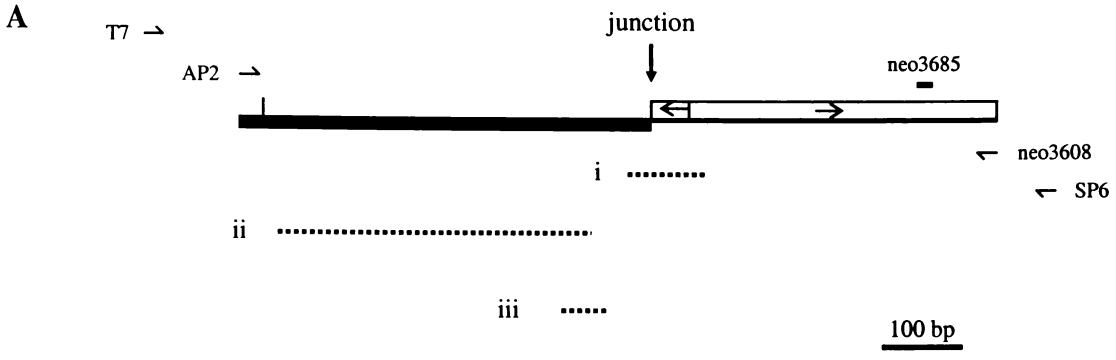
6.5 *Subcloning and sequencing of PCR products*

The amplification products from clones 1b-1 (*Sample 1*) (950 bp and 550 bp) and 3b-4 (*Sample 11*) (450 bp) were subcloned and sequenced. Each product was re-amplified by taking a 'stab' sample of each template band from the agarose gel following secondary amplification (described in Chapter Two). Stab amplification of the 450 bp product of clone 3b-4 also produced a secondary product of approximately 650 bp as well as the original 450 bp product. All products were confirmed as neomycin specific by hybridisation to the neo3685 oligonucleotide (data not shown) and were subcloned into pGEM-T Easy (Promega). Following transformation of XL1-blue cells, positive clones were identified for the 950 bp and 550bp 1b-1 products and the 650 bp 3b-4 product by restriction endonuclease digestion and hybridisation to the neo3685 oligonucleotide. No positive clones were identified however for the 450 bp 3b-4 product (data not shown). All clones were sequenced utilising either the T7 or the SP6 primer, which are external to the cloning site of pGEM-T Easy.

6.5.1 *Clone 1b-1*

Sequencing from both ends using the T7 and SP6 primers revealed that the larger clone was 939 bp in length. It consists of 30 bp of adaptor, 478 bp of genomic DNA and 431 bp of plasmid pneo-tk DNA sequence. Sequencing of the smaller clone revealed that it was 369 bp, of which 30 bp was the adaptor sequence and the remaining sequence was identical to the plasmid pneo-tk DNA (data not shown). This suggests that the adaptor had ligated to a site within the plasmid sequence. As there are no *Sca*I restriction sites within the plasmid sequence, either a new *Sca*I site has been created during the integration of the plasmid, or alternatively, an artificial blunt end was produced during DNA preparation.

There are three interesting features of the larger 939 bp clone (*Figure 6.7*). Firstly, a rearrangement of 40 bp (nucleotides 5128-5167 of pneo-tk) of the plasmid backbone had occurred at the junction between the genomic DNA and pneo-tk. This short piece of DNA had recombined in the opposite orientation.



B

(i) gcagaggcagagccactacaccacacaacaCCTGACGAGCATCACAAAA
ATCGACGCTCAAGTCAGAGGTGGCGGAGCCTATGGAAAAA
CGCCAGCAACGC

(ii) caatgcttcagaggcaagtatttgagctatttcttgattgataactgatatagtgagagagcccagataactgtgggca
atgccacctttgggcaagtggtcctggatgaaaagaaaaaaaagtaagccatgaggaacaagttattaagcagcct
taatggccttaattaatggtgaaatcacttaattaatggtgaaatcactttgggtctccaggtccctgttctgacttcatca
aggatggactatgctatagaaatacacaacaaaacaaacgcttttctccccaagtagcttttggtcatggtgttttatca
ttgaaacaggaaccctaagacatctataaccgccgggcaatggtggcacacgcctttaatcccagcacttgg

(iii) cgggcaatggtggcacacgcctttaatcccagcacttgggaggcggaggcagaggca

Figure 6.7 **(A)** *Schematic representation of clone 1b-1 (total length = 939 bp).* The integration junction is indicated with a vertical arrow between pneo-tk (box) and genomic DNA (solid box). Horizontal arrows within pneo-tk show plasmid orientation. The location of primers used for Siebert PCR (AP2 and neo3608), sequencing (T7 and SP6) and the oligonucleotide probe (neo3685) are as shown. Three fragments of this clone are marked with dashed lines (i, ii and iii) and the DNA sequence of these fragments is shown in panel B. **(B)** (i) Sequence flanking the integration junction. (ii) Exon 16 of the mouse gene for DNA dependent protein kinase catalytic subunit (100% identity). (iii) Mouse B1 repeat consensus sequence (81% identity). Genomic DNA sequence is in lowercase and plasmid DNA sequence is in uppercase. The wavy underline in B(i) indicates that the plasmid is in an inverted orientation.

Downstream from this site, the clone sequence had 100% identity to the pneo-tk sequence. Secondly, the 478 bp of genomic DNA was analysed by comparing the sequence to the NCBI mouse database using the BLAST search tool. Nucleotides 15 to 403 (389 bp) of this sequence have 100% identity with the 5' end of exon 16 of *Mus musculus* DNA for DNA dependent protein kinase catalytic subunit (nucleotides 392 to 4) (Accession number AB005160.1). Thirdly, nucleotides 365 to 421 of the genomic sequence are 81% homologous to nucleotides 1 to 57 of the mouse B1 repeat consensus sequence.

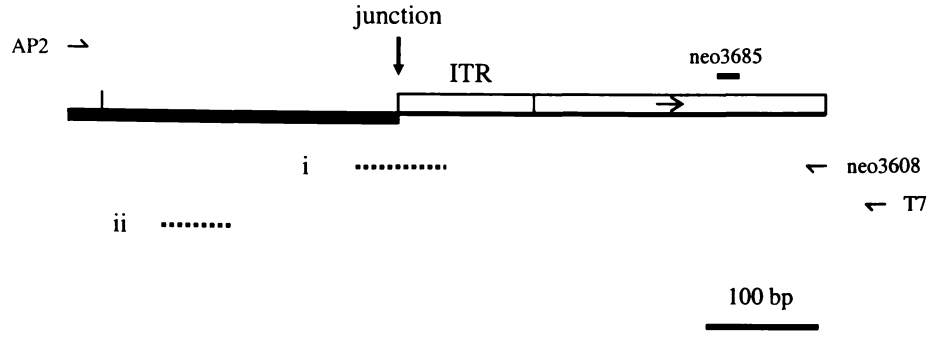
6.5.2 Clone 3b-4

The complete sequence of this clone was obtained with the T7 primer. This clone is 671 bp in length consisting of 30 bp of adaptor, 262 bp of genomic DNA and 379 bp of plasmid pITR-neo-tk DNA sequence (data not shown). *Figure 6.8* summarises the features of the sequence. The junction between genomic DNA and pITR-neo-tk occurs in the middle of the upstream ITR flanking the neomycin cassette (nucleotide 95 of pITR-neo-tk). Downstream from this site, the clone sequence is 100% homologous to the expected plasmid sequence. Analysis of the 262 bp of genomic sequence by comparison to the NCBI mouse database using the BLAST search tool revealed no overall homology to one particular sequence, but rather homology to a specific region within over 60 mouse sequences: again this specific sequence contained the mouse B1 repeat. Alignment of the B1 consensus sequence to the cloned sequence gave 73% homology over a 62 bp region (nucleotides 51 to 112 of the genomic sequence aligned to nucleotides 74 to 13 of the complement of mouse B1 repeat consensus sequence).

