Development of a gene therapy approach for the treatment of human mammary carcinoma using tissue specific retroviral vectors

A dissertation

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by

Feride Öztürk-Winder

from Ankara, Turkey

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1st supervisor: P.D. Dr. Brian Salmons

2nd supervisor: Prof. Dr. Elisabeth Weiß

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'Anyone who has never made a
mistake has never tried anything new.'

Albert Einstein.

....to my family and David

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1. INTRODUCTION

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1.1 Gene Therapy

Gene therapy is defined as the transfer of a heterologous gene into an organism for the purpose of correcting a genetic defect, providing a new therapeutic function to the target cell, inducing a cure or improving associated symptoms (Anderson *et al.,* 1984).

The idea that replacing the defective genes with functional ones could treat genetic disease has long since existed (Friedman and Tublin, 1972). Advances made in the field of virology and biotechnology in the late 1960s and early 1970 allowed a better understanding of how viruses replicate and integrate into the host genome. These suggested that they could be used to perform gene transfer. This was aided by the development of recombinant DNA techniques, which enabled the isolation of large quantities of genetic material for use. Having defined both a delivery system and possessing the technology to manipulate DNA, research became more focused on the inherited genetic diseases that could be potentially treated. Initial attempts were made to treat the haemoglobinopathies, the first group of genetic diseases to be characterised at the molecular level. However, in 1980 following a failed attempt to treat β-thalassaemia, focus turned towards Severe Combined Immune Deficiency (SCID) disease, such as adenosine deaminase (ADA) deficiency. This type of diseases should be easier to treat using gene therapy replacement, as, unlike the haemoglobinopathies, no complex regulation of gene expression is required.

Retroviral mediated gene transfer was developed in the early 1980s in animals models (Miller *et al.*, 1983). It was concluded that the retroviral vector system possessed the properties suitable for the first attempts at gene therapy in humans (Anderson *et al.*, 1984), and this conclusion has had a major influence in directing initial gene therapies towards the use of retroviral systems.

There are currently two types of gene therapy capable for human implementation. Germinal gene therapy consists of introducing new genetic material into the germ line cells. This type of gene therapy affects not only the individual receiving the treatment but also has the ability to affect future generations through the gametes. Germinal gene therapy has not yet been executed on humans. Somatic gene therapy consists of introducing new genetic material into the cells of the body whose chromosomes will not be passed on to future generations. This type of gene therapy affects only the individual receiving the treatment, and has proved successful in treating human genetic disorders.

In September 1990, a four year old girl suffering from adenosine deaminase (ADA) deficiency received an infusion of her own T lymphocytes into which a normal copy of the ADA cDNA had been introduced (Blaese *et al.*, 1990). ADA deficiency is a very rare genetic disease in which affected children lack an enzyme (the ADA protein) that is necessary for the normal function of their immune system. ADA is an enzyme of the hydrolase class that catalyses the deamination of deoxyadenosine to deoxyinosine. It is a member of the purine salvage pathway and when it is absent, deoxyadenosine can accumulate to levels that are toxic in certain cell types (*e.g.* T lymphocytes). Children without the ADA enzyme live in the constant threat of developing common infections.

A large number of candidate disorders are being considered for gene therapy (Tab. 1.1). Conceptually the most simple disease to treat would be a monogenic recessively inherited disease, such as haemophilia (Snyder *et al.*, 1997), whereby a functional form of the gene would be added to the cell restoring it to a normal phenotype. However, research is leaning towards the treatment of monogenic dominantly inherited diseases such as hypercholesterolamia (Gerad and Collen, 1997), acquired genetic diseases such as cancers (Roth and Cristiano, 1997), regulation of cellular proliferation e.g. to prevent arteriosclerosis following angioplasty (Kim *et al.*, 1997), promotion of cellular repair following trauma to the CNS (Federoff *et al.*, 1992) and protection from infectious disease (Caruso and Bank, 1997).

System	Disease targeted
Cardiovascular	Artherosclerosis Myocardial infarction Aortic anuerysms Transplant rejection Restenosis after angioplasty Myocarditis
Respiratory	Cystic fibrosis Alpha-1-antitrypsin deficiency Malignant mesothelioma
Neuro-muscular	Parkinson‘s disease Huntington‘s disease Duchenne muscular dystrophy (DMD)
Metabolic	Familial hypercholesteraemia Tay-Sachs disease Fabry disease Mucopolysaccharidosis type VII Gaucher‘s disease Analbulminaemia Niemann pick type A & C Phenylketonuria Refractory Diabetes mellitus Adenosine deaminase (ADA) deficiency
Haematological	Fanconi anaemia Haemophilia type-B Hypercoagulative states Blood cell disorders Inherited coagulant deficiency states (*e.g.* clotting factor V & VII-XI)
Tumour	Malignant gliomas Prostate Breast Melanoma NSCLC (Non- small -cell- lung- carcinoma) Pancreatic Kidney Mesothelioma Lung Cervical Liver Colorectal Head and Neck
Miscellaneous	Genetic liver diseases Hepatitis B HIV

Table 1.1: Candidate Diseases For Gene Therapy

1.2 Methods of Gene Delivery

There are presently two gene transfer strategies to accomplish gene therapy; *ex vivo* (Fig. 1.1) where cells are removed, genetically modified and transplanted back into the donor and *in vivo* therapy accomplished by transfer of genetic material directly into the patient.

The advantage of *ex vivo* gene therapy include transfection of a specific cell population, better control and monitoring of transfection and protein expression and a reduced risk of immunogenicity because the patient´s immune system is not directly exposed to the vector. Disadvantages include the fact that such protocols are labour-intensive, require skilled technicians, and are both expensive and time consuming and need high tech facilities. Targeted, injectable *in vivo* therapy will be the goal for most gene therapy protocols, with the exception of therapies aimed at bone marrow stem cells and circulating progenitor cells.

To accomplish *in vivo* gene transfer, a vector must possess certain intrinsic characteristics. It must ensure high rates of gene transfer, target the gene to specific cell types, regulate the expressed gene product and allow temporary or permanent expression. The delivery system should have no pathogenic effects itself and induce no unwanted immune response.

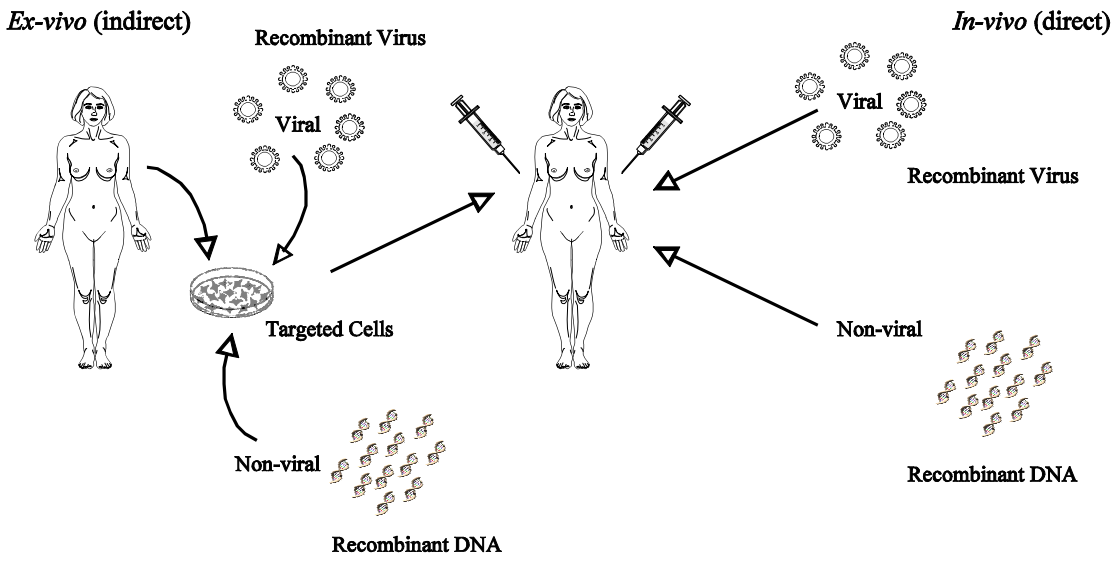

Figure 1.1: Gene Transfer Strategy
Gene therapy applied *ex vivo* or *in vivo*. *Ex vivo* where the cells are removed, genetically modified and transplanted back into the donor and *in vivo* therapy accomplished by the transfer of genetic material directly into the patient.

Many different types of vectors have been developed for gene transfer applications. These include both viral and nonviral vectors (Tab. 1.2). In all of these strategies, in order to accomplish effective gene transfer, the delivered gene must cross multiple subcellular barriers. These barriers include: transit through the eucaryotic cell plasma membrane, cytoplasmic to nuclear transport, nuclear entry and functional maintenance within the nucleus.

Gene Transfer Applications			
Non-viral			Viral
	in vitro	*in vitro* & *in vivo*	*in vitro* & *in vivo*
Chemical	Calcium phosphate transfection	Liposomes Naked plasmid DNA injection	
Physical	Electroporation Particle bombardment		
Biological			Retrovirus Adenovirus Adeno-associated virus Herpes virus Vaccinia viruses Measles viruses

Table 1.2: Gene Transfer Applications

1.2.1 Non-Viral Methods

Non-viral gene transfer vectors have been developed as an alternative to viral systems. This form of gene transfer utilises distinct mechanisms to accomplish gene delivery. Such methods of DNA transfer only require small amounts of genetic material, have a virtually infinite capacity, have no infectious or mutagenic capability and large scale production is possible using pharmaceutical techniques.

There are four common methods of non-viral DNA transfer; uncomplexed plasmid DNA, DNA-liposome complexes, DNA-protein conjugates and DNA-coated gold particles.

1.2.1.1 Purified Uncomplexed Plasmid DNA

Purified DNA or mRNA can be injected directly into tissues and results in transient gene expression. Although direct injection of plasmid DNA has been shown to lead to gene expression, the overall level of expression is much lower than with either viral or liposomal vectors. Naked DNA is also unsuitable for systemic administration due to the presence of serum nucleases. Consequently, direct injection of plasmid DNA seems to destined to be limited to only a few applications involving tissues that are easily accessible to direct injection such as skin and muscle cells. This has been illustrated in muscle tissue, where direct injection of uncomplexed DNA is most effective. It has also been demonstrated that purified plasmid DNA or mRNA encoding a reporter gene could mediate transgene expression following direct injection into the quadricaps muscle of a mouse (Wolf *et al*., 1990). DNA injection results in longer gene expression than mRNA injection.

1.2.1.2 DNA Coated Gold Particles

Plasmid DNA can be affixed to gold particles and then "shot" into superficial cells. The DNA is co-precipitated onto the gold particle and then propelled from a myler sheet using an electric spark or pressurised gas as the motive force. This so called gene-gun can be used to accelerate the DNA-coated particles into the superficial cells of the skin or into skin tumours. Gene expression lasts a few days, which may be more a function of the cells targeted than the method of delivery. In animal models, gene delivery of DNA vaccines is highly effective (Fynan *et al.*, 1993). Gene-gun delivery is ideally suited to gene-mediated immunisation, where only brief expression of an antigen is necessary to achieve an immune response. This technique is limited in its use to surface cells that can be accessed directly because of the limited depth of DNA penetration. Furthermore, the epidermal layers are a preferred target for vaccination. The simplicity, safety, and technical ease of preparation of this DNA transfer system make its large application more feasible than available viral DNA delivery systems.

1.2.1.3 Liposomes

Liposomes have been used extensively as a technology for delivering drugs experimentally to the interior of cells. The premise is that by surrounding hydrophilic molecules with hydrophobic ones, agents otherwise impermeable to cell membranes might be escorted into the cell. Potential advantages of such a delivery system include targeting drugs to an intracellular location and reducing toxicity. The basic challenge in *in vivo* gene therapy is to deliver a transgene, itself a large hydrophilic molecule, across the plasma membrane and into the nucleus where it can access the cells transcription machinery (Fig. 1.2).

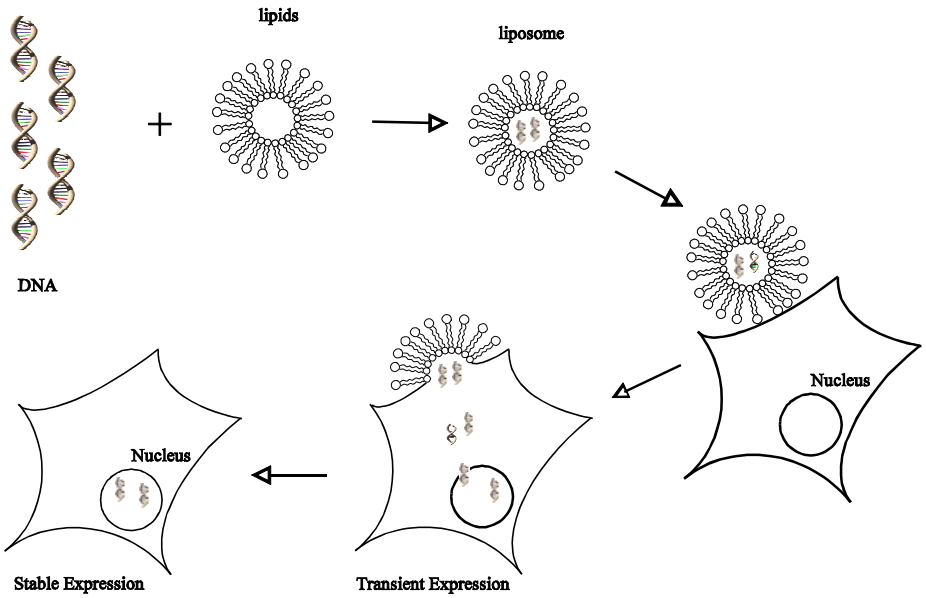

Figure 1.2: Liposomal Gene Delivery
Liposomes are phospholipid-like membranes or vesicles in which the DNA is covered. The vesicles set the DNA free after fusion with the cell membrane.

Liposomes are either unilamellar or multilamellar spheres that are manufactured using a variety of lipids. Their structure can be influenced by the choice of lipid composition and manufacturing process. Proteins and other nonlipid molecules can be incorporated into the lipid membrane. Liposomes are classified as either anionic or cationic, based on their net negative or positive charge, respectively.

Anionic liposomes were the first type to be used in *in vivo* delivery of genes (Nicolau *et al.*, 1983). The DNA transgene coding for insulin was encapsulated in anionic liposomes and the complex injected into rats. The transfected rats had increased circulating levels of insulin and decreased blood glucose concentration. Various proteins can be inserted into the external layer of liposomes to alter their *in vivo* behaviour, including selective cell delivery. This approach can enable liposomes given intravenously to evade the reticuloendothelial system. Protein ligands, transferin (Stravidis *et al*., 1986), virus envelope proteins (*e.g* Sendai virus, Vainstein *et al.*, 1983) or antibodies to cell surface molecules (Heath *et al.*, 1987) incorporated into the liposome surface also can target liposomes to specific cell surface receptors on desired cell populations (Wu and Wu 1987). The substance to be delivered must be encapsulated within the liposomes, and this makes the manufacturing process complex. Most DNA constructions necessary for gene therapy are large compared with the liposome, so that encapsulation efficiency is very low.