6.6 Amplification of integration junctions in HEK-293 clones by Siebert PCR

Genomic DNA isolated from 12 different HEK-293 clones was used as templates for Siebert PCR. The methods for amplification, screening, subcloning and sequencing of positive clones were identical to that used for HC11 clones. From the 12, sequence data was only obtained from two clones. These were clones 1a-1 and 2b-4. For both, the sequence was identical to plasmid DNA and adaptor

A



B

(i) tagtcttgggtgtctacaaataggttagtttttcctcagtGCCCGGCCTCAGTGAGCGAGCGAGCGCG
CAGAGAGGGAGT

(ii) gttcttaggctaagaaatntgcctgcttctgtctcccaggtactggaatgaaaagcatgcgc

Figure 6.8 **(A)** *Schematic representation of clone 3b-4 (total length = 671 bp).* The integration junction is indicated with a vertical arrow between pITR-neo-tk (box) and genomic DNA (solid box). Horizontal arrow within pITR-neo-tk shows plasmid orientation. The region of the plasmid encoding the ITR is labelled. The location of primers used for Siebert PCR (AP2 and neo3608), sequencing (T7) and the oligonucleotide probe (neo3685) are as shown. Two fragments of this clone are marked with dashed lines (i and ii) and the DNA sequence of these fragments is shown in panel B. **(B)** (i) Sequence flanking the integration junction (ii) Mouse B1 repeat consensus sequence (73% identity). Genomic DNA sequence is in lowercase and plasmid DNA sequence is in uppercase.

sequence only. No genomic DNA sequence was obtained (data not shown). This suggests that the adaptor had ligated to a site within the plasmid sequence. As there are no *Sca*I restriction sites within the plasmid sequence, either a new *Sca*I site has been created during the integration of the plasmid, or alternatively, an artificial blunt end was produced during DNA preparation.

6.7 Discussion

6.7.1 Southern blot analysis – HC11 cells

All of the HC11 clones analysed by Southern hybridisation in *Figures 6.1* and *6.2* had been selected with G418 for the presence of the neomycin resistance gene. They had not been selected with FIAU for loss of the thymidine kinase gene present on the plasmid backbone of pneo-tk and pITR-neo-tk; therefore it is possible that a complete copy of the respective plasmids may have integrated. The different hybridisation pattern shown by each clone suggests that the site of integration for each clone is unique. It is possible however that multiple copies have integrated into the same genomic location, as concatemers of head-to-head, head-to-tail or tail-to-tail arrangements of all or part of the transfected plasmid.

An interesting feature of these Southern blots is the presence of hybridisation bands smaller than the molecular size of a complete copy of the transfected plasmid DNA, suggesting that only part of the plasmid, including the neomycin resistance gene has been integrated. This supports the observation made in Chapter Five that, in approximately half of the integration events where the neomycin resistance gene was incorporated into the genome, a functional copy of the HSV-tk gene was not expressed. An alternative possibility is that transfected plasmid DNA has been maintained in an episomal state within cells, and that the size of the observed restriction fragments correspond to such DNA. Throughout the expansion of clones, selective pressure with G418 was maintained. Furthermore, DNA was prepared using a procedure that favours the isolation of high molecular weight DNA. These two factors make it unlikely that episomal plasmid DNA could either be present in cells, or detected by the procedures

described in this thesis, however it cannot be completely discounted. A further explanation may be that degradation of chromosomal DNA could have occurred during genomic DNA isolation, giving rise to smaller sized fragments than expected.

Four out of twelve clones which were transfected with pneo-tk and pBR322 (*Figure 6.1*), and five out of fifteen clones which were transfected with pITR-neo-tk and pHIVrep (*Figure 6.2*) had multiple bands hybridised to the neomycin probe, suggesting that these clones have more than one site within the genome where the neomycin transgene had integrated. Incomplete digestion of genomic DNA could alternatively lead to such a result, however this is unlikely as reaction conditions in these experiments were optimised for complete digestion, and analysis of ethidium bromide stained gels suggested that this had occurred (data not shown). Furthermore, it could not be determined from these results if bands of similar size represent the same genomic site where the plasmid DNA has integrated. The use of pulse field gel electrophoresis would allow better separation of restriction fragment greater than 12 kb and hence better size estimation of hybridisation products.

The higher signal intensity shown by clones 1a-8, 1a-10 and 1a-11 (lanes 7, 8 and 9 respectively) in *Figure 6.1*, and clones 3a-1 and 3a-2 (lanes 3 and 4) in *Figure 6.2*, can partly be explained by a greater amount of digested DNA loaded onto the agarose gels for these clones (data not shown). However it cannot be discounted that the transgene copy number in these clones may be greater than in other clones. Furthermore, hybridisation to the neomycin probe was not detected for clone 3a-15 (lane 15 of *Figure 6.2*). It is possible that single or very low copy number integration events had occurred in this clone, resulting in signal intensity below the detection level. Longer exposure of the hybridisation membrane to autoradiographic film did not improve signal intensities (data not shown). Although the copy number was not quantified, the sensitivity of the chosen method would allow identification of single copy integration events.

6.7.2 Southern blot analysis – HEK-293 cells

Hybridisation products specific to the neomycin gene probe were not observed in all clones analysed. The amount of digested DNA loaded onto the agarose gel was similar between samples (as confirmed by ethidium bromide staining). This suggested that the number of copies of the transgene, which have integrated into HEK-293 genomic DNA of different clones, may have been heterogeneous. Furthermore, the range of unique sized hybridisation bands obtained with the neomycin gene probe could result from differences in copy numbers of the transgene integrating at a single genomic site. Alternatively, the unique size range of hybridisation bands could result from integration into different genomic sites.

To assess if site-specific integration of the neomycin resistance gene into the AAVS1 locus had occurred in any of these clones, the same membrane was stripped and re-probed with an AAVS1 probe. Upon analysis of the two autoradiographs, only one clonal line derived from transfection with AAV-based plasmid DNA had hybridisation bands visible with both probes (lane 14). Furthermore, these hybridisation bands were of different size. This suggests that the neomycin resistance gene did not integrate into the AAVS1 locus of this clone. Site-specific integration into AAVS1 has been previously observed at frequencies of between 40% and 75% following transfection of HEK-293 with AAV-based plasmid DNA in the presence of Rep78 and/or Rep68 protein (Shelling and Smith, 1994; Balague *et al.*, 1997; Surosky *et al.*, 1997). Analysis of a greater number clones would be required to determine if site-specific integration into AAVS1 is occurring in the experiments described in this thesis.

6.7.3 Siebert PCR

The 'Siebert' PCR technique utilised in these experiments used adaptor-specific primers. Rather than just the desired adaptor-primed and transgene specific-primed PCR reaction products, adaptors ligated to both ends of the template DNA allowed amplification to proceed via the adaptor primer alone, resulting in creation of non-specific products. These products were seen as common bands

and also as a background smear in all samples, including the control HC11 DNA (H_A). This non-specific amplification can be avoided by attaching an amine group to the 3' end of the lower strand of the adaptor. This amine group stops non-specific amplification by blocking polymerase catalysed extension of the lower adaptor strand, preventing the generation of the primer binding site unless a defined, distal, gene-specific primer extends a DNA strand opposite the upper strand of the adaptor. However, while this modification could be beneficial, it is not essential (Siebert *et al.*, 1995).

In these present experiments, specificity was improved with a second round of amplification using the nested primer pair of AP2 (adaptor specific) and neo3608 (transgene specific). However non-specific AP2-AP2 amplification can still occur. Thus, it was essential to identify specific products by screening for hybridisation to the neomycin specific oligonucleotide neo3865 with Southern blot analysis.

Multiple specific products from the same DNA template can result from either multiple integration sites of the transgene into the genome or, alternatively, incomplete digestion of the genomic DNA with *Sca*I. In the experiments described here, when more than one specific product was detected in a single clone, the largest product was chosen to be subcloned and sequenced. Smaller products were not selected for subcloning because it was thought that larger candidates would reveal a greater amount of information about the site of integration.

6.7.4 Analysis of junctions

It has been demonstrated with sequence analysis of integration junctions, that liposome-mediated transfection of AAV-based plasmid DNA into human cell lines can result in site-specific integration into AAVS1 on human chromosome 19 (Surosky *et al.*, 1997; Lamartina *et al.*, 1998; Pieroni *et al.*, 1998; Rinaudo *et al.*, 2000; Tsunoda *et al.*, 2000). Recombinant AAV viral infection of human cell lines can also result in site-specific integration; however only a few researchers

have carried out detailed analysis of the integration junction at the DNA sequence level (Samulski *et al.*, 1991; Palombo *et al.*, 1998). Other researchers have obtained limited information on site-specific integration into AAVS1 from co-localisation of transgene and AAVS1 specific probes in Southern blot hybridisation (Young *et al.*, 2000). Alternatively, fluorescent *in situ* hybridisation methods have been used to demonstrate that integration sites co-localise to the expected chromosomal integration sites (Miao *et al.*, 1998; Palombo *et al.*, 1998).

Research presented in this thesis identified that genomic DNA from other species, including mouse, have homology to a small 37 nucleotide region of the human AAVS1 locus. However, this evidence alone is not conclusive enough to allow assumptions about potential AAV-mediated site-specific integration into these loci.

In a study by Nakai *et al.* (1999), a recombinant AAV (rAAV) vector expressing human coagulation factor IX was administered to mice via the portal vein. Sustained expression of the transgene was detected in the liver for up to 10 months. Furthermore, analysis by Southern blot suggested that the rAAV vector had integrated into the host genome, forming mainly head-to-tail concatemers. They isolated rAAV vector-cellular DNA junctions from 18 clones. Sequencing of these junctions revealed homology to four different mouse genes in four separate clones, with the remainder of clones having no apparent homology to any known mouse sequence (NCBI GenBank database). Of the four known genes, no sequence identity to any features of AAVS1 was present. This suggested that the integration of recombinant AAV DNA into the mouse genome in this study was occurring randomly.

Given the paucity of published data, the results from this chapter describing analysis of integration at the DNA sequence level of AAV-based plasmid DNA into mouse cells, do present some interesting new information.

Clone 1b-1 was isolated from a G418 resistant clone (1b-1), derived from HC11 cells co-transfected with the pneo-tk and pBR322. These plasmids do not contain any AAV sequence and were included as negative controls for analysis of

integration frequency (Chapter Five). The integration of the neomycin gene into the host genome should therefore be random. Alignment of clone 1b-1 genomic DNA to the NCBI mouse database using the BLAST search tool revealed that nucleotides 15 to 403 (389 bp) had 100% identity with the 5' end of exon 16 of *Mus musculus* DNA for the DNA dependent protein kinase catalytic subunit (nucleotides 392 to 4) (Accession number AB005160.1).

Rep78 and/or Rep68 protein has been shown to be essential for site-specific integration into the AAVS1 site on human chromosome 19 (Surosky *et al.*, 1997). In the absence of Rep78 and/or Rep68, DNA flanked by ITRs will still integrate, but randomly into the human genome (Xiao *et al.*, 1997). Linden *et al.* (1996a) showed that a 33 nucleotide region of AAVS1 containing a Rep Binding Element (RBE) and terminal resolution site (trs) was the only sequence required for specific targeting for AAV virus integration *in vitro*. However, it remains to be determined whether this sequence alone is sufficient for targeting in a chromosomal locus *in vivo*. Two groups have produced transgenic mice by inserting a 2.7 kb or a 3.5 kb AAVS1 fragment from human chromosome 19. Both transgenic sequences have RBE and trs present. Site-specific integration into these loci was observed with AAV-based plasmid transfection (Rizzuto *et al.*, 1999a) or with rAAV infection (Young *et al.*, 2000).

In the present experiments, Clone 3b-4 was isolated from a G418 resistant clone (3b-4) derived from HC11 cells co-transfected with the pITR-neo-tk and pHIVrep. No RBE-like sequences were identified in clone 3b-4 genomic sequence immediately upstream of the junction. However, it could not be determined from these data whether or not any RBE-like sequences exist at a greater distance from the junction site in this clone.

For the human cell lines analysed to date, the location of the junction sites have all been observed within the ITR of the AAV vector for both transfected (Surosky *et al.*, 1997; Lamartina *et al.*, 1998; Pieroni *et al.*, 1998; Rinaudo *et al.*, 2000; Tsunoda *et al.*, 2000) and infected (Samulski *et al.*, 1991; Palombo *et al.*, 1998) cells. In contrast, Nakai *et al.* (1999) infected mice *in vivo* with a rAAV vector and found that in 14 out of 18 integration events analysed, the junction sites were

within the ITR. Of the remaining four, the junction was in close proximity, not more than 45 bp from the end of the ITR. The exact location of junction sites within the ITR was different in all integration events that they analysed. The junction site in clone 3b-4 from the experiments described in this work occurs at a unique position, 65 bp from the 5' end of the ITR (*Figure 6.8*). Analysis of junction sites from a greater number of clones may help elucidate whether or not this location within the ITR is important for integration in these present experiments.

6.7.5 B1 homology

A notable feature of both clones 1b-1 (*Sample 1*) and 3b-4 (*Sample 11*) is homology of genomic sequence in the integration region to the sequence of the mouse B1 repeat. The mouse B1 repeat sequence belongs to the short interspersed element (SINE) family of repeats which are present in all higher eukaryotes (Jelinek and Schmid, 1982). The 129 bp B1 repeat is estimated to be present in between 130,000 and 150,000 copies in the murine genome (Bennett *et al.*, 1984) and is frequently located at intron-exon junctions within genes (Krayev *et al.*, 1980). The function of B1 is unknown. The B1 consensus sequence is homologous to the human *Alu* repeat (Jelinek and Schmid, 1982).