1.2.1.4 Cationic Liposomes

Felgner and co-workers (1987) synthesised cationic liposomes and demonstrated that they would efficiently bind nucleic acids (which are anionic) by electrostatic interactions upon simple incubation of liposomes with nucleic acids at room temperature for brief periods. The DNA or RNA complexed to cationic liposomes readily entered cells in culture without perceptible injury to the cells (Fig. 1.3). Little is known about the structure of the plasmid-liposome complex. Likewise, the processes affecting cell entry and transport to the nucleus are yet to be clarified. The circular plasmid DNA does not readily incorporate into the host genome and does not replicate in mammalian cells, thus transgene expression is apparently episomal in nature.

In vivo, cationic liposomes properties are quite different from those of anionic liposomes. Intravenous injection of cationic complexes has been shown to effect transgene expression in most organs if the liposome-DNA complex is injected into the afferent blood supply to the organ. In addition, the liposome-DNA complexes can be administered by intra-airway injection or aerosol to target epithelium. In experimental animals, neither intravenous injection nor aerosol delivery of cationic liposome-plasmid complexes appears to be toxic (Brigham *et al.*, 1989). Cationic liposomes have been used to deliver DNA gene constructs in several experimental models *in vivo*. Nabel and colleagues (1994) delivered a foreign histocompatibility gene by direct injection of plasmid-liposome complexes into tumors and showed attenuation of tumour growth in murine models. Hyde and colleagues (1993) showed that cationic liposome mediated gene delivery could correct cystic fibrosis transmembrane conductance regulator (CFTR) protein, cyclic AMP stimulated chloride conductance to normal levels in transgenic mice homozygous for a null mutation in CFTR. Rabbits that had been intravenously given the gene coding for the proximal enzyme in prostanoid synthesis as a plasmid-cationic liposome complex produced increased amounts of endothelium-derived protanoid in their lungs. This protected the

lungs of the transfected animals from the effects of endotoxemia (Conary *et al.*, 1994).

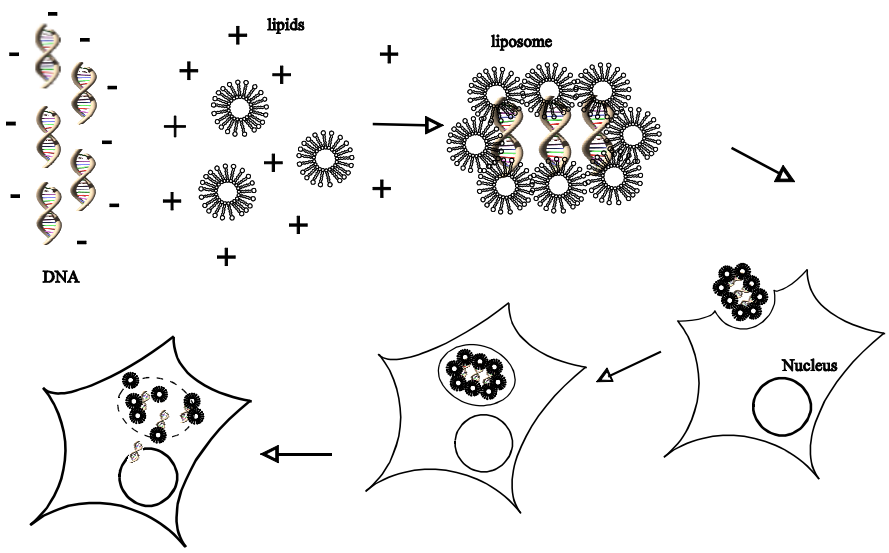

Figure 1.3: Cationic liposomes
Cationic liposomes are positively charged and hence can form a complex with the negatively charged. These complexes can be taken up by the cell via endocytosis. Targeting of this cellular entry can be achieved through the incorporation of antibodies or ligands that recognise specific proteins on the target cell surface.

At present, liposome-mediated transfection offers a non-toxic, non-immunogenic means to deliver DNA to a variety of tissues. Current use of this strategy is limited by generally lower levels of gene transfer than can be obtained with viral vectors.

1.2.1.5 DNA-Protein Conjugates

Methods have also been developed to deliver DNA via the receptor mediated endocytosis pathway. These vectors are known as molecular conjugate vectors (Wu *et al.*, 1987; Wu *et al.*, 1989; Ferkol *et al.*, 1993). The basic design of molecular conjugates is to attach plasmid DNA to a macromolecule ligand that can be internalised by the target cell type. To accomplish this, a molecular conjugate vector possesses two distinct functional domains: a DNA binding domain which is composed of a polycation such as polysine and a ligand domain which binds to a particular cell surface receptor. The polysine domain is chemically linked to the ligand (Wu *et al*., 1991). The polycation not only binds DNA through electrostatic forces but also condenses the DNA allowing it to mimic the compact structure of macromolecules (Wagner *et al.*, 1991). This facilitates the entry of DNA into the cell vesicle system.

Conjugates vectors, which internalise by a normal cellular pathway were first used to achieve gene transfer in hepatocytes (Wu *et al.*, 1991). In this strategy, gene transfer was achieved using the internalisation mechanism for clearance of asialoglycoproteins, which are internalised by hepatocytes. Other groups have also accomplished gene transfer via receptor-mediated endocytosis (Fig. 1.4A) using transferrin-polylysine molecular conjugate vectors (Zenke *et al.*, 1990).

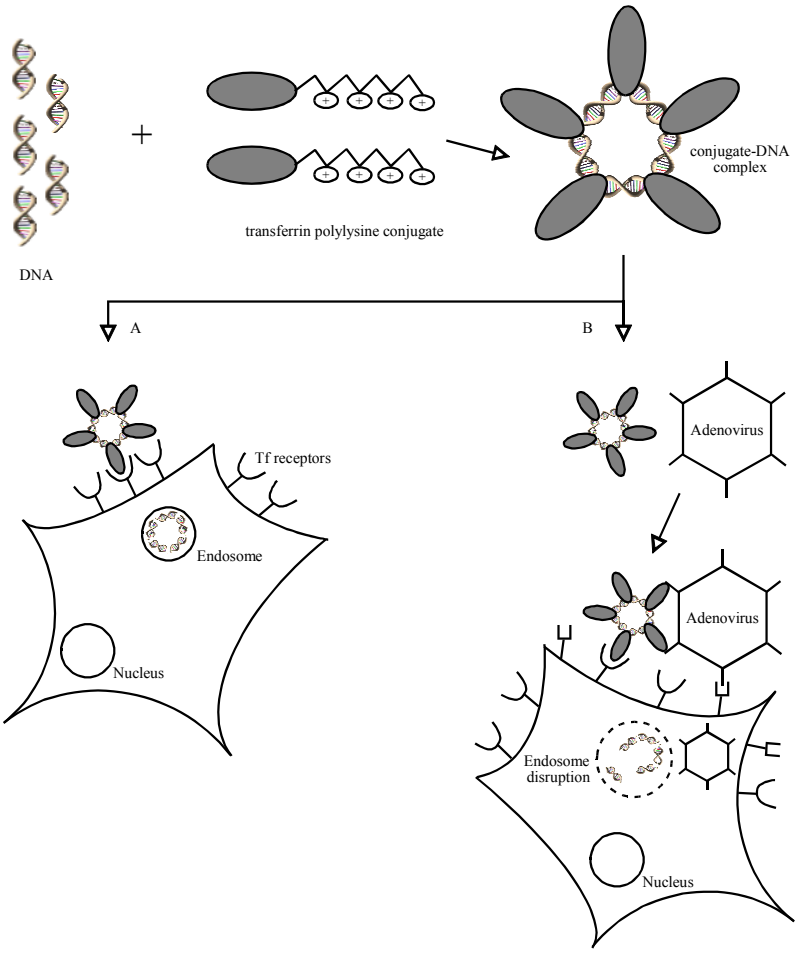

Figure 1.4: Gene Transfer via The Receptor Mediated Endocytosis Pathway
Ligand mediated gene transfer is a transfer method in which DNA is linked to a transferrin-polylysine conjugate. This complex is taken up via receptor mediated endocytosis either with (a) or without (b) the presence of inactivated adenoviral particles. In the presence of the adenoviral particle the DNA is both protected from endosomal degradation and released more freely into the cytoplasm.

In order to target respiratory epithelial cells, other groups have achieved gene transfer via the immunoglobulin A (IgA) transcytosis pathway (Ferkol *et al.*, 1993). This vector system uniquely allows for the capacity of cell-type specific targeting, but early DNA-ligand complexes were inefficient for DNA transfer because most of the endocytosed complex was shuttled to the lysosomal compartment and the DNA hence degraded. Although several lysosomatropic agents have been used to block this type of degradation (Cotten *et al.*, 1990), the efficiency of transfection is still low compared with other DNA-delivery methods.

Cytoplasmic delivery of endocytosed polymer/DNA complexes (or Ligand/DNA) has also been enhanced through the attachment of inactivated adenovirus to the complex (Fig. 1.4B). However, this method is unlikely to be used *in vivo* as it has been shown that systemic delivery of recombinant adenoviruses leads to a prominent host immune response (Smith *et al.*, 1993). It has also been demonstrated that the transfection efficiency of these is much lower *in vivo* than *in vitro*.

1.2.2 Viral Methods

The delivery of nucleic acids into mammalian cells can be made more efficient than physical methods permit through the use of viral vectors that are capable of infecting virtually every cell in a target population (Gluzmann and Hughes, 1988).

The natural life cycle of viruses has made them a logical starting point for the design of therapeutic gene transfer vehicles. The modification of viruses for the delivery of exogenous genes was first reported in 1968. These early attempts using the tobacco mosaic virus showed that viruses could be used to transfer specific genetic material into cells. Studies rapidly shifted to viruses capable of infecting mammalian cells. The first viruses used as gene transfer vectors for mammalian cells were transforming DNA viruses, including the papovaviruses (simian virus 40 and polyoma). These were followed by the retroviruses (Wei *et al.*, 1981; Shimotohno *et al.*, 1981; Temin, 1989). In the meantime, other recombinant viral vector systems have been both developed and improved. Recently, the most popular model vectors for the efficient targeted introduction of foreign genes into mammalian cells have been derived from murine and avian viruses.

The list of viruses that have been studied as gene transfer vectors is large. The most common vectors can be divided into three categories: (1) vectors previously used in patients such as retroviruses (Blaese *et al.*, 1990), adenoviruses (Zabner *et al.*, 1993; Bellon *et al.*, 1999), vaccinia viruses (Hill *et al.*, 1999; Eder *et al.*, 2000), adeno associated virus (Wagner *et al.*, 1998; Wagner *et al.*, 1999) (2) vectors under development such as herpes simplex viruses (Fink *et al*., 1996) including HSV Typ I, Epstein Barr Virus (EBV; Wang *et al*., 1996), Cytomegalavirus (Mocarski *et al.*, 1988) and lentiviruses (Poznansky *et al.*, 1991); (3) future prospects (papilloma virus (Shillitoe *et al.*,1994; Sawamura *et al.*, 2000)), sindbis/semliki forest virus (Berglund *et al.*, 1993) and others. Each of these viruses has both advantages and disadvantages as a gene transfer vector. Vectors, in general, have to be non pathogenic, be able to access the target cells and the expressed gene product must be correctly processed and produced at levels sufficient to complement the disease phenotype.

1.2.2.1 Adenovirus/Adenoviral vectors

The adenoviruses are a family of DNA viruses that can infect both dividing and non-dividing cells, causing benign respiratory infections in humans (Field *et al.*, 1978). They are non-enveloped icosahedral linear double stranded DNA viruses with the ability to infect many cell types. Viral replication is in the nucleus of the cell without integration into the host DNA, although this can happen on occasion. DNA replication is mediated by both viral and cellular proteins. The wild type adenovirus genome is approximately 36-38 kb, of which up to 30 kb can be replaced with foreign DNA (Smith *et al.*, 1995; Verma and Somia, 1997). The virus has both early and late genes. The early genes can be divided into 4 regions, E1 (A,B), E2 (A,B), E3 and E4, which have regulatory functions whereas the late transcript codes for structural proteins.

Adenoviral infection is a highly complex process. Each stage is mediated by the interaction between different components of the viruses with different host cells receptors. After binding to a target cell through the capsid "fibre" protein, the virus is taken up into endosomes where the

low pH leads to its disassociation and release of DNA from the endosomes.The DNA then makes its way to the nucleus where the early proteins are transcribed, leading to DNA replication and transcription of the late genes which give rise to capsid proteins. The proteins are assembled into the cytocapsid. Viral assembly takes place in the cytoplasm and host cell lysis allows their release.

Several adenoviral serotypes are known, with serotypes 2 and 5 being the types most extensively used for vector construction because their molecular composition is well characterised (Van Ormondt, *et al.*, 1984; Chroboczek *et al*., 1992). The serotype 5 vector system is based on bacterial plasmids containing the adenovirus genome with deletions of the E1 and E3 genes. Deletion of E1 renders the virus replication defective. In addition, all or part of the E3 region, which is not essential for virus function, is deleted in order to accommodate the genes of interest, and the plasmid vector can then be grown in bacterial culture. The purified plasmid DNA subsequently is transfected into the 293 line of human embryonic kidney cells. This cell line was derived following transformation of 293 cells (Graham *et al*., 1977) and can thus transcomplement the E1-deficient viral genome. The virus can be isolated from 293 cell media and purified using the limiting dilution plaque assay (Graham and Prevek, 1991). The problems associated with the use of recombinant adenoviruses in gene therapy are mainly due to the hosts cellular and humoral immune response. A second generation of recombinant adenovirus vectors has been generated to overcome this problem. These vectors additionally use an E2a temperature sensitive mutant (which at non-permissive temperatures fails to express late gene products even when E1 in expressed *in trans*) (Engelhardt *et al.*, 1994) or an E4 deletion (Armentano *et al.*, 1997). The most recent "gutless" vectors contain only the inverted repeats (ITRs) and packaging sequence around the transgene with all the necessary viral genes being provided *in trans* by a helper virus (Chen *et al.*, 1997).

The main advantages of adenoviral vectors is that the transduction efficiency is high, as is the level of gene expression, although this is only transient and deteriorates rapidly within a few weeks or months. One of the other main disadvantages of adenoviral vectors is that cell-specific targeting is difficult to achieve as the virus has no envelope to attach cell-specific ligands to, as can be achieved with retroviruses. Furthermore, the adenovirus receptor is virtually ubiquitous and consequently systemic administration is likely to lead to adenoviral uptake in cell types other than the target cell thereby reducing the specificity of the gene therapy. It has been demonstrated that although repeat administration of adenovirus is possible, the gene transfer becomes progressively less efficient. Adenoviruses have also been implicated in causing cardiotoxicity and brain damage, as well as causing neurogenic and pulmonary inflammation at high doses and over longer periods of time.

1.2.2.2 Adeno-Associated Viruses

Adeno-associated viruses (AAV) are non-pathogenic human parvoviruses, dependant on a helper virus, usually an adenovirus or herpes virus, to proliferate. They are capable of infecting both dividing and non-dividing cells. The AAV genome is single stranded and 4.68 kb in length (Green and Roeder, 1980; Hermonat and Muzyczak, 1984). Structurally, it is composed of two open reading frames (rep and cap) flanked by inverted repeat (ITR) sequences. The rep region encodes four proteins which mediate AAV replication, viral DNA transcription and endonuclease

functions used in host genome integration. The rep genes are the only AAV sequences required for viral replication. The cap region encodes structural proteins that form the viral capsid. The ITRs, 145 bp in length, are located at the extreme ends of the genome. They contain the viral origin of replication, provide encapsidation signals, and participate in viral DNA integration. Integration of wild type virus is specific for chromosome 19 (19q13.3-qter) at a high frequency (Kotin *et al.*, 1990), or at least shows preferential integration at this site.