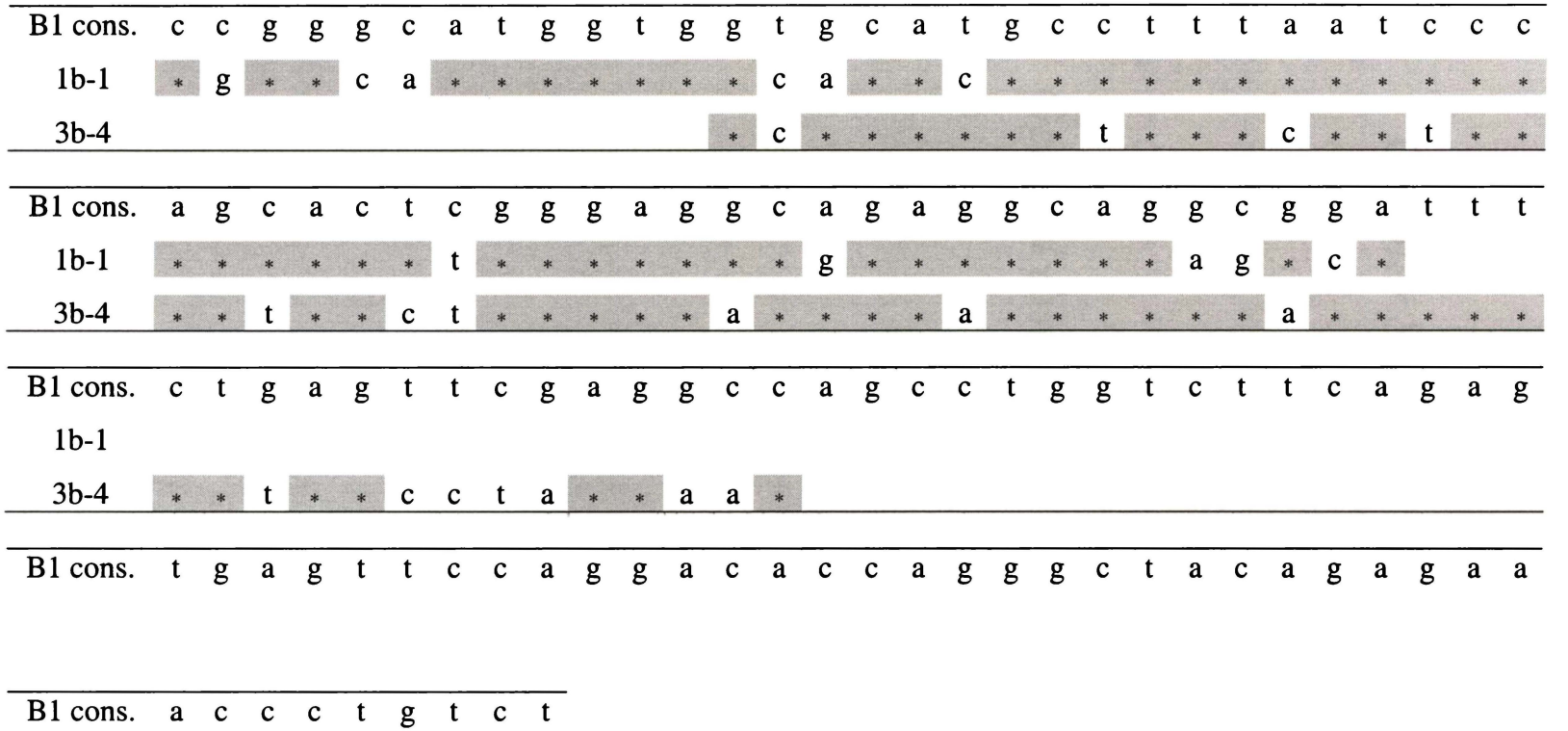
Clone 1b-1 has 81% homology over 57 bp (nucleotides 365 to 421) and clone 3b-4 has 73% homology over 62 bp (nucleotides 51 to 112) to B1. The regions of homology overlap, and are predominantly at the 5' end of B1 consensus sequence (*Figure 6.9*).

As clone 1b-1 was derived from cells initially transfected with plasmids containing no AAV features (pneo-tk and pBR322), the integration site of the neomycin transgene into the HC11 genome is assumed to be random. Kang *et al.* (2000) demonstrated that an increase in the frequency of transgene integration could be achieved by homologous recombination of a construct containing flanking B1 repeat sequences with genomic B1 sequences. However, as neither

pneo-tk, nor pITR-neo-tk contain B1-like sequence, homologous recombination is unlikely to be the mode of integration in these present experiments.

Overall, the PCR technique used in these experiments was successful at amplification of junction regions between the transfected plasmid DNA and the genomic DNA at the site of integration. However, the efficiency of amplifying unknown genomic DNA sequence, and not just plasmid DNA sequence was poor. This could possibly be improved by including a 3' amine group on the adaptor, reducing the number of non-specific amplification products.

Figure 6.9 *Alignment of the 129 bp mouse B1 consensus sequence* to clones 1b-1 (nucleotides 365 to 421) and 3b-4 (nucleotides 112 to 51). An asterisk (*) indicates exact identity to the B1 consensus (B1 cons.) sequence. Differences between the 1b-1 and 3b-4 sequences and the B1 consensus are as indicated.



Chapter Seven

General discussion

7.1 Mammary epithelial cell transfection in vitro

The overall goal of this work was to express foreign genes in mammary epithelial cells by chemical-mediated delivery of plasmid DNA. At the commencement of this study, cationic liposome-mediated delivery of plasmid DNA was the most promising method available to achieve this goal. To assess the efficacy of this approach and to identify a suitable cationic liposome, an *in vitro* model was established. Both the mouse mammary epithelial cell line HC11 and primary cultures of bovine mammary cells were employed.

LipofectAMINE, which consists of a 3:1 ratio of the polycationic lipid DOSPA and the neutral lipid DOPE respectively, was chosen to be used because of evidence suggesting that it was the most effective commercial liposome available at the time. It was shown to provide higher efficiencies of transfection in several different experiments with different cell lines, as compared to earlier generation cationic liposomes (Hawley-Nelson and Shih, 1993). In experiments reported in this thesis, the efficiency of plasmid DNA delivery to HC11 cells with LipofectAMINE was greater than that achieved with any of the other cationic lipids tested. In comparison, although calcium phosphate co-precipitation attained a similar level of efficiency, it was not persevered with as a viable option for DNA delivery because of its non-practicality for use *in vivo*. LipofectAMINE was subsequently used for all cell transfections *in vitro* and *in vivo* in this study.

In order to optimise the efficiency of LipofectAMINE-mediated plasmid DNA delivery to HC11 cells and primary bovine mammary cells, the transfection parameters were refined. Specifically, the ratio of LipofectAMINE to DNA, and the overall concentrations of each of these respective components was altered to achieve the maximal expression of a β-galactosidase reporter gene. The greatest percentage of cells that were detected to express β-galactosidase 48 hours post-transfection was 2.1% for HC11 cells, and 0.3% for primary bovine mammary cells. In contrast, 75% of Neuro-2a cells stained positive for β-galactosidase expression 48 hours after LipofectAMINE-mediated delivery of the same reporter construct.

There are two main facets of the transfection process that are crucial for efficient delivery of plasmid DNA to the cell nucleus. These are cellular uptake and nuclear entry. More specifically, there are several physiological and biochemical factors that may contribute to different cells having different efficiencies of transfection. Particular attention has been paid to proteoglycans on the cell membrane, as these appear to play an important role in the uptake of cationic liposome transfection complexes. Cells that are deficient in proteoglycan synthesis have been demonstrated to be more difficult to transfect (Mounkes *et al.*, 1998). Furthermore, there is evidence to suggest that the role of proteoglycans is to protect cells from potential cytotoxicity of cationic lipids (Belting and Petersson, 1999). Proteoglycans perform a wide variety of functions ranging from the formation of extracellular matrix to mediating cell-cell contact and communication. Thus, different cell types may have quite distinct proteoglycan composition (Kjellen and Lindahl, 1991). The exact proteoglycan composition of HC11, primary bovine mammary, or Neuro-2a cells however is not known.

Previous studies, in which either the plasmid DNA or the chemical utilised for delivery was labelled, show that cellular uptake of the transfection complex is an efficient process. A high percentage of cells accumulate significant concentrations of these foreign molecules within the cytoplasm as a consequence of internalisation by endocytosis. In one study, Amstutz and Schanbacher (1995) incubated COMMA-1D mouse mammary and primary bovine mammary epithelial cells in culture with a plasmid DNA-polyion complex. The plasmid DNA was labelled with ^{33}P and the polyion contained fluorescein-labelled poly-L-ornithine. Within minutes following delivery, a fluorescent signal was associated with virtually all cell surfaces and, after 6-8 hours, fluorescence was detected within the cytoplasm of most cells. Radiolabelled plasmid DNA co-localised with the fluorescent signal indicating that the transfection complex was intact in the cytoplasm. However, the majority of signal remained within the cytoplasm, with little being detected in the nucleus. This suggested that nuclear transport was the limiting step to successful transfection in this model.

Other researchers have also found that inefficient nuclear entry is a major limitation in transfection experiments. Mortimer *et al.* (1999) found that nuclear

transport limited successful transfection, and suggest that mitotic activity is essential for successful nuclear transport to occur. Furthermore, inclusion of nuclear localisation signals (NLS) into the transfection complex can enhance nuclear transport of foreign DNA molecules (Branden *et al.*, 1999; Subramanian *et al.*, 1999).

Neither cellular uptake nor nuclear entry of plasmid DNA delivered to HC11 cells with LipofectAMINE was analysed in this thesis. However, evidence presented in these other studies suggests that nuclear entry, rather than cellular uptake, may be the main barrier to efficient transfection. Analysis of the fate of transfected DNA, using labelled plasmid DNA, may indicate whether poor nuclear transport was also a contributing factor to the low transfection efficiencies observed in this study using HC11 and primary bovine mammary cells.

What is apparent from the present and other studies, is that a ubiquitous cationic lipid ideal for gene transfer to all cell types is not yet available. Several advances however have been made during the course of this study in cationic liposome production, with several "new and improved" formulations now available. In general, these improvements have been aimed at increasing transfection efficiency, broadening the range of cell types able to be transfected, and reducing the toxicity of cationic liposomes to cells.

The low efficiency of mammary epithelial cell transfection achieved in these *in vitro* models is not sufficient for high-level expression of foreign genes in the mammary gland *in vivo*. This problem of low transfection efficiency is generally recognised as the main limitation of liposome-mediated delivery of DNA to cells (Luo and Saltzman, 2000). Despite this, liposome-mediated transfection is a popular option for human gene therapy. The Wiley Journal of Gene Medicine Clinical Trials Database currently lists 532 gene therapy trials, 68 of which use lipofection as the mode of delivery (12.8%). In comparison, there are 204 protocols using retroviral (38.3%) and 136 protocols using adenoviral (25.6%) vectors (www.wiley.co.uk).

7.2 Transfection of the rat mammary gland in vivo

Despite the low transfection efficiency of cationic liposome-mediated delivery achieved *in vitro*, it was important to assess the efficacy of this approach for *in vivo* gene transfer to the mammary gland. Lactating Sprague-Dawley laboratory rats were used as a model. Rats, as opposed to mice, were chosen for these *in vivo* experiments primarily because of the larger size of rat teat canals, which would make the successful infusion of transfection complexes technically more feasible. Infusion through the teat canal was the preferred route of delivery since this is the least invasive approach. However, direct injection into the mammary gland was also performed as an alternative approach to assess whether this was a viable route for administration. Lactating rats were used for several reasons. The number of mammary epithelial cells available to target for gene transfer during lactation is greater than at any other developmental stage. Furthermore, these cells are actively expressing and secreting milk proteins during lactation. One potential application of successful gene transfer to the mammary gland is the production of novel proteins; therefore secretion into milk is desirable.

Based on results from *in vitro* experiments, LipofectAMINE-mediated transfection was chosen as the best available method to deliver a eukaryotic expression vector containing a β-galactosidase reporter gene driven by the CMV promoter. The lipid-DNA complex was delivered to the mammary glands of a total of eleven animals, at a range of lactational stages from day seven to day seventeen. The route of administration was either by infusion through the teat canal or by direct injection into the gland.

Analysis of mammary tissue following infusion or injection revealed β-galactosidase expression in only three of the eleven rats, and only in a very small number of mammary epithelial cells that were heterogeneously distributed throughout the transfected mammary glands. It was not possible to estimate the proportion of cells that had been successfully transfected because the number of positive cells was very low as compared to the total number of mammary epithelial cells that were originally targeted.

There have been very few reported attempts at chemical-mediated delivery of DNA to the mammary gland. Hens *et al.* (2000) delivered a human growth hormone (hGH) reporter gene construct, complexed with various chemicals, to the guinea pig mammary gland by infusion through the teat canal at various developmental stages of the mammary gland including pre- and post-parturition. Transgene expression was quantified by a hGH radioimmunoassay (RIA) of milk samples from the transfected glands collected 2 - 14 days after delivery. No hGH was detected in any of the milk samples from glands transfected in late pregnancy with the hGH plasmid when delivered with LipofectAMINE. However, the same construct delivered at a similar stage of development with DEAE-dextran expressed hGH at detectable levels. This interesting observation suggests that LipofectAMINE was not as effective as DEAE-dextran for gene transfer in the physiological environment encountered within the guinea pig mammary gland at this stage of development. Furthermore, DEAE-dextran was also more efficient at transfecting the guinea pig mammary gland *in vivo* than a charge-shifting mixed polyion complex that was specifically developed for delivery to mammary epithelial cells *in vitro*. In contrast, this charge-shifting mixed polyion was more efficient than DEAE-dextran at transfecting cells *in vitro* (Schanbacher and Amstutz, 1997). These data demonstrate that transfection efficiencies achieved *in vitro* and *in vivo* with the same delivery vehicle do not always correlate. This lack of correlation between *in vitro* and *in vivo* has also been observed in human airway epithelial cells (Fasbender *et al.*, 1997; Matsui *et al.*, 1997).

Hens *et al.* (2000) also observed that the stage of mammary development at which the transfection complex was delivered to the guinea pig mammary gland had an effect on the efficiency of transgene expression. They detected hGH in milk following delivery of the transfection complex within 5 days before parturition at levels several fold higher than that obtained at any other stage of development, ranging from early pregnancy to involution. In comparison, the transfection complex used for rat mammary gland transfection described in the present study was only delivered postpartum, during lactation. Interestingly, from eleven rats that had the transfection complex delivered, only three showed positive β-galactosidase staining, all of which were transfected on day 12 or earlier of lactation. In one other study, an unsuccessful attempt was made to transfect the

bovine mammary gland, with a charge-shifting mixed polyion complex, during lactation (Schanbacher and Amstutz, 1997).