Recombinant, replication defective viruses that have been developed for gene therapy lack rep and cap sequences. Production of the recombinant vector requires that Rep and Cap are provided *in trans*, along with helper virus gene products. The conventional method is to cotransfect two plasmids, one for the vector and the other for rep and cap, into 293 cells infected with wild -type adenovirus or HSV (Samulski *et al.*, 1989). Infection with either adenovirus or HSV provides helper functions that induce the synthesis of capsid proteins, which in turn transactivate the synthesis of capsid proteins. The transgene flanked by the ITRs is then packaged into viral particles that can be isolated and purified. The cells produce mature recombinant AAV vectors as well as wild-type adenovirus or HSV. The wild type adenovirus or HSV is removed by either density/gradient centrifugation or heat inactivation. This method is cumbersome, low yielding ($<10^4$ particles/ml) and prone to contamination with adenovirus and wild type AAV. One of the reasons for the low yield is the inhibitory effect of the rep gene product on adenovirus replication (Vincent *et al.*, 1997). More recent protocols remove all adenoviral structural genes and use rep resistant plasmids (Xiao *et al.*, 1997) or conjugate a rep expression plasmid to the mature virus prior to infection (Fisher *et al.*, 1996). In the absence of Rep, the AAV vector will only integrate at random, as a single viral genome or head to tail concatamers, once the terminal repeats have been slightly degraded (Rutledge and Russel, 1997).

Interest in AAV vectors has been due to their integration into the host genome which allows prolonged transgene expression. Gene transfer into vascular epithelial cells (Maeda *et al.*, 1997), striated muscle (Fisher *et al.*, 1997; Herzog *et al.*, 1997) and hepatic cells (Synder *et al.*, 1997) has been reported, with prolonged expression when the transgene is not derived from a different species. Neutralising antibodies to the AAV capsid may be detectable, but this does not prevent readministration of the vector or shut down promoter activity. There is no inflammatory or immune response.

AAV vectors have produced some promising results and have a wide variety of theoretical advantages but there are numerous problems. They can only carry a fairly small therapeutic gene insert, with certain foreign DNA sequences appearing to inhibit AAV DNA replication. Mechanisms of AAV integration are poorly understood. AAV can also be rescued from the human genome by means of co-infection with a helper virus and such a rescue into the general population may have serious consequences.

1.2.2.3 Herpes Simplex Virus

The herpes simplex virus 1 (HSV-1) is a natural pathogen of humans, causing recurrent oropharyngeal cold sores. The virus itself is transmitted by direct contact and infects epithelial cells, gains access to the sensory neurone endings supplying the infected area of skin and travels by retrograde axonal flow to neuronal cell bodies within the respective dorsal root ganglia

(Lachmann *et al.*, 1997). It exhibits both a lytic and latent function. It is an enveloped virus with a double-stranded DNA genome of 152 kb that replicates in the nucleus. The genome consist of two unique sequence stretches, U_L and U_S which are flanked in either orientation by internal repeats sequences (IRL and IRS). At the non-linker end of the unique regions are terminal repeats (TRL and TRS). There are up to 81 genes (Marconi *et al.*, 1996), of which about half are not essential for growth in cell culture. Once these non-essential genes have been deleted, 40-50 kb of foreign DNA can be accommodated within the virus (Glorioso *et al.*, 1995). Three main classes of HSV-1 genes have been identified, the immediate-early (IE or alpha) genes, early (E or beta) genes and late (L or gamma) genes. The viral gene products are synthesised in a highly co-ordinated temporal and functional sequence (Kennedy *et al*., 1997). IE genes are transcribed first after activation by Vmw65, a structural protein. Protein products of IE genes (ICPO, ICP4, ICP22, ICP27 and ICP47) act as transacting factors that are involved in the regulation of subsequently expressed viral genes. E genes code enzymes, such as thymidine kinase (TK), DNA polymerase and ribonucleotide reductase, needed for DNA replication and nucleotide metabolism. L genes code for viral structural proteins.

The molecular events leading to the establishment of latency have not been fully determined. Gene expression during latency is driven by the latency- associated transcripts (LATs) located in the IRL region of the genome. Two LATs (2 and 1.5 kb) are transcribed in the opposite direction to the IE gene ICPO. LATs have a role in HSV-1 reactivation from latency (Steiner *et al*., 1989) and establishment of the latency (Sawtell and Thomson, 1992). Two latency active promoters which drive expression of the LATs have been identified (Marconi *et al.*, 1996) and may prove useful for vector transgene expression.

Interest in HSVs as a vector relates primarily to their ability to establish long-term latent infections in neural cells. Gene therapy has been suggested as a potential therapeutic approach to a vast range of a number of neurological disorders, ranging from inherited metabolic disorders to degenerative diseases and malignancy.

Two basic approaches have been used for production of HSV-1 vectors; amplicons and recombinant HSV-1 viruses. Amplicons are defective plasmid derived vectors (Stow and McMonagle, 1982). They consist of sequences required for propagation [col E1, an Escherishia coli origin of replication)] and selection in bacteria. In addition, they contain an HSV-1 origin of replication (OriS) for propagation in mammalian cells (Valzny *et al.*, 1981), the HSV-1 packaging signal (Valzny *et al.*, 1982; Stow *et al.*, 1983) and the transgene under control of an immediate-early promoter (Federoff *et al.*, 1992). The amplicon is transfected into a cell line containing a helper virus (a temperature sensitive mutant) which provides all the missing structural and regulatory genes *in trans*. Viral particles of both helper and amplicon origin are delivered to the recipient. More recent amplicons include an Epstein-Barr virus derived sequence for plasmid episomal maintenance (Wang and Vos, 1996).

Recombinant viruses are made replication deficient by deletion of one the immediate-early genes (e.g ICP4), which is provided *in trans* in a producer cell. The transgene of interest is introduced by transfection of an expression plasmid flanked by HSV-1 sequences. After super infection, recombination occurs between the HSV genome and the transfected plasmid. They are less pathogenic and can direct transgene expression in brain tissue, although they are toxic to neurons

in cell culture (Marconi *et al.*, 1996). Deletion of a number of immediate-early genes substantially reduces cytotoxicity and also allows expression from the promoters that would be silenced in the wild type latent virus. These promoters may be use in directing long term gene expression.

Replication-conditional mutants are only able to replicate in certain cell lines. Permissive cell lines are all proliferate and supply a cellular enzyme to complement for a viral deficiency. Mutants include thymidine kinase (During *et al.*, 1994), ribonucleotide reductase (Kramm *et al.*, 1997), UTPase or the neurovirulence factor g34.5 (Kesari *et al.*, 1995). These mutants are particularly useful for the treatment of cancers, killing the neoplastic cells (Andreansky *et al.*, 1996, 1997).

The advantages of HSV-1 vector systems include their wide host range, the ability to infect non-replicating cells, the availability of high titre stocks and a capacity to carry large transgenes. Although HSV-1 vectors might appear to be a good candidate for gene therapy approaches, especially in central nervous systen (CNS), such vectors have several disadvantages which makes it unsuitable for gene delivery. Experiments indicate that HSV-1 derived vevtors have a limited duration of transgene expression (Geller and Freese 1990; Geller, 1991; Geller *et al.*, 1993) as well some cytotoxicity (Johnson *et al.*, 1992).

1.2.2.4 Vaccinia Virus

Vaccinia virus (VV) is a member of the poxvirus family. Infectious virus particles are brick shaped and 300-400 nm in diameter. Lipoprotein membranes surround a complex core structure that contains a linear 200 kb DNA molecule. The virus replicates in the cytoplasm of infected cells.

The extensive clinical experience with vaccinia vaccines and their ease of manipulation have led to efforts to develop gene therapy vectors from pox viruses (Moss *et al.*, 1996). Vectors are constructed by inserting the gene of interest into a non-essential gene sequence of a plasmid vaccinia expression vector followed by transfection into cells that are already infected with vaccinia virus. Recombination events result in the generation of a recombinant viral vector that can be identified by several methods. The identified recombinant virus is then plaque purified. These vectors offer the potential to developed a large variety of gene therapy based vaccinations. Recombinant VV has been used for the delivery of suicide genes for murine liver metastases (Gnant *et al.*,1999) and it has been experimentally used to express interleukin-12 and induce tumour regression (Meko *et al.*, 1996). The system is also being examined in the treatment of HIV.

Vaccinia virus can infect a large variety of cell types and can carry large amounts of foreign DNA under the control of VV promoters. Levels of transgene expression are relatively high but short (1 to 4 weeks). One of the main safety problems of VV vectors is the demonstrated toxicity of replication competent virus in immunosuppressed individuals. In addition, vaccinia vaccination can cause disease in healthy individuals at an overall rate of approximately 1 in 20,000 vaccinations.

1.3 Retroviruses

Retroviruses are a class of RNA viruses that are capable of converting their RNA into DNA, and integrating into the host genome. The so-called provirus, the integrated form of the viral DNA, serves as the basis for the synthesis of viral RNA which will be packaged into newly formed viral particles, as well as forming the template for mRNA synthesis which subsequently leads to viral protein production (Varmus, 1982).

Retroviruses were first discovered in 1904 by Ellermann and Bang. They described "as a filtrable agent that causes cancer in chickens". Only decades later was the Rous Sarcoma Virus (RSV) named and classified as a retrovirus. Retroviruses have been found in all vertebrate animals in which they have been sought including fish, birds, rodents, cats, ungulants, non human primates and humans (Varmus, 1988). Examples have been found in lower organisms such as insects (Drosophila, gipsy-element) and annelids (Varmus, 1983; Dahlberg, 1988). Only since the 1980's have infectious retroviruses shown to exist in humans (such as HTLV-1, HTLV-2 and HIV).

1.3.1 Retroviral Classification

Retroviruses belong to the family of the *Retroviridae*. The have been divided into the subfamilies of the *Oncovirinae*, *Spumaviriniae* and *Lentivirinae.* (Fig. 1.5).

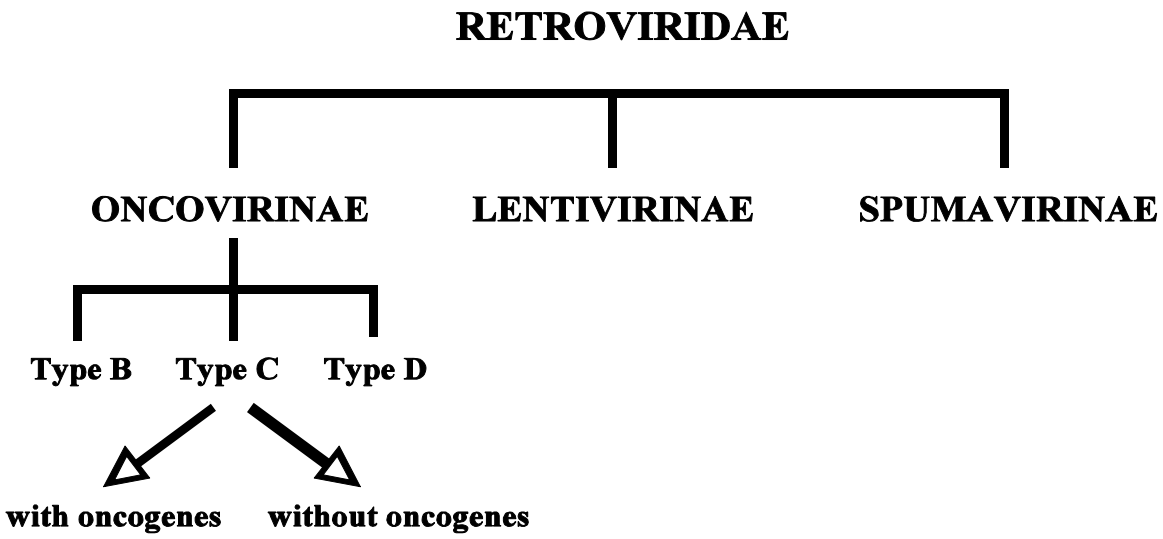

Figure 1.5: The Family of the Retroviridae

The largest subfamily, the *Oncovirinae*, is divided into those viruses that carry oncogenes and those that do not. Retroviruses, during their lifecycle (see retroviral lifecycle), are capable of picking up cellular genetic information. Viral oncogenes (*v-onc*) are those sequences that have been acquired by retroviruses and are homologous to known regulatory genes in the cell (Stehelin et al., 1976; Bishop, 1985). The cellular analogues themselves are mainly genes that are involved in the regulation of growth in the cell and are termed proto-oncogenes (*c-onc*). Through the

relatively strong enhancer activity of the viral LTR, the viral oncogenes become constitutively and resultantly uncontrollably expressed. Expression that occurs at the wrong time or to the wrong extent could lead to a change in growth of single cells within a tissue. It has also been shown *in vitro* that these genes are capable of transforming cells after infection by a retrovirus carrying them. This is also the case in animals that have been infected by such retroviruses. In the case of the *ras* oncogene (Weinberg, 1989) expression of the mutated protein leads to a neoplastic transformation of the infected cell.

The uptake of cellular genes can, however, lead to the loss of viral sequences that code for structural proteins and are therefore required for viral replication. Such uptake events lead to the creation of replication defective viruses. Helper viruses, that the supply structural genes *in trans*, are required to produce infectious particles. The Rous Sarcoma Virus has also taken up additional cellular sequences, in the form of *src*, but has done so without the loss of the genetic information required for the production of the structural proteins.

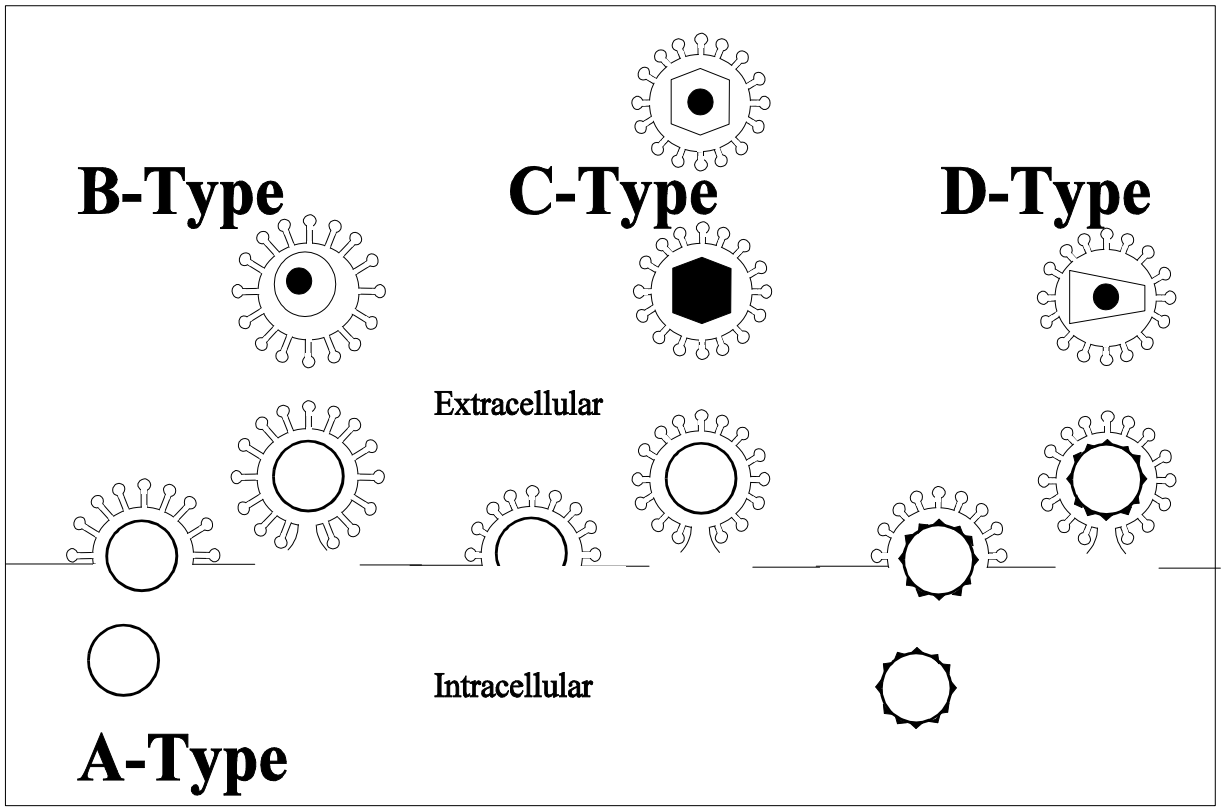

Figure 1.6: Morphological Classification of Retroviruses
Historically, retroviruses divided into groups based on their morphology in negatively-stained E.M. pictures.