These results suggest that delivery of the transfection complex during lactation may be especially problematical as compared with prepartum delivery, although further studies would be required to confirm this point. There are two main physiological differences between the pre- and postpartum mammary gland, which may affect transfection efficiency. Firstly, the intramammary environment encountered in the lactating gland, specifically the presence of milk, may be detrimental to successful delivery of the transfection complex to target cells. Secondly, the mammary gland prior to parturition undergoes extensive development involving mitosis, and it has been suggested that mitotic activity is essential for nuclear transport of foreign DNA to occur (Mortimer *et al.*, 1999). Transfection efficiencies may consequently be greatest in these actively dividing cells encountered in the prepartum mammary gland.

As the transfection efficiency attained in this *in vivo* model was extremely low following LipofectAMINE-mediated plasmid DNA transfection, it was obvious that this vehicle and method of delivery was not adequate. While a number of researchers are currently investigating ways of enhancing DNA delivery using different chemicals, further work in this area was outside the scope of the research in this thesis. Any potential increase in the efficiency of transfection would be beneficial to a chemical-mediated gene transfer approach. Therefore an alternative way of enhancing transfection efficiency was sought in this present study, to complement advancements made by others in the devopment of new chemicals.

7.3 Enhancement of transfection efficiency using AAV-based plasmid DNA

In general, the major problem with liposome-mediated gene transfer of plasmid DNA is low transfection efficiency. The use of AAV-based plasmid DNA is an attractive alternative to potentially alleviate this problem. This approach combines the safety and simplicity of liposome-mediated delivery of plasmid

DNA with the features of AAV. The experiments described in this study entailed the delivery of a plasmid containing a β-galactosidase reporter gene construct flanked by AAV ITRs (pITR-CMVlacZ) to the mouse mammary epithelial cell line HC11. LipofectAMINE was used to deliver both this construct and a control β-galactosidase plasmid without ITRs (pCMVlacZ) to these cells, utilising previously optimised transfection conditions. The transfection efficiency, as determined by staining for the percentage of cells positive for β-galactosidase 48 hours post-transfection, was enhanced by 1.4-fold when the β-galactosidase gene was flanked by ITRs. Although not remarkable, this increase in transfection efficiency of HC11 cells was statistically significant.

Other researchers have similarly utilised the ITRs of AAV to enhance transient transfection efficiencies. Philip *et al.* (1994) recorded an increase, of up to 10-fold, in interleukin 2 (IL-2) protein expressed from a construct with ITRs as compared to an ITR minus construct, when they transfected cells from a rat prostate cell line using a combination of the cationic lipid DDAB and the neutral lipid DOPE. IL-2 protein was quantified by ELISA 48 hours post-transfection. Furthermore, Vieweg *et al.* (1995) transfected cells from the same rat prostate cell line with LipofectAMINE and measured expression 48 hours post-transfection from constructs with and without ITRs flanking a β-galactosidase reporter gene. They found that up to 78% of cells transfected with plasmids containing ITRs were positive for β-galactosidase, whereas only 5% of cells transfected with plasmids without ITRs showed positive staining. However, it must be recognised that the β-galactosidase reporter gene in the control plasmid, without ITRs, was driven by the SV40 promoter, as opposed to the CMV promoter for the plasmid with ITRs. Hence it is difficult to make a direct comparison between these two constructs since expression from the SV40 promoter is generally less than that from the CMV promoter. In another study, with mouse C2C12 muscle and human embryonic kidney 293 cells, between a 1.6- and 3.5-fold increase in expression was observed from plasmids containing ITRs, flanking either the human apolipoprotein AI (apoA1) or lecithin-cholesterol acyltransferase (LCAT) genes (Fan *et al.*, 1998). All three studies described above speculate that the

mechanism for this observed enhancement results from the ITRs facilitating nuclear accumulation of the transfected plasmid.

The level of enhancement that was attained in these three reports is greater than what was observed with the experiments presented in this thesis. The reason for this disparity is not immediately obvious. However, a lack of an appropriate control in the second study did not allow an accurate quantification of the effect that ITRs may have had on transfection efficiency. Radiolabelling of plasmid DNA prior to transfection, and subsequent observation of its intracellular fate could assist in elucidating any effect ITRs have on nuclear accumulation of the plasmid.

It should be emphasised that in the liposome-mediated AAV-based plasmid DNA transfection experiments described above, DNA was delivered to either mouse or rat cell lines. In other studies, several researchers have utilised cationic liposomes to deliver AAV-based plasmid DNA to human cell lines *in vitro* (Shelling and Smith, 1994; Surosky *et al.*, 1997; Lamartina *et al.*, 1998; Pieroni *et al.*, 1998; Rinaudo *et al.*, 2000; Tsunoda *et al.*, 2000). These experiments were all focussed on investigation of the site of integration into the human genome and unfortunately none have addressed the question of the effect that AAV ITRs might have on the efficiency of plasmid DNA transfection. This would require comparisons with transfections using control plasmids without ITRs.

Furthermore, these studies with human cell lines were all performed in the presence of Rep78 and/or Rep68 protein. The AAV Rep78 and/or Rep68 protein was shown to be required for site-specific integration to occur at the human AAVS1 locus (Surosky *et al.*, 1997). A model for the integration process was proposed by Linden *et al.* (1996a, 1996b), which involves binding of the Rep78 and/or Rep68 protein to a sequence motif, known as the Rep Binding Element (RBE), which is present in both the human AAVS1 locus and also the AAV ITR sequences. In the absence of Rep78 and/or Rep68, DNA flanked by ITRs will still integrate, but at random sites within the human genome (Xiao *et al.*, 1997).

In seeking to evaluate what role the Rep78 and/or Rep68 protein may have on integration into the genome of cells other than human, an attempt was made in the present experiments to identify whether or not a sequence that is homologous to human AAVS1 exists in other species. Previously, Samulski *et al.* (1991) demonstrated by Southern blot analysis that genomic DNA from only human and green monkey, but not mouse, rat, cat, cow, rabbit, chicken, or yeast, hybridised to a 500 bp probe based on the human AAVS1 sequence. This suggested that a homologous locus to AAVS1 does not exist in these other species. However, the conditions for hybridisation were stringent, with a hybridisation temperature of 68°C. Such a temperature would presumably only allow hybridisation, and hence, detection of genomic sequences with high sequence identity with the probe sequence. Recently, following results using an *in vitro* model, it was suggested that a 33 nucleotide region of AAVS1, which includes the RBE, was the only sequence required for integration into AAVS1 (Linden *et al.*, 1996a; Linden *et al.*, 1996b). Therefore, experiments were performed in this thesis to analyse whether or not this 33 nucleotide sequence existed in any other species.

Mouse, rat, sheep and cow genomic DNA, as well as human genomic DNA, was screened by Southern blot analysis with an oligonucleotide probe containing this 33 nucleotide region. Hybridisation to this probe was observed in all species, including the expected size restriction fragment from human genomic DNA, suggesting that some degree of identity to this 33 nucleotide sequence exists in all of these species analysed. However, the probe has a GC content of approximately 80% and the hybridisation was at 60°C in this experiment. The binding specificity of the probe under these non-stringent hybridisation conditions is difficult to determine and binding to divergent target sequences may occur. Hybridisation at higher temperatures may provide further information about the conservation of AAVS1 sequences in these other species.

Subsequent to this observation, Young *et al.* (2000) demonstrated that, not just the 33 nucleotide region of AAVS1, but the entire AAVS1 locus is essential for site-specific integration of recombinant AAV *in vivo*. Therefore, although evidence presented in this thesis suggests that other species may have sites within their genome that are similar to this 33 nucleotide sequence, it is unlikely that

integration will occur specifically into these loci by a mechanism involving the Rep78 and/or Rep68 protein. Nonetheless, it cannot be discounted that Rep78 and/or Rep68 protein may have a role in the integration of plasmid DNA containing ITRs into the mouse genome or the genomes of other species besides human. This remained to be determined and was investigated further in this study.

7.4 Stability of expression from transfected AAV-based plasmid DNA

Transient expression, following low transfection efficiency, is a secondary disadvantage of liposome-mediated gene transfer. A technique to enhance the longevity of gene expression following transfection would be beneficial. One potential mechanism, which may lead to greater stability of transgene expression, is integration of plasmid DNA into the host genome. AAV-based plasmid DNA transfected into human cells has been shown to integrate site-specifically into the host cells genome at the AAVS1 locus (Shelling and Smith, 1994; Surosky *et al.*, 1997; Lamartina *et al.*, 1998; Pieroni *et al.*, 1998; Rinaudo *et al.*, 2000; Tsunoda *et al.*, 2000). However, very little information exists regarding the fate of AAV-based plasmid DNA after transfection into mouse, and the genomes of other species.

To further investigate the integration characteristics of AAV-based plasmid DNA, the mouse mammary epithelial cell line HC11 was transfected with plasmid DNA containing the neomycin resistance gene flanked by ITRs. Furthermore, a *rep* expression construct was co-transfected with this neomycin construct to evaluate the role Rep78 and/or Rep68 may have on integration into the HC11 genome. Cells were subsequently selected for integration of the neomycin resistance gene using the drug G418. There was no significant difference observed in the number of colonies that resulted from transfections with the addition of ITRs, *rep* or a combination of both, as compared to the control transfection without ITRs or *rep*. These results suggested that firstly, the presence of the ITRs in the transfected plasmid flanking the neomycin resistance gene does not change the frequency of integration of this gene into HC11 mouse genomic DNA. Secondly,

co-transfection of a *rep* expression plasmid also does not have an effect on the integration frequency of the neomycin resistance gene into HC11 genomic DNA. It should be noted however that although *rep* mRNA production was confirmed by Northern blot analysis, the presence of Rep78 and/or Rep68 protein was not confirmed. The optimal intracellular concentration of Rep78 and/or Rep68 protein has been demonstrated to be important for efficient integration into the AAVS1 locus of human cells. Excessive Rep78 and/or Rep68 protein can cause non-specific genomic rearrangements at the AAVS1 locus (Balague *et al.*, 1997; Surosky *et al.*, 1997; Rinaudo *et al.*, 2000).

To evaluate whether this observation was unique to this mouse cell line, a human embryonic kidney cell line, HEK-293 was also co-transfected with plasmid DNA containing the neomycin resistance gene flanked by ITRs, and a *rep* expression construct. Cells were similarly selected with G418 to determine the integration frequency of the neomycin resistance gene and the numbers of colonies were compared to those generated using control constructs. Unfortunately due to technical difficulties with these cells, not enough data was obtained to make any inference about what effect the ITRs flanking the neomycin resistance gene, or co-transfection of a *rep* expression plasmid had on the frequency of integration.

Several liposome-mediated AAV-based plasmid DNA transfection studies have been performed with a range of human cell lines *in vitro*, with the aim of elucidating the site of AAV integration. Results have demonstrated that AAV-based plasmid mediated transfection can lead to site-specific integration into the AAVS1 locus on human chromosome 19. Shelling and Smith (1994) transfected AAV-based plasmids containing ITRs and *rep* genes into human HEK-293 and HeLa cell lines. They observed site-specific integration into the AAVS1 locus in 75% of cell lines analysed by Southern blot analysis. More recently, site-specific integration has been confirmed by isolation and sequencing of AAVS1-plasmid DNA junctions (Surosky *et al.*, 1997; Lamartina *et al.*, 1998; Pieroni *et al.*, 1998; Rinaudo *et al.*, 2000; Tsunoda *et al.*, 2000).

In an attempt to recreate an animal model to characterise the molecular aspects of site-specific integration and to test the efficacy of targeted integration of

AAV-based vectors, Rizzuto *et al.* (1999a) created transgenic rats containing a 3.5 kb AAVS1 fragment from human chromosome 19. Subsequent transfection of a plasmid, which contained the neomycin resistance and the green fluorescent protein genes flanked by ITRs and also a *rep* gene on the plasmid backbone, into primary rat fibroblasts derived from these transgenic animals resulted in site-specific integration into the AAVS1 transgene locus. However, the frequency was low, with Southern blot and fluorescent *in situ* hybridisation (FISH) analysis only demonstrating integration in one of the seven (14%) clones analysed. Thus, this data provided evidence that site-specific integration into the AAVS1 transgene locus could occur following AAV-based plasmid DNA transfection, albeit at a low frequency. This frequency of site-specific integration was similar to what was observed in another study with the same AAV-based plasmid, when it was transfected into human cell lines (Pieroni *et al.*, 1998).

7.5 Site of integration of AAV-based plasmid DNA

Experiments were undertaken in this thesis to determine what effect AAV ITRs and Rep78 and/or Rep68 protein had on the fate of plasmid DNA transfected into mouse cells. Although the frequency of integration was not changed, it was of interest to identify and characterise the genomic site at which integration had occurred following delivery of these AAV-based plasmids.

Several clonal HC11 cell lines were created earlier in this study from cells that were co-transfected with an AAV-based plasmid DNA construct containing a neomycin resistance gene flanked by ITRs, and a *rep* expression construct (+ITR +*rep*). In parallel, HC11 cells were also co-transfected with a construct containing a neomycin resistance gene, without ITRs, and a *rep* "minus" control construct (-ITR -*rep*). Subsequent selection with the drug G418 identified colonies in which integration had occurred. Individual colonies were expanded so that genomic DNA could be isolated for analysis of the integration characteristics.

Firstly this isolated genomic DNA was subjected to Southern blot analysis, whereby DNA was digested with the restriction endonuclease *Hind*III, which does

not have any recognition sites in either of the transfected neomycin constructs (+ITR or –ITR), and hybridised to a neomycin gene probe. Different patterns of hybridisation products were detected for all clones analysed from both +ITR *+rep* (fifteen clones) and -ITR *–rep* (twelve clones). The differences observed could result from either heterogeneous sites of integration, from multi-copy integration, or a combination of both.

Parallel transfection and selection experiments, using the same plasmids, were performed with the human embryonic kidney cell line HEK-293. Southern blot analysis of seven +ITR *+rep* and eight -ITR *-rep* clonal cell lines with the neomycin gene probe revealed no common hybridisation products. Hybridisation products of different sizes could result from either heterogeneous sites of integration, multi-copy integration, or a combination of both, and is similar to what was observed in the HC11 cells. Furthermore, upon hybridisation of the HEK-293 membrane with a probe based on the human AAVS1 locus, no co-localisation of hybridisation products between the neomycin and AAVS1 probes was observed in any of the clones analysed. This suggested that site-specific integration of the neomycin transgene into the AAVS1 locus had not occurred in any of these clones.