The *Oncovirinae* are also divided into the groups A, B, C and D based upon their morphology (Bernhard, 1958; Fig. 1.6). Type A particles are the predecessors of type B particles. They are naked, intracellular viral capsids that are uninfectious and reside in the cytoplasm. Type B particles form their core in the cytoplasm but not directly on the plasma membrane. (Bernhard,

1960). After leaving the host cell they exhibit an eccentric core and prominent spikes (surface proteins). An example of type B retroviruses is the Mouse Mammary Tumour Virus (MMTV). The majority of viruses that are classified within the *Oncovirinae* belong to the type C retroviruses. e.g. Moloney Murine Leukaemia Virus (MoMuLV). They possess a concentric capsid. The particle itself is formed directly at the cell membrane where it will later leave the cell.

Type D particles have the characteristics of both B type and C type viruses and are represented by primate specific types eg. Mason Pfizer Monkey Virus (Chopra and Mason, 1970; Jensen *et al.,* 1970). However, after budding, the resultant particle exhibits a rod-shaped core (Fig. 1.6).

The second subfamily is the *Lentivirinae*, to which the so-called "slow viruses" belong. They cause diseases, which are not classified as neoplastic changes, after long periods of latency following the initial infection event. A typical example of this subfamily is HIV (Human Immunodeficiency Virus) whose infection is followed by a chronic degenerative disease of the immune system.

A further subfamily is the *Spumavirinae*, which are also know as the "foamy" viruses (e.g. HSRV, Human Spuma Retrovirus). They cause chronic infections which are not accompanied by any obvious clinical symptoms. Although such viruses have been isolated from tissues that have been taken from both patients with nasopharangeal carcinoma (Achong *et al.,* 1971), and chronic myelogenic leukaemia, no correlation between infection and disease has been shown. However, studies have shown a link between foamy virus infection and neurodegenerative disease (Aguzzi, 1993).

1.3.2 Host Specificity

The MoMuLV retroviruses, which are the most common basis for retroviral vectors, can also be classified depending on the type of their host specificity. Ecotropic viruses are those that can only infect the same species of cell as they originated from. Virus that originate from one species of cell but can subsequently only infect another species are known as xenotropic. Amphotropic viruses have the potential to infect and actively replicate in cells of many species including the species from which they originated (Teich, 1985).

1.3.3 Retroviral Structure

There is considerable diversity between the various types of retrovirus. Retroviruses are spherical particles between approximately 90 and 120 nm in diameter, with a nucleoprotein core wrapped in an envelope (Fig. 1.7). The envelope carries a virus-encoded glycoprotein, which forms spikes in the membrane. There are certain structural and functional similarities between the envelope glycoprotein and influenza haemagglutinin. The mature protein is cleaved into two polypeptides. The outer envelope glycoprotein (SU) (Tab. 1.3), the major antigen of the virus, responsible for the receptor binding is linked by disulfide bands to the transmembrane protein (TM) which holds the SU protein in the envelope and is responsible for membrane fusion.

Inside the membrane is the matrix (MA) protein, which is rather amorphous. This largely obscures the capsid (CA), which is believed to be icosahedral. The capsid is the most abundant protein in the particle. Inside the capsid is the core which contains the RNA genome; nucleocapsid protein (NC), reverse transcriptase (RT) and integrase (IN). The protease (PR) is localised between the capsid and the envelope.

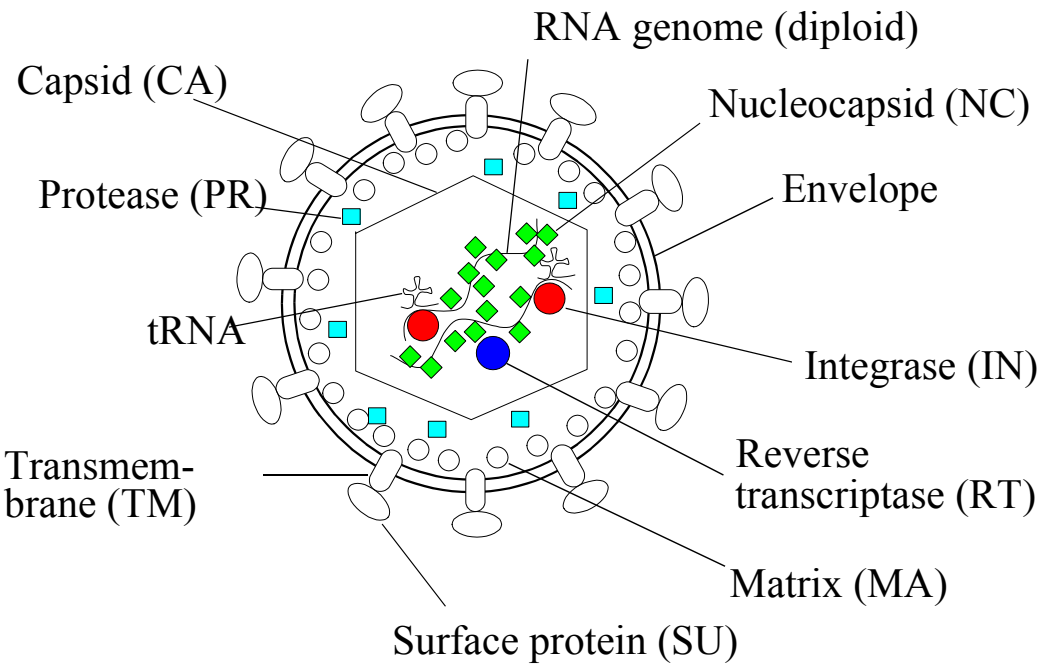

Figure 1.7: Structure of Retroviruses
Retroviruses are viruses that carry two copies of positively charged single-stranded genomic RNA and possess an envelope. Each of the genomic RNA strands is associated with a cellular tRNA. The genomic RNA is also associated with the viral coded reverse transcriptase (RT) and integrase (IN) and is protected by the nucleocapsid (NC). The capsid protein (CA) forms a shell around the nucleoprotein complex. The matrix protein (MA) like the viral protease (PR) lies outside the capsid and links it with the outer envelope. The envelope is formed from the cellular membrane of the host cell and also contains the transmembrane protein (TM) as well as the protruding surface protein (SU).

Name	Protein	Function
MA	Matrix	*gag* gene matrix protein, lines envelope
CA	Capsid	*gag* gene capsid protein, protects the core
NC	Nucleocapsid	*gag* gene RNA binding protein, forms the core
PR	Protease	Essential for *gag* protein cleavage during maturation
RT	Reverse Transcriptase	Reverse transcribes RNA genome, transcriptase also has RNAse H activity
IN	Integrase	Encoded by the *pol* gene, needed for integration of provirus
SU	Surface Protein	The outer envelope glycoprotein
TM	Transmembrane Protein	The inner part of the mature envelope protein

Table 1.3: The Function of Retroviral Proteins
All these proteins are essential for replication; some retroviruses also encode additional essential and non-essential proteins.

1.3.4 The Retroviral Lifecycle

The retroviral lifecycle can be divided into several different stages (Fig. 1.8); the penetration of the host cell, reverse transcription of the RNA into DNA, integration of the DNA into the host cells' genome and transcription of the provirus to produce viral RNA which, after packaging together with other virally produced proteins leaves the cells as a new virus particle.

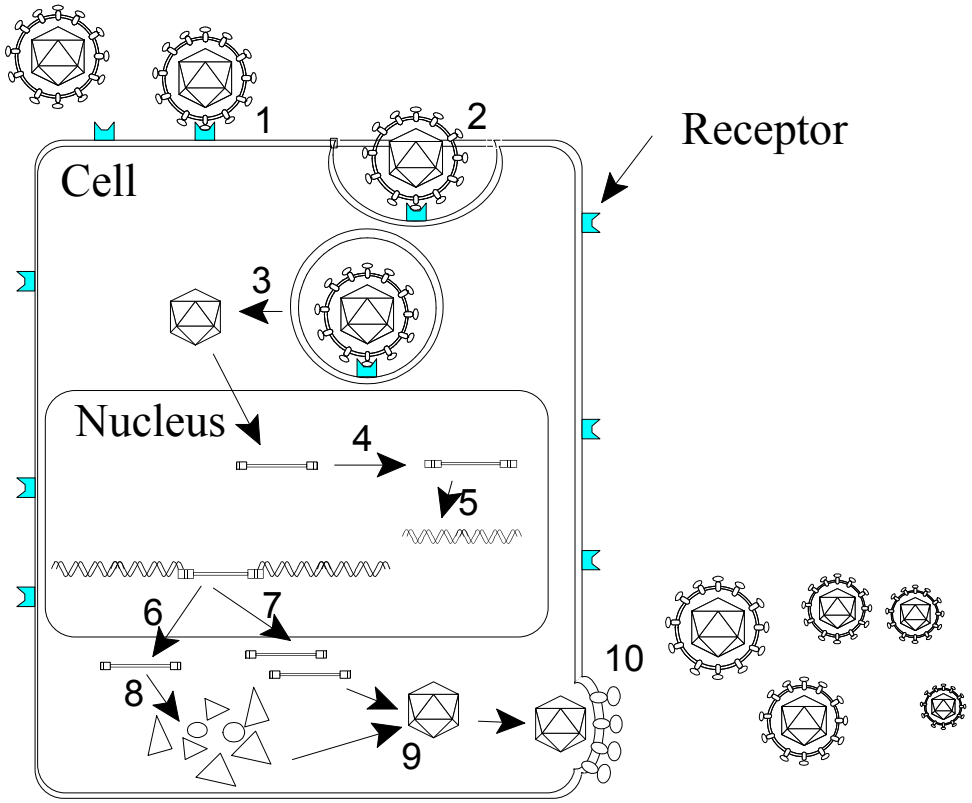

Figure 1.8: The Retroviral Lifecycle
Receptor binding (1), endocytosis (2), beginning of reverse transcription (RT) (3), end of reverse transcription (4), integration (5), transcription of mRNA (6) and genomic RNA (7), synthesis of viral proteins (8), nucleocapsid formation (9) release of viral particles via budding (10).

1.3.5 Host Cell Invasion

Entry into the host cell is achieved via a specific interaction between viral glycoproteins and cellular membrane proteins. The cellular proteins act as receptors to the viral proteins and their interaction is followed by endocytosis (Weiss, 1993). However, HIV can enter the cell through both receptor mediated endocytosis (Bauer *et al*., 1987), as well as via membrane fusion (Stein *et al*., 1987). Evidence has also been found that indicates that D-type Mason Pfizer Momkey Virus, C-type Simian Sarcoma Associated Virus (Sommerfeld und Weiss, 1990) and B-type Mouse Mammary Tumour Virus (Redmond *et al*., 1984) are also capable of gaining entry to the cell via membrane fusion.

After entry into the cell, the membrane proteins are thought to be partly removed by cellular enzymes (uncoating) and the nucleocapsid moves into the nucleus. The movement of the nucleocapsid into the nucleus is also probably accompanied by the degradation of the nuclear membrane as the nuclear pores are too small to accommodate a particle of the size of the capsid. This would also explain the fact that retroviruses require actively dividing host cells in order to infect efficiently (Roe *et al*., 1993). Once more HIV-1 is an exception in this case as it can also infect mitotic and nerve cells in G_0 (Bukrinsk *et al*., 1993). This capability is due to two redundant mechanisms. In the first a nuclear localisation signal (NLS) in the matrix protein (MA) is recognised by the Integrase (IN) after it has been phosphorylated. This complex is then actively transported into the nucleus. (Gallay *et al*., 1995). The HIV-specific protein Vpr is central to the second mechanism. It is incorporated into the viral particle via an interaction with the gag proteins. The Vpr can also drive the migration of the pre-integration complex to the nucleus using a, as yet, unexplained mechanism.

1.3.6 Reverse Transcription and Integration

Retroviruses carry a diploid genome. This means the viral particles contain two identical RNA molecules, which are themselves similar to cellular mRNAs in that they have a 5' cap site and a 3' polyadenylation. An RNA dependent DNA polymerase reverse transcribes the RNA into a double stranded DNA (Temin und Baltimore, 1972). During this process the unique regions (U), which lie at the ends of the viral RNA, are copied and duplicated to both ends of the newly synthesised DNA. Thus, the long terminal repeats (LTRs) are created (Fig. 1.9).

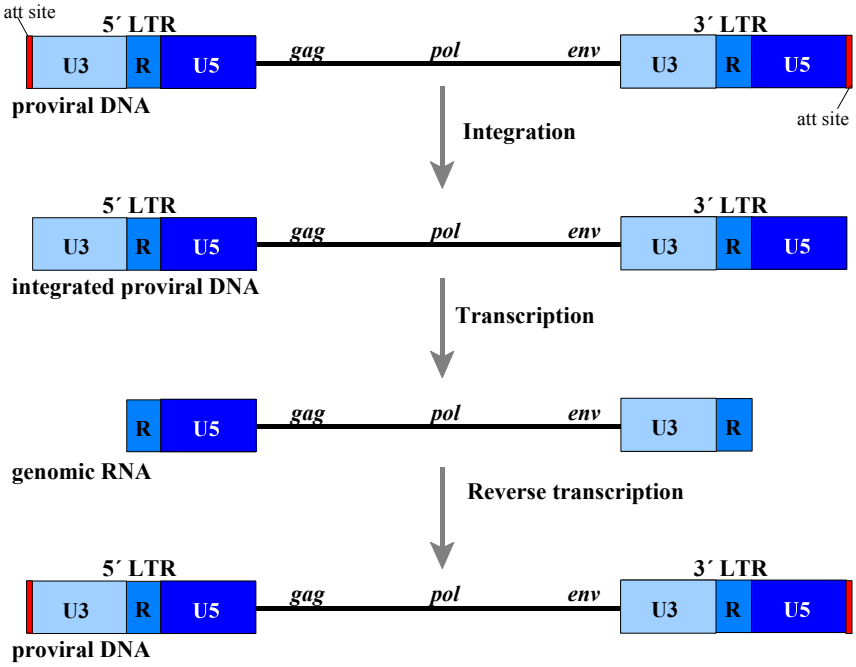

Figure 1.9 Comparison of Viral RNA and Proviral DNA

Specific tRNA molecules, which differ between retroviruses (*e.g* Leu or Gly for MoMLV), serve as primers for DNA synthesis and are delivered in the retroviral capsid. Short RNA fragments function as primers in the (+) DNA synthesis. During reverse transcription the end regions (U3 of the 3' end, U5 of the 5' end) are duplicated and the LTRs formed. As a result, the viral DNA is longer than the RNA genome. Viral integrase, which possesses both endonuclease and polymerase activity, completes the integration of the linear DNA into the host cell (Panganiban and Temin, 1984).

The first integration events can be detected after 8 hours, with the majority of the molecules completing the process between 12 and 48 hours after contact with the cell (Varmus *et al.*, 1973). Integration itself begins with the recognition of the nucleotides at the ends of the LTRs by the integrase (IN). This is followed by the processing of the last two bases of the LTRs to a single stranded status thus leading to a two base pair overhang at the end of the reverse transcribed DNA. At the same time, the IN cuts the cellular DNA at a seemingly random position. However, there does seem to be some preference for areas of transcriptionally active chromatin or areas of DNA containing large grooves (Goff *et al.*, 1992). The overhangs at the ends of both the viral and cellular DNA are joined together and ligated, even if the ends do not have complementary sequences. After ligation, such non-complementary joins are repaired by cellular mechanisms (Fig. 1.10).

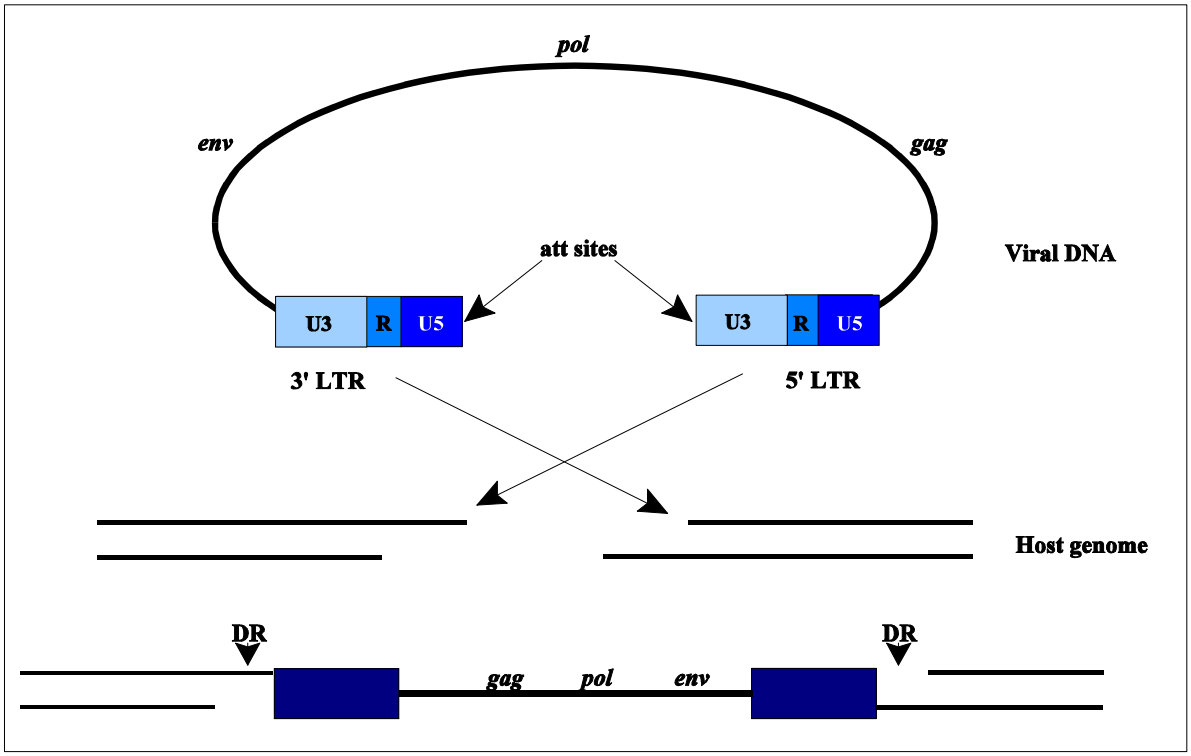

Figure 1.10: Integration of the Provirus into the host cell Genome
The integration is catalysed by the viral integrase (IN).The enzyme has both endonuclease and polymerase activity and coded for from the *pol*-region. During integration both ends of the viral DNA are cut by the integrase (IN) at the attachment sites (att sites, inverse repeats (IR), in such a manner that and overhang from 4-6 base pairs occurs. Simultaneously, the host cell genome is cut at the future site of integration (also creating an overhang) after which the viral and cellular DNA is joined. Cellular repair mechanisms remove unpaired bases from the attachment sites of the viral DNA, fill in the overhangs and thus connect the viral with the cellular DNA. This leads to the creation of the direct repeats (DR) at the sites of integration in the host genome (Shoemaker *et al.*, 1980).

The result is an integrated viral DNA (Provirus), that is a total of four base pairs shorter (two from each end) is than before integration took place (Fig. 1.11). The provirus always integrates in a colinear fashion. This means in the form of LTR - retroviral genes - LTR, which is crucial for the correct transfer of the genetic information.

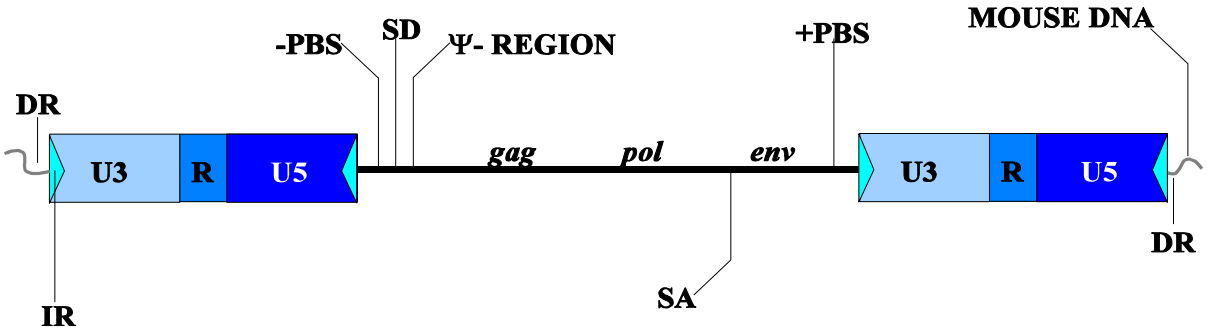

Figure 1.11: Retroviral Structural Elements

DR (Direct Repeat): These direct sequence repeats flank the integrated provirus, though they are not of retroviral origin. They arise during integration when cellular mechanisms repair overhangs/mismatches between the proviral DNA and the host cells' genome. They arise after the filling up of the overhangs created after the joining of the viral with the host DNA. Their length is dependent upon the retrovirus (Goff, 1992), e.g. B MLV and BLV have direct repeats of 4 base pairs whereas MMTV has 6 base pairs for its' repeats.

IR (Inverted Repeats): These reverse sequence repeats form the borders of the LTRs. They are important for the recognition of the LTRs by the integrase.

U3-region (Unique 3'-region): The unique 3'-Region carries an IR at its' 5' end, the promoter/enhancer as well as regulatory elements for the transcription of the viral RNA (beginning at the position +1). It is present at the 3' end of the viral RNA, but is duplicated and copied to the 5' end during reverse transcription to be present in both LTRs.

R-region (Repeat region): The sequences are between 18 and 250 nucleotides long and are present at both ends of the viral RNA. These repeated sequences play an important role during the first jump (DNA strand transfer) of reverse transcription.

U5-region (Unique 5'-region): This sequence does not code for any viral proteins, but plays important roles in packaging of the genomic viral RNA (in conjunction with the ψ-region) as well as in reverse transcription and subsequent integration of the viral DNA (Murphy and Goff, 1989). It is only present once in the viral RNA but is duplicated during the process of reverse transcription.

-PBS (-Primer Binding Site): This region is found at the 3' end of the U5-region and contains a sequence that is complementary to the 3' end of the tRNA (Peters and Glover, 1980). It is therefore essential for reverse transcription.

SD-site (Splice Donor): This site lies in the 5' part of the *gag*-gene (Hackett *et al*., 1982) and is necessary for the production of the subgenomic *env* mRNA.

ψ-region (Packaging Signal Region): This region is between 100 and 300 nucleotides long and lies after the 3' SD site and the 5' AUG *gag* start codon. It is an obligatory recognition signal required for the selective packaging of full-length genomic retroviral RNA. The hairpin structure formed by the RNA is recognised by the retroviral proteins as a packaging signal and leads to packaging of the viral RNA (Mann *et al.*, 1983). RNA without this signal is either not packaged at all or packaged very inefficiently into budding viral particles.

***gag*-region:** The *gag*-region codes for an 80 KD polyprotein which gives rise to viral proteins which form the capsid after post-translational processing (Dickson *et al*., 1982). They include: matrix protein (MA), that is found between the nucleocapsid and the envelope as well as the capsid protein (CA) and the nucleocapsid protein (NC).

***pol*-region:** The *pol*-region is transcribed together with the *gag*-Region as a *gag-pol* precursor polyprotein. Following post-translational processing reverse transcriptase (RT) and integrase (IN) are formed.

***env*-region:** The 1.8 kb *env*-region codes for a polyprotein, which, after glycosylation, can be modified and incorporated in the cell membrane of the host. The resultant mature proteins are the transmemnbrane protein (TM) and the surface protein (SU), which form the main part of the viral coat (Dickson *et al*., 1982). The surface proteins determine the host range specificity and act as antigens for the primary immune response of the host.

+PBS (+Primer Binding Site): This primer binding site lies in a purine rich region at the end of the *env*-region (Polypurine tract, PPT). Due to the RNAse-H activity of the reverse transcriptase, a (-) DNA complementary RNA sequence is formed which acts as a primer for the synthesis of the (+) DNA strand.

1.3.7 Expression of Retroviral Genes

Retroviruses use cellular transcriptional machinery for expression of their proteins (although a few encode additional transcriptional and post-transcriptional regulatory factors *e.g.* HTLV and HIV). Therefore all viral proteins are expressed in the same manner as cellular genes.

The LTRs are not only involved in integration, but also play a role in the transcription of the provirus. The enhancer and promoter present in the 5' LTR control expression via the RNA Polymerase II. RNAs for both the synthesis of viral proteins and for incorporation into newly formed particles are produced (Fig. 1.12). After synthesis, the genomic RNA and the full length mRNA used for protein translation exhibit different secondary structures, which in the case of the mRNA, are used during translation (Darlix, 1986).

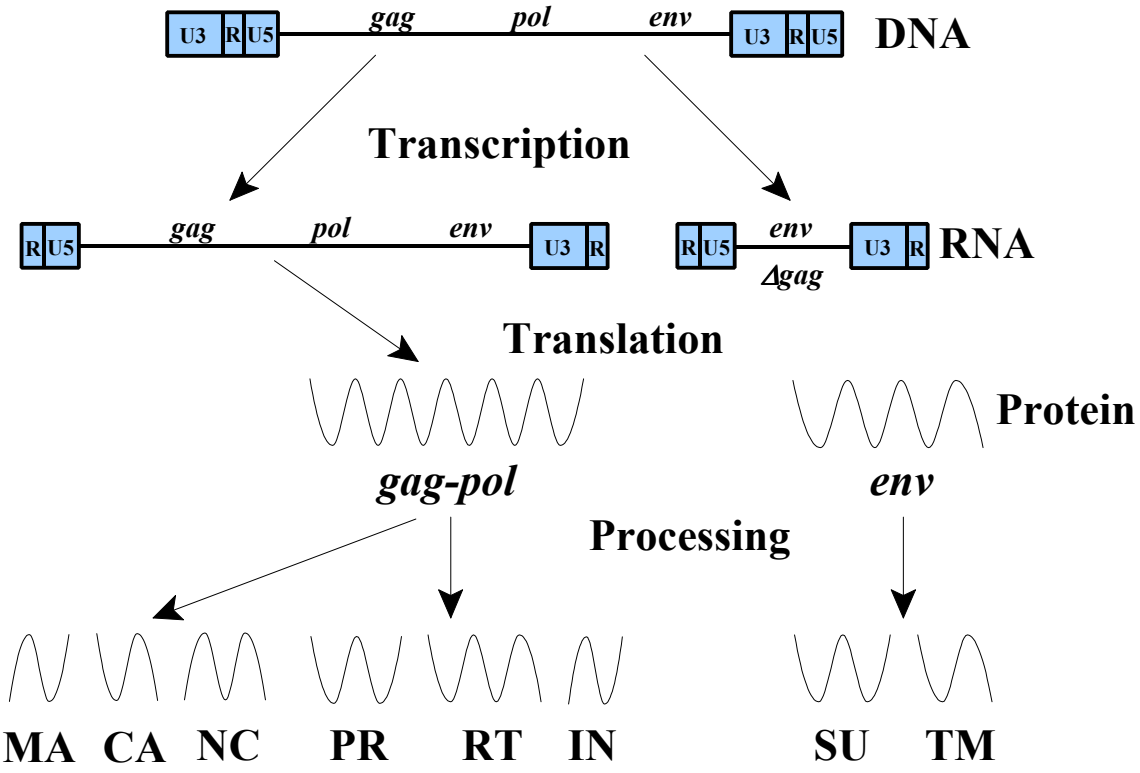

Figure 1.12: Transcription and Translation of Viral Genes
Transcription starts at the first nucleotide of the R region at the 5' end of the Provirus and ends at the last nucleotide of the 3' R region. This transcript of genomic length (mRNA) is used for the translation of the *gag* and *pol* proteins. A second spliced transcript, in which the *gag* and *pol* sequences are not transcribed between the splice donor (SD) and splice acceptor, is translated into the coat proteins.
The *gag* and *gag-pol* proteins are cleaved by the viral protease whereas the *env* protein is cleaved by cellular proteases into its' components. i.e. capsid (CA), matrix (MA), and nucleocapsid (NC) proteins in the case of the *gag*. Similarly to the *gag* polyprotein, the *pol* products (i.e. PR, RT, IN) are created after post translational processing. The *env* mRNA is translated and processed by membrane bound ribosomes in such a manner that, in addition to the signal peptide, the transmembrane (TU) and surface (SU) proteins are created. Intracellularly, they are linked to each other and are anchored in the plasma membrane.

The *gag* and *pol* proteins are translated from genomic length transcripts in the form of a polyprotein (Fig. 1.12). This precursor protein is proteolytically processed into the mature *gag* (MA; CA; NC) and *pol* (RT; IN) proteins. The *env* proteins (SU and TM) are translated from a subgenomic spliced viral RNA. Cellular proteases are responsible for the cleavage of the precursor protein into the mature SU and TM proteins.

Following the association of the viral genomic RNA with the capsid proteins, integrase and reverse transcriptase the core moves towards the cell membrane where it buds from an area where a high concentration of *env* proteins have associated.

1.3.8 Establishment of Retroviral Vectors

Most retroviral vectors are derived from murine or avian retroviruses. Retroviral vectors based on Moloney murine leukaemia virus (MoMuLV) are the most commonly used, particularly for gene therapeutic experiments in rodents and humans. The first retroviral vector was described in 1981 (Wei *et al*., 1981) and the first patient was treated (for adenosine deaminase deficiency) in 1990 (Andersen *et al*., 1990). They are the most popular vectors for gene transfer in clinical trials, being used in 60-70% of human gene therapy trials.

Almost all retroviral vector systems consist of two components: (i) the recombinant retroviral vector molecule that carries the gene or genes of interest and (ii) constructs providing retroviral structural proteins in *trans*. Together, these two components allow the production of recombinant viral particles capable of infecting target cells.

Retroviral vectors are derivatives of wild-type, replication competent retroviruses in which part of the retroviral coding information (*gag, pol,* and *env*) has been replaced by the gene or genes to be transferred to the target cell (Fig. 1.13A). Usually these vectors contain at least two heterologous genes: (i) a drug selectable marker, and (ii) the therapeutic gene to be transferred.

The two genes can be expressed from the same retroviral promoter (Gilboa *et al*., 1986) where the natural splicing mechanism of the retrovirus is utilised to generate different RNAs from which the gene products are separately expressed (Fig. 1.13$B_{1\text{-}2}$). Alternatively, the genes can be expressed from different promoters, usually one from the retrovirus itself and one introduced along with the construct (Levine *et al*,. 1991; Koo *et al*., 1992).

A wide variety of vector types have been described including those with LTRs as the major promoters (Miller *et al*., 1983) and those with internal promoters (Episkopou *et al*., 1984; Overell *et al*., 1988). It has been observed that introduction of internal, heterologous promoters can be problematic due to interference that can occur between the heterologous and viral promoters (Xu *et al*., 1989; Wu *et al*., 1996). However, the retroviral promoter is not particularly strong and additionally is susceptible to downregulation and/or silencing in many different cell types over different periods of time (Xu *et al*., 1989). Silencing of the expression can be avoided by either modifying the retroviral promoter or replacing it altogether.

The reverse transcription of the viral genomic RNA into a double stranded DNA can be utilised to this end. During this process sequences from the 5' end of the RNA are duplicated and copied to the 3' end of the DNA as well as 3' sequences being similarly copied and transferred to the 5' end. This process leads to two identical LTR structures at both ends of the viral genome.

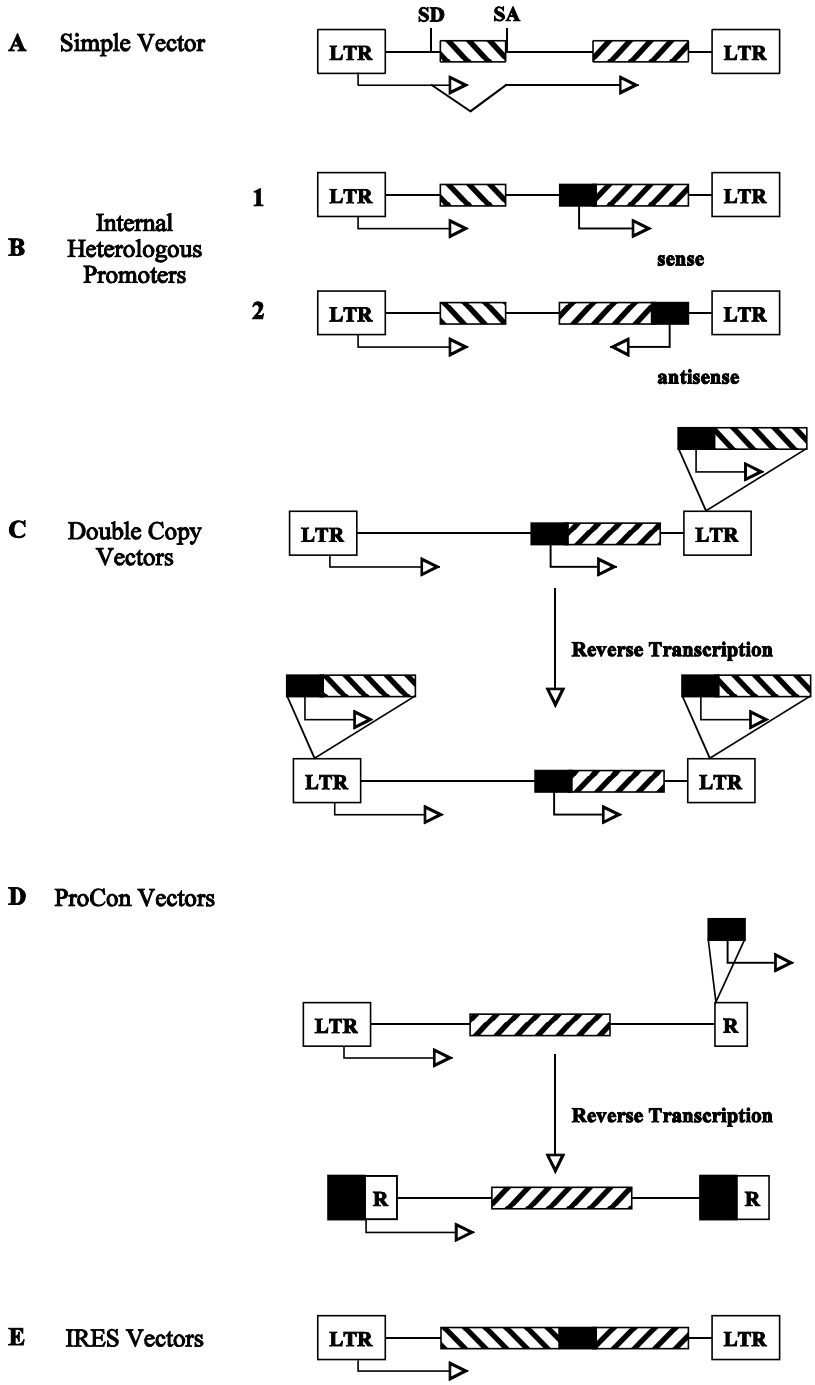

Figure 1.13: Common Retroviral Vector Constructs
1) Simple type of RV vector; 2A- B) RV vector with a internal heterologous promoter; 3) Double Copy RV vector; 4) RV vector with promoter converted; 5) IRES RV vector

The first vectors to utilise this property were the self-inactivating vectors (SIN) (Yu *et al*., 1986; Cone *et al*., 1987), in which the enhancer in the U3 region of the 3' LTR was removed. After reverse transcription and the resultant copying of the deleted U3 region from the 3' end of the genomic RNA to the 5' LTR, expression from the retroviral promoter in the infected cell was lost. This strategy has been used to minimise the inhibiting effect of the retroviral promoter upon the heterologous promoter present (Soriano *et al*., 1991). Incorporation of an expression cassette into the 3' U3 region, which is duplicated during reverse transcription, leads also to duplication of the expression cassette in the infected cell (double-copy vector, DC; Fig. 1.13C) (Hantzopoulos *et al*., 1989). A similar strategy has been used in a series of vectors in which either a heterologous promoter enhancer element or other regulatory elements were incorporated into the LTR (Ferrari *et al*., 1995; Vile *et al*., 1995). It has also been shown that whole promoter regions can be replaced (ProCon vectors; Fig. 1.13D) (Mrochen *et al*., 1997; Saller *et al*., 1998). This type of vector is preferable with respect to safety as the frequency of recombination events with viral sequences present in the producer cell line or the target cell should be reduced due to the missing viral sequences that are normally present in the U3 region. A different approach to the elimination of promoter interference is to make use of an internal ribosomal entry site (IRES). They allow the translation of two or more genes from the same transcript, which is under the expressional control of a single promoter (Koo *et al*., 1992; Zavada *et al*., 1972). These vectors should produced both higher titres and more stable expression (Fig. 1.13E).

The vector is then introduced into a packaging cell line that has been engineered to produce *gag, pol,* and *env* proteins in which no RNA molecules that encode complete retroviral genomes nor RNA molecules that may be encapsidated are produced. The introduction of a vector into a packaging line gives rise to a producer cell line that makes only replication-defective vector particles capable of introducing their genes into target cells without the capacity for replication.

1.3.9 Packaging Cell Lines

The first generation of packaging cell lines were the ψ-2 (Mann *et al*., 1983) and ψ-am (Cone and Mulligan, 1984) cell lines. Ecotropic virus is produced from ψ-2 cells, whereas the ψ-am cells produce amphotropic virus that shows an extended host range. Unfortunately, such cell lines still give rise to wild type virus at a relatively high frequency following because a single recombination event between the packaging construct and the vector construct is sufficient to do so (Fig. 1.14A).

To reduce the risk of generating wild type, replication competent, virus, a second generation of retroviral system carrying mutations in the LTR of the packaging construct was developed (Fig. 1.14B). In this case, at least two recombination events must occur to give rise to a replication competent virus (RCV). The packaging cell line PA317 is an example of this type of system. The packaging construct contains a deletion in the 5'-LTR, a mutated ψ-signal and an SV-40 polyadenylation signal in place of the 3'-LTR (Miller *et al*., 1986).

The third generation is the current generation of "safe" packaging cell lines, which carry two separate constructs, one of which expresses the *env* proteins (Fig. 1.14C) from a heterologous promoter. In such systems at least three recombination events are necessary to give rise to RCV. The most well known of this generation of packaging cell lines are the GP+E86 cells, producing

an ecotropic virus, GP+envAm12 cells, that produce amphotropic particles (Markowitz *et al*., 1988) and PG13 cells that produce gibbon ape leukaemia virus based particles.

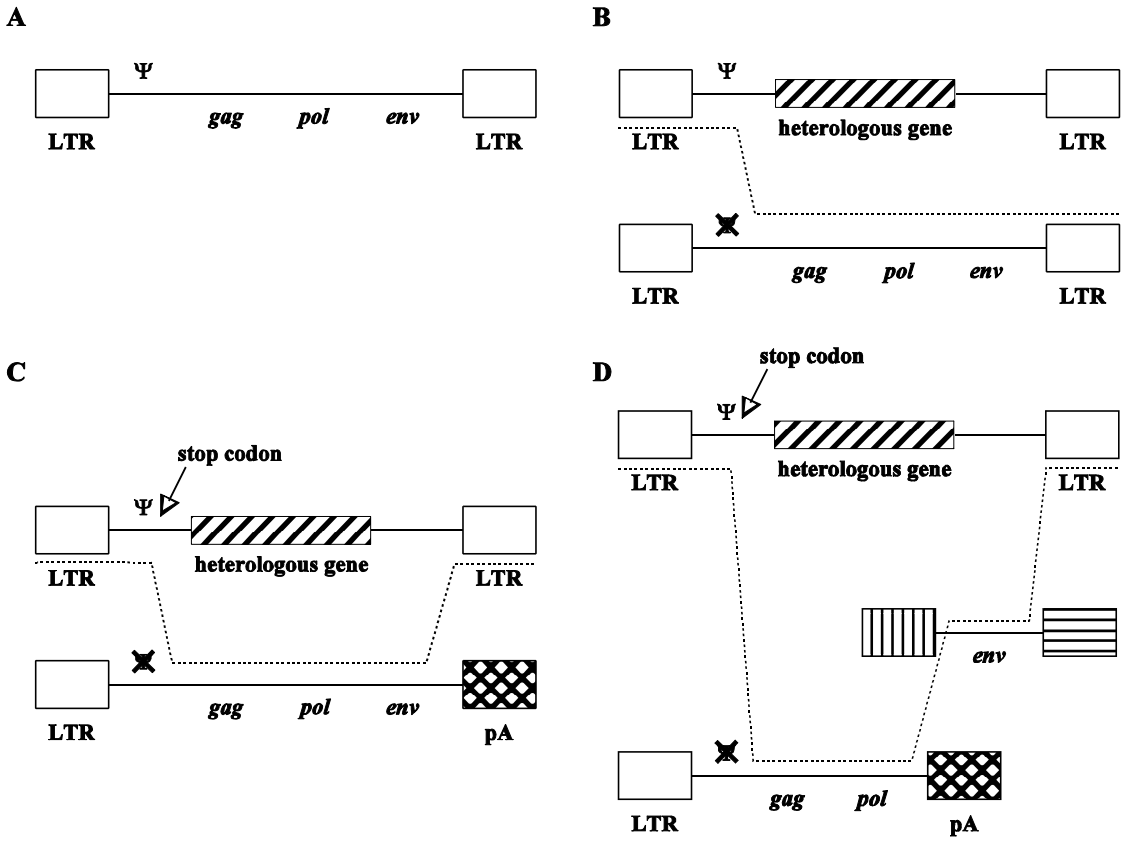

Figure 1.14: Packaging Cell Lines
A) Wild type replication competent retrovirus, B) First generation packagings system, C) Second generation packagings ystem D) Third generation packagings ystem

The use of non-murine cells as the basis of retroviral packaging cells is also a way in which it is possible to reduce the possibility of RCV production. As it is possible to create RCV via recombination with endogenous retroviral elements it would be wise to use cells from non-murine species as their endogenous retroviral sequences have little or no homology to murine retroviruses (Rigg *et al*., 1996). Human embryonal kidney cells (293) have been used to create both the ecotropic BOSC-23 (Pear *et al*., 1993) as well as the amphotropic PROPAK-A packaging cell lines (Rigg *et al*.,1996; Forestell *et al*., 1997). These cells contain separate expression constructs for the *gag/pol* and *env* proteins. Several commonly used packaging cell lines are shown below (Tab. 1.4).

System	Key Characteristics
PA 12	3T3 based, single helper genome
Ψ am	3T3 based, single helper genome
PA317	3T3 based, single helper genome, good titre
Ψ CRIP	3T3 based, split genome, good titre
gp+am12	3T3 based, split genome, good titre
DAN	D17 (dog) based, split genome
DA, CFA	D17, CF12 (dog) based, good titre, split genome, no LTRs
HX	HT1080 (human) based, xenotropic, split genome, good titre, no LTRs
Gibbon Ape Leukaemia Virus based	3T3 based, GALV envelope
VSV-G system	293 (human) based, transient
Kat	293 (human) based, transient
BOS-23	293 (human) based, stable
PROPAK	993 (human) based, stable

Table 1.4: Most Common Packaging Cell Lines

1.3.10 Targeting of Gene Therapy

Targeting of the transfer and expression of genes is essential for future gene therapy. The advantages of targeting genes to specific tissues are readily apparent. Firstly, gene delivery to non-target tissue is avoided. For example, if the gene to be delivered is toxic, the ability to transduce only the target cells will reduce non-specific killing. Therefore, unwanted side effects of the delivery of a toxic gene could be avoided. Secondly, by limiting delivery to a specific cell type, the overall efficiency of the vector is increased and the therapeutic dose can be lowered. Development of tissue specific vectors would make targeted gene delivery possible. The administration of tissue specific vectors could allow *in vivo* therapies to be performed in place of the expensive and time consuming *ex vivo* approaches.

1.3.10.1 Targeting of Retroviral Gene Transfer

Retroviral vectors have been the vehicles of choice for gene transfer and represent at present the majority of the vehicles used in a clinical setting. The most commonly used retroviral vectors are based upon the mouse retrovirus MoMuLV as this virus is able to infect a wide range of cell types. The *env* protein of ecotropic MoMuLV, gp70, interacts with a cationic amino acid transporter, which serves as the host cell receptor (Kim *et al.*, 1991; Wang *et al.*, 1991) and is expressed in many tissues with the notable exceptions of liver, heart and muscle (Kim *et al.*, 1991). It has been also shown that it is possible to limit the infection spectrum of this virus and

consequently of MoMuLV based vectors. Several targeting strategies have been developed and it has also been demonstrated with multiple vector constructs.

1.3.10.2 Modification of the Infection spectrum

One approach involves the coupling of antibodies, directed against known proteins that are expressed on the surface of the target cell, to antibodies specific for the virus *env* protein via streptavidin (Fig. 1.15A). This bi-specific complex was able to direct the retrovirus to human cells expressing the MHC class I or MHC class II receptors (Roux *et al.*, 1989). A similar linkage strategy using an anti-envelope antibody and an anti-EGF receptor antibody showed that retroviruses could be directed to transduce EGF receptor expressing cells (Etienne-Julan *et al.*, 1992). A second strategy for targeting the infection spectrum of retroviral vectors involves chemical linkage of lactose to the retrovirus envelope (Neda *et al.*, 1991) and the transferrin receptor, using an anti-transferrin antibody (Goud *et al.*, 1988). This approach has been used successfully to enable a chemically modified ecotropic MoMuLV to infect human hepatocytes (Fig.1.15B).

Another approach that has been used to target retroviral vectors, is the co-expression of other ligands on the virus surface along with the wild type envelope SU proteins (Cosset and Russell, 1996).

The infection spectrum of the viral *env* protein could also be altered by modification of the *env* gene (Fig. 1.15C). This is achieved by manipulation of the *env* gene in the packaging construct and results in vector virus carrying a modified *env*, which then interacts with the target cell via a novel, specific receptor.

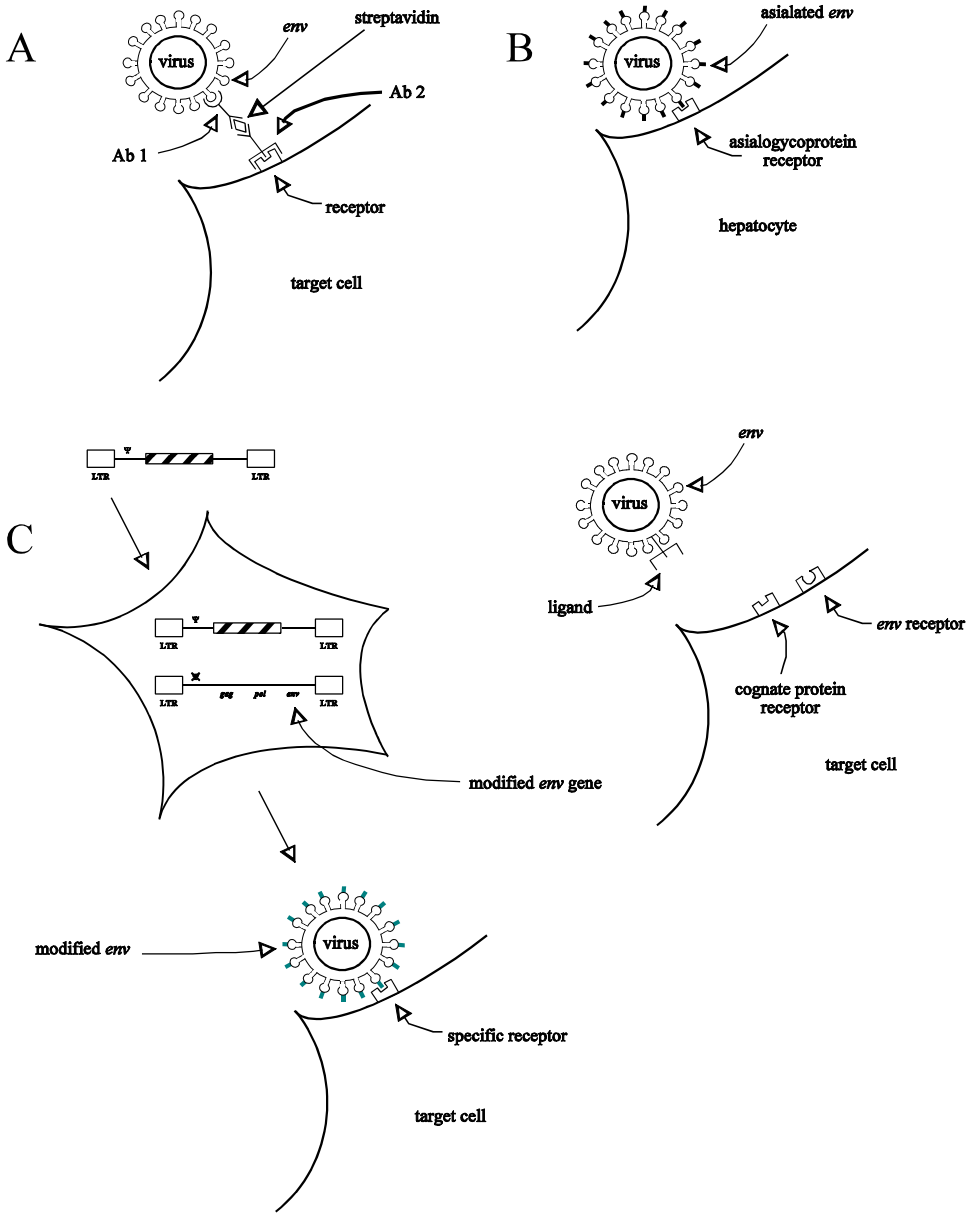

Figure 1.15: Modification of the Infection Spectrum

A) Antibodies directed against a specific, known protein that is located on the target cell surface are linked by streptavidin to antibodies against viral *env* proteins. Thus, the antibody-streptavidin complex functions as a bridge to link the virus to its target cell via the novel, specific receptor. B) The retrovirus *env* proteins are chemically modified (*e.g.,* asialated by coupling of lactose) so that they are recognized by receptors expressed on the surface of the target cell (*e.g*., hepatocyte-specific asialoglycoprotein receptors; Neda *et al.*, 1991). C) The *env* gene of the retroviral vector is redesigned by replacing segments with epitopes that recognize receptors other than those normally used by virus. This is achieved by manipulation of the *env* gene in packaging construct and results in vector virus carrying modified *env*, which then interacts with the target cell via a novel, specific receptors. D) Specific ligands are coexpressed together with the retroviral *env* proteins on the virus surface. The cognate receptor for the ligand is expressed on the surface of the target cell so that the retroviral vector and target cell can interact. Coexpression of the retroviral *env* protein may results in preferential targeting of the retroviral vector to cells that express both the *env* and ligand receptors.

1.3.10.1.2 Chimeric Retroviral Vectors

The infection spectrum of retroviral vectors may also be modified by producing pseudotyped virions (Weiss *et al.*, 1984) consisting of cores (*gag, pol* and vector genome) derived from one type of retrovirus and the *env* of a second, non-related retrovirus (Fig. 1.16). MoMuLV derived pseudotyped vectors have been obtained carrying the *env* of an avian retrovirus, Rous sarcoma virus (RSV; Landau and Littman, 1992), the *env* of Gibbon ape leukaemia virus (GaLV; Miller *et al.*, 1991) or the *env* of the MMTV retrovirus (Baumann, 2000). The heterologous *env* protein responsible for the modified and/or extended infection spectrum of such pseudotyped viruses need to be of retroviral origin but can be supplied by other enveloped viruses, such as vesicular stomatitis virus (Burns *et al.*, 1993).

A replication competent RSV-derived retrovirus vector has been constructed in which the gene encoding the haemaglutinin (HA) protein of influenza virus was inserted in place of the RSV *env* gene. The resultant chimeric avian virus was able to infect both human and avian cells with similar efficiency (Dong *et al.*, 1992). RSV is the only retrovirus so far that permits the construction of replication competent retroviral vectors.

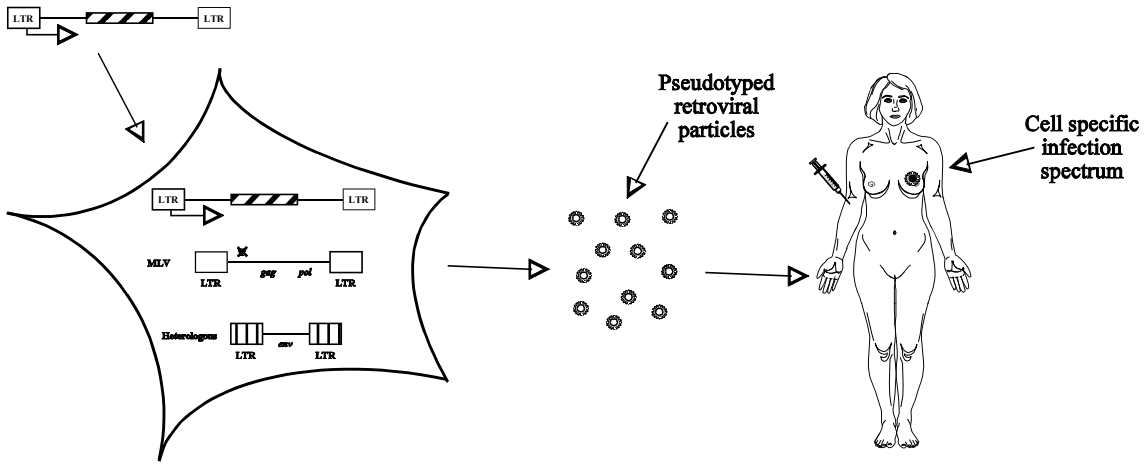

Figure 1.16: Pseudotyped Vectors

1.3.10.1.3 Expression Level Modification

Tissue or cell specific regulation of expression of the therapeutic gene may be realised by co-introducing sequences that have been defined as gene regulatory elements into retroviral vectors (Fig. 1.17). The promoter of MoMuLV is relatively promiscuous in that it directs expression in most cell types. The inclusion of tissue specific regulatory elements or promoters in retroviruses (RVs) have been shown to limit the expression of indicator genes to predefined cell types (Salmons and Günzburg, 1993). A number of tissue specific promoters have been compared with the MLV promoter for their ability to direct high level expression of an indicator gene after transfer to target cells in a retroviral vector. RVs carrying genes that can specifically be expressed in cells of hepatic origin are already being developed. The liver is of particular interest for gene therapy because of the many metabolic disorders that are the result of genetic defects in genes that encode liver specific enzymes. The promoter of the phosphoenolpyruvate carboxylase (PEPCK) gene contains regulatory elements that direct the expression of heterologous genes to the liver and kidney (McGrane *et al.*, 1988). This promoter has been incorporated into retroviral vectors. The resultant virus was injected into rats, with indicator genes subsequently being expressed in the livers of more than half of the successfully injected animals. Another interesting study of the targeting of gene expression was with the α-fetoprotein (AFP) gene coupled with the *tk* gene of varicella zoster virus (VZV). Infection of hepatoma cell lines with a retroviral vector carrying the AFP-VZVtk construct resulted in the expression of VZVtk and was not expressed in infected cell lines derived from organs other than the liver (Huber *et al.*, 1991).

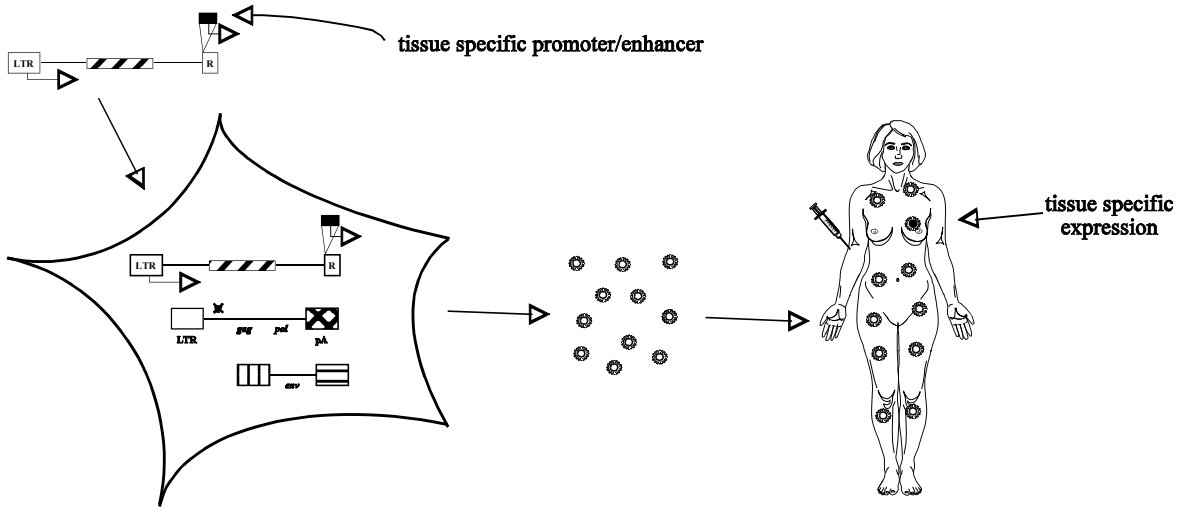

Figure 1.17: Tissue Specific Expression

Tumour specific promoters are also being used in retroviral vectors in order to develop cancer therapies, including tyrosine kinase for B16 melanoma (Diaz *et al.*, 1998) with the therapeutic gene HSV-tk or IL-2 and *erb*B2 (Slamon *et al.*, 1987) for breast cancer with the therapeutic cytosine deaminase (CD) gene. The tyrosinase promoter is also active in other pigment producing cell types, such as normal melanocytes, and could therefore prove ultimately unsuitable for the

treatment of melanoma. A number of other interesting tissue specific promoters as well as breast specific promoters are being used such as those from the DF3/MUC 1 (Chen *et al.*, 1995) or whey acidic protein (WAP; Kolb *et al.*, 1994) genes.

The temporal expression of the transgene construct can also be controlled by drug inducible promoters. The introduction of cAMP response element enhancers in a promoter would allow cAMP modulating drugs to be used (Suzuki *et al.*, 1996). Spatial control of expression has been developed using ionising radiation (radiotherapy) in conjuction with the erg1 gene promoter (Hallaham *et al.*, 1995). Retroviral encoded autoregulatory factors themselves may be used, both to target and to control the expression of retroviral vectors. The diphtheria toxin A (DT-A) chain gene was inserted, together with RRE containing HIV *env* sequences, into an amphotropic Mo-MuLV vector under the transcriptional control of the HIV-LTR carrying the trans activating response (TAR)-element. This DT-A carrying retrovirus was the used to infect the human H9 cell line and then either superinfected with HIV or transfected with a complete HIV provirus, leading to the expression of Tat and Rev. The DT-A retrovirus infected H9 cells were impaired in their ability to support HIV production when compared to non-infected H9 cells, presumably due to the induction of DT-A expression leading to the selective elimination of HIV-infected cells (Harrison *et al.*, 1992). As an alternative, inducible viral promoter, the mouse mammary tumour virus (MMTV) promoter may be considered as is regulated by glucocorticoids hormones and its inducibility with reporter genes has already been shown (Salmons *et al.*, 1995).

The *E.coli* tetracycline (tc) repressor fused with the C-terminal domain of eucaryotic transcriptional activator VP16 is another regulatable promoter system in use today. This tetracycline-regulatable transcription factor (tTA) specifically *trans*-activates artificial minimal promoters carrying multiple tc operator sites (tc-Op) (Gossen and Bujard, 1995).

Combinations of these strategies may ensure strict tissue specific expression of therapeutic genes delivered by retroviral vectors.

1.4 Human Breast Biology

1.4.1 Human Breast Anatomy

The secretory apparatus of the breast consists of approximately 10 to 15 ducts extending from the nipple, and coursing through the mammary fat pad to terminate in clusters of alveoli. Each duct serves a specific lobule (Fig. 1.18). Lobules are separated and supported by thick connective tissue, septae and, in the non-pregnant**,** non-lactating breast, by large amounts of adipose tissue. Blood vessels, nerves and lymphatics run in the septae, which merge imperceptibly with the fascia at the anterior thoracic wall.

The nipple, which serves as the termination point for the lactiferous ducts, is surrounded by an area of pigmented skin, the areola containing both sebaceous and sweat glands. The areola serves as the termination point for the fourth intercostal nerve, which carries the sensory information about suckling to the spinal cord and brain. This is extremely important in the regulation of secretion of oxytocin from the posterior pituitary and prolactin from the anterior pituitary. The mammary ducts expand slightly to form sinuses beneath the areola.

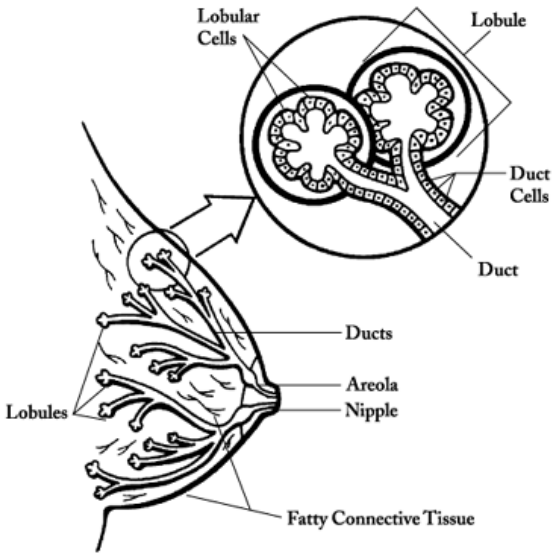

Figure 1.18: Breast Anatomy
The mature female breast is composed of essentially four structures: lobules or glands; milk ducts; fat and connective tissue. The lobules group together into larger units called lobes. The protruding point of the breast is called the nipple and the darkened tissue surrounding the nipple called the areola.

1.4.2 Development of the Human Mammary Gland

Mammary gland development is probably one of the most fascinating and puzzling biological phenomena. The most important element in this puzzle is the fact that the mammary gland seems to be only organ that is not fully developed at birth (Vorher *et al.*, 1974). No other organ presents such dramatic changes in size, shape and function as does the breast during growth, puberty, pregnancy and lactation (Fig. 1.19).

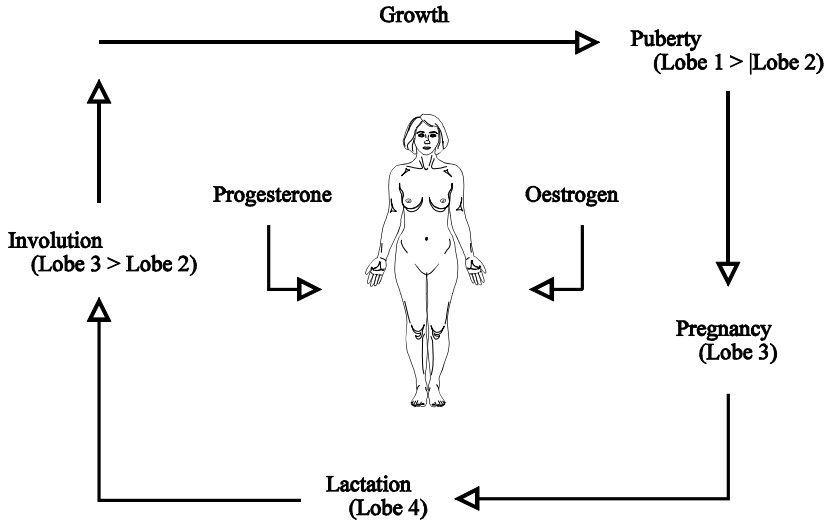

Figure 1.19: Development Stages of the Mammary Gland
The structure of the mammary gland is dependent on the development stage. The adult female mammary gland experiences recurrent cycles of regulated growth, differentiation and apoptosis. Estrogen and progesterone play a central role in this process. The cycles that occur in the mammary gland can be divided into several stages: puberty, pregnancy, lactation and involution. Each stage can be further described by the structure of the glands, called lobules or lobes (Russo and Russo, 1998).

1.4.2.1 Prenatal

The structure of the mammary gland is essentially similar in both sexes until puberty. The first visible indication of mammary gland development can be found during day 35 of pregnancy (Dawson, 1934) with the proliferation of paired areas of epithelial cells in the epidermis of the thoracic region. During week 5, these areas of proliferation have extended in a line between the foetal axilla and inguinal region and form two indistinct ridges called the mammary ridges or milk lines (Dawson, 1934; Dabelow, 1957). By the end of the week 6, the mammary ridges have regressed back in to two areas in the thoracic region, where two solid epithelial masses begin to grow downwards into the underlying mesenchyme. During week 10, solid secondary epithelial buds grow and branch off the main mammary bud, establishing the future lobed structure of the mature gland. In the epidermis overlying the developing gland, the nipple begins to form. During the same period, the mesenchymal cells differentiate to form fibroblasts, smooth muscle cells,

capillary endothelial cells and adipocytes. Around week 20 the solid mammary sprouts canalise and the epidermis in the region of the nipple becomes depressed, forming the mammary pit. The epithelial cells lining the ductules first appear as a biolayer of cuboidal cells. The luminal layer rapidly gains the characteristic of secretory cells while the basal layer becomes myoepithelial. By six months, the basic tubular architecture of the gland has become established. The tubules are separated by "fat islands" within a dense fibroconnective tissue stroma and rudimentary secretory epithelial cells have become functional.

Perinatally the gland can exist as primitive ducts with solid end buds, through ducts with bud like projections, to ducts with budding primitive alveoli. Postnatally maternal hormones may cause a transient secretory activity in the rudimentary breast (Hiba *et al.*, 1977). The secretory activity of the newborn gland subsides 3-4 weeks postnatally. During the same period, the nipple is formed by proliferation of the the underlying mesenchyme, causing inversion of the embryonic mammary pit. The glands normally remain at this rudimentary stage until puberty.

1.4.2.2 Puberty

The changing hormonal environment at the onset of puberty is the controlling factory for the sexually dimorphic growth and development of the mammary gland. In the male, testosterone acts on the mesenchymal cells to inhibit further growth of the mammary gland. In the female, estrogen acts on the mesenchymal cells to stimulate further development. The gland increases in size, mainly due to the deposition of interlobular fat. The ducts extend and branch into the expanding stroma. The accompanying epithelial cell proliferation and basement membrane remodeling is controlled by interactions between the epithelium and the intralobular hormone sensitive zone of fibroblasts. By adulthood the complete ductal architecture of the glands has been established.

The mammary glands remain in this mature but inactive state until pregnancy, which brings about the next major change in the hormonal environment. However, there are relatively small cyclical changes brought about by ovarian hormones released during the menstrual cycle. During the follicular phase, the stroma is less dense, the epithelial cells of the smaller ducts are cuboidal, there is no secretion and early in the follicular phase the duct lumens are not apparent. During the luteal phase, epithelial cells become more columnar, the lumens open up and there is some secretion. There is a moderate level of cell proliferation, increasing to a maximum towards the end of the luteal phase. This followed by abrupt involution and apoptosis during the last few days, before the onset of menstruation.

1.4.2.3 Pregnancy and Lactation

During pregnancy, the mammary gland attains its maximum development. This occurs in response to hormones initially from the corpus luteum (estrogen and progesterone), followed by placental hormones (estrogen, progesterone and somatotropin), pituitary hormones (prolactin) and adrenocorticoids from the adrenal gland. Estrogen exerts its effects mainly on the ductal system, while progesterone promotes alveolar development. This occurs in two distinctly dominant phases characteristic of early and late stages of pregnancy (Dabelow, 1957; Salazar and Trobon, 1974; Vorherr, 1987). During the first trimester, the terminal ductules branch and

elongate. The epithelial cells proliferate from stem cells distributed throughout the gland. During the second trimester, differentiation of alveoli from terminal end buds predominates. The alveoli possess two layers of cells, the luminal cells are the prospective secretory cells, while the basal cells are myoepithelial and extend contractile processes in a network around the alveolus. There are low levels of secretion from cells in the ductules and more mature alveoli. During the third trimester, the alveoli mature. The epithelial cells become cuboidal, with an extensive basal endoplasmic reticulum, basally situated nuclei, apical granules and cytoplasmic lipid droplets. The functional gland architecture is maintained by prolactin, released from the anterior pituitary in response to a suckling stimulus from the infant.

Lactation ceases at the end of the suckling period. Epithelial cells numbers are reduced through apoptosis, the remaining cells become inactive and reduced in size and alveoli and ducts regress back to a resting state. The stromal fibroblasts reconstruct the collagenous interlobular connective tissue and the gland becomes reinvaded with adipose tissue. Though the post lactational breast returns to a resting state, the architecture of the gland and composition of the supporting tissues are not identical to their structures before pregnancy.

1.4.2.4 Menopause

The resting state is maintained in a manner similar to that of the post-pubertal gland, with the potential to re-enter the fully functional state during subsequent pregnancies. The amount of elastic tissue increases, the stroma becomes more fibrous and less cellular and adipose tissue is lost. The levels of circulating ovarian hormones falls, the ductal elements degenerate and dense connective tissue replaces the intralobular loose connective tissue.

1.5 CANCER

1.5.1 Breast Cancer

Breast cancer is the most common form of cancer among women. It occurs in one in every 11 women in Western Europe and in 1 every 14 in the United States. The incidence rate of breast cancer is highest in Western Europe and North America where the risk of developing it is approximately 10%. Furthermore, the incidence of breast cancer appears to be inexorably increasing (McPherson *et al.*, 1994; Broeders *et al.*, 1997), with an annual worldwide incidence of over one million predicted at the beginning of the 21st century (Miller *et al*., 1986).

Hereditary breast cancers account for about 5-10 % of all breast cancers and a large percentage of them are early onset breast cancer (Agnorsson *et al.*, 1998; Hall *et al.*, 1990). Inherited mutations in several genes including BRCA-1 (Miki *et al.*, 1994), BRCA-2 (Wooster *et al.*, 1995; Tautigian *et al.*, 1996), ataxia-telangiectasia mutated (ATM) (Cortez *et al.*, 1999; Broeks *et al.*, 2000) and p53 (Livingstone *et al.*, 1992), have been found to increase a women's risk of developing breast cancer**.** BRCA-1 and BRCA-2 are estimated to account for more than half of all hereditary breast cancers (Easton *et al.*, 1993; Wooster *et al.*, 1994). Women who inherit a single defective copy of either of these tumour suppressor genes, have a significantly increased lifetime breast cancer risk compared to the general population.

Cancer results from a series of genetic alterations that confer a loss of growth control plus the development of invasive, angiogenic, and metastatic capabilities.

The vast majority of breast cancers are carcinomas, the malignant tumours of the epithelia. Based upon histological evaluations, development of the breast cancer has been postulated to be a multi-step process and follows a defined sequence of qualitatively different events, as documented for a number of other malignancies (Russo *et al.*, 1993; Russo *et al.*, 1997).

In the human breast, ductal hyperplasia and atypical ductal hyperplasia represent the initial stages of neoplastic growth and progress gradually to ductal carcinoma *in situ*, invasive ductal carcinoma and ultimately metastasis, even though normal cells could directly give rise to ductal carcinoma *in situ* or invasive ductal carcinoma (Fig. 1.20).

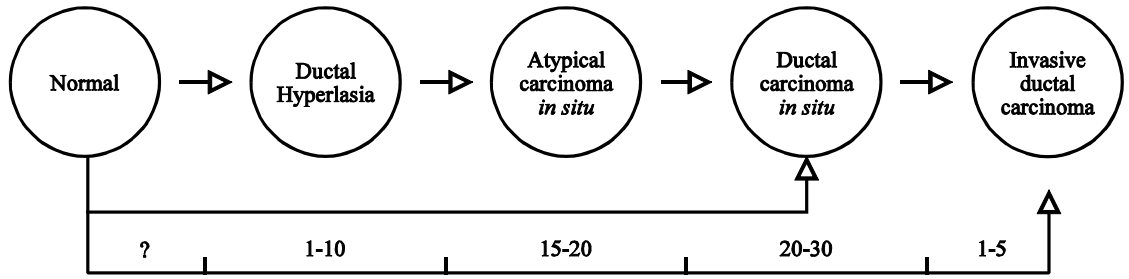

Figure 1.20: Stages of Tumour Development
In the human breast, ductal hyperplasia and atypical ductal hyperplasia represent the initial stages of neoplastic growth and progress gradually to ductal carcinoma *in situ*, invasive ductal carcinoma and ultimately metastasis, even though normal cells could directly give rise to ductal carcinoma *in situ* or invasive ductal carcinoma. Indicated is the timescale in years.

The goal of any breast cancer treatment is to achieve effective local-regional control of the tumour in order to maximise the chance for cure. There are four main kinds of conventional treatment; surgery (removing the cancer with an operation), radiation therapy (killing the cancer cells with radiation), chemotherapy (killing the cancer cells with drugs) and hormone therapy (stopping the cells from growing with hormone blocking agents). The type of treatment that will work best for the patient depends the stage of their cancer. Primary tumour treatment is relatively successful but it is still difficult to treat metastases.

The development of new treatments for breast cancer during the past decade has been actively progressing. The ability to characterise tumours at the molecular level and to use modern biological and chemical technologies to identify and develop novel molecules of therapeutic potential has opened several different possible avenues for the development of new therapeutic modalities such as immunotherapy or gene therapy.

Gene therapy aims to engineer a new vector system with a therapeutic gene in it that would be ideally suited for arresting metastatic progress of breast cancer. Such vectors would be designed to delivery a therapeutic gene only to target breast tumour cells.

1.5.2 Male Breast Cancer

Although it occurs infrequently, breast cancer can affect men as well as women. Male breast cancer is rare. Less than 1% of all breast carcinomas occur in men (Crichlow, 1990; Borgen *et al.*, 1992; Crichlow, 1990). About 1,600 new cases were diagnosed in the United States in 1998 (Hultborn *et al.*, 1997). According to the National Cancer Institutes (NCI) Surveillance, Epidemiology, and Results (SEER) Program, breast cancer affects 14 black men in every million and 8 men in every million overall (Ardyce *et al.*, 1978). In Egypt, male breast cancer represents 6% of all breast cancer, and in Zambia it accounts for 15%. The mean age at diagnosis is between 60 and 70, although men of all ages can be affected with the disease.

The breast of the adult male is similar to the breast of a pre-adolescent girl (Roswit and Edlis, 1978; Willsher *et al.*, 1997). It consists primarily of a few branching ducts lined by flattened cells surrounded by connective tissue. In girls, these cells and ducts develop in response to hormones secreted during puberty.

In males, too, breast tissue is capable of responding to hormonal stimulation. Enlargement of the male breast due to growth of the ducts and supporting tissues is known as gynocomastie. Approximately 40 percent of all adolescent boys experience temporary breast enlargement, probably in response to hormones being secreted by the testes.

Predisposing risk factors (Jaiyesimi *et al.*, 1992) appear to include radiation exposure, estrogen administration and diseases associated with hyperestrogenism, such as cirrhosis