Site-specific integration into AAVS1 has previously been observed at frequencies of between 40% and 75% following transfection of HEK-293 with AAV-based plasmid DNA in the presence of Rep78 and/or Rep68 protein (Shelling and Smith, 1994; Balague *et al.*, 1997; Surosky *et al.*, 1997). In comparison, wild type AAV integrates site-specifically into the AAVS1 locus in approximately 70% of cases (Kotin *et al.*, 1990; Kotin *et al.*, 1991; Samulski *et al.*, 1991; Kotin *et al.*, 1992). Analysis of a greater number clones would be required to determine whether site-specific integration into AAVS1 is occurring in the experiments described in this thesis.

It could not be conclusively determined if different clones had similar integration sites within the mouse genome by Southern blot analysis alone. This is because no common site of integration has been identified in the mouse genome to date. Therefore a novel PCR method was utilised to amplify regions flanking the

integration junction between plasmid and genomic DNA (Siebert *et al.*, 1995). Amplified DNA was then subcloned and the sequence determined. From twelve clonal HC11 cell lines screened using this method of Siebert, sequence information was obtained for two clones: 1b-1 and 3b-4.

Clone 1b-1 was derived from HC11 cells co-transfected with pneo-tk and pBR322, which do not contain any AAV sequence (- ITR, - *rep*). Alignment of sequence data from this clone to the NCBI mouse database, using the BLAST search tool, revealed that nucleotides 15 to 403 (389 bp) had 100% identity with the 5' end of exon 16 of the *Mus musculus* DNA for DNA dependent protein kinase catalytic subunit (nucleotides 392 to 4) (Accession number AB005160.1). Clone 3b-4 was derived from HC11 cells co-transfected with pITR-neo-tk and pHIVrep (+ ITR, + *rep*). No significant matches to the NCBI mouse database using the BLAST search tool were detected for the sequence of this clone, hence it had integrated into an as yet unknown region of the mouse genome.

Of particular interest however from alignment of both clones 1b-1 and 3b-4 to the NCBI mouse database was the similarity to the mouse B1 repeat. Clone 1b-1 has 81% sequence identity over 57 bp (position 365 to 421) and clone 3b-1 has 73% sequence identity over 62 bp (position 51 to 112) to B1. The regions of sequence identity overlap and are predominantly at the 5' end of the B1 consensus sequence.

The mouse B1 repeat sequence belongs to the short interspersed element (SINE) family of repeats which are present in all higher eukaryotes, including the human *Alu* repeat (Jelinek and Schmid, 1982). The 129 bp B1 repeat is estimated to be present in between 130,000 and 150,000 copies in the murine genome (Bennett *et al.*, 1984) and is frequently located at intron-exon junctions within genes (Krayev *et al.*, 1980).

Interestingly, Surosky *et al.* (1997) reported the presence of a short region of sequence, 95% (19/20 nucleotides) homologous to *Alu* at the integration junction in one of their HEK-293 clones which had been transfected with an AAV-based plasmid. Adjacent to this *Alu* sequence was an unknown region of DNA. They

demonstrated by inverse PCR that the *Alu* sequence is not present in this unknown genomic DNA of non-transfected cells and suggest that the junction is the result of two crossover events; between the unknown DNA and *Alu*, and between *Alu* and the plasmid.

As sequence data was only obtained for two clones, it could not be determined whether the presence of B1 repeat sequence identity at the site of integration was a function of a targeted integration event or by chance random integration. Wu *et al.* (1998) reported a successful method, using *Alu*-PCR and B1-PCR, to identify recombinant AAV vector-mediated DNA integration into several human cells lines *in vitro* and into the rat brain *in vivo* respectively. A similar approach using mouse B1 repeat, and transgene specific primers could possibly elucidate more information about the occurrence of B1 homologous sequences near the site of integration in these experiments.

7.6 Summary

The aim of the work outlined in this thesis was to develop a method for delivery of plasmid DNA to the mammary gland *in vivo*. Chemical-mediated delivery of plasmid DNA is an attractive method for gene transfer. Features, which make it a preferential choice over viral-mediated delivery to introduce foreign DNA to cells, include safety, simplicity and cost. Unfortunately, one of the main drawbacks of this technique is the low transfection efficiency, and in attempting to accomplish the original goal of this work, two approaches were explored to overcome this problem.

Firstly, an *in vitro* mouse mammary epithelial cell culture model was developed for assessment of various cationic liposomes to identify an ideal vehicle for plasmid DNA delivery. Upon testing several cationic liposomes and optimising their efficiencies, LipofectAMINE was chosen as the best available, however the level of transfection efficiency achieved was relatively low. Subsequent to these *in vitro* trials, *in vivo* transfection experiments with rats were undertaken with LipofectAMINE as the delivery vehicle. Unfortunately, very low transfection

efficiencies were also obtained. These observations suggest that a modified approach will be required for *in vivo* transfection. Specifically, it may be necessary to tailor a delivery vehicle for the unique circumstance encountered in the mammary gland. This is in agreement with other researchers who have addressed a similar question (Hens *et al.*, 2000). Furthermore, the timing of delivery to the developing mammary gland appears to be important to the efficiency of transfection. Future attempts at *in vivo* transfection should investigate this further.

The second approach was based on the use of AAV-based plasmid DNA to enhance transfection efficiency. Inclusion of ITRs in the transfected plasmid flanking the transgene enhanced transient transfection efficiency by 1.4-fold. However, the mechanism by which this is occurring is unknown. Other researchers have suggested that the mechanism of enhancement may involve the ITRs facilitating nuclear accumulation of the transfected plasmid (Philip *et al.*, 1994; Vieweg *et al.*, 1995). Labelling of AAV plasmid DNA before delivery, so that the intracellular location can be determined, may provide further information about the effect that ITRs have on nuclear transport of foreign DNA.

Further investigations in this thesis, on any effects that ITRs and AAV *rep* expression have on the fate of plasmid DNA transfected into HC11 cells, revealed that the frequency of integration was unchanged in the presence of ITRs and/or *rep* expression. Additional studies with the Rep78 and/or Rep68 protein could provide further insight as to their role, if indeed they have any. Specifically, analysis of the concentration of Rep78 and/or Rep68 protein, and regulation of this concentration may be informative.

Analysis of the site of integration and the characterisation of integration junctions revealed that two unique genomic sites were identified in the clones examined, however clearly a greater number of clones need to be evaluated before definitive conclusions can be drawn. These new data would also indicate whether the presence of B1 sequences near the integration junction were significant in the two clones that were analysed, or whether their occurrence close to the site of

integration merely reflects the fact that there are numerous B1 sequences in the genome.

In summary, this project has contributed toward the understanding of cationic liposome-mediated transfection of mammary epithelial cells *in vitro* and *in vivo*. Furthermore, it was demonstrated that the use of AAV-based plasmid DNA could enhance transient transfection efficiency in the HC11 mouse mammary epithelial cell line. Investigation of the integration characteristics of this plasmid DNA into the mouse genome has provided insight into the molecular fate of AAV-based plasmids in a species other than human.

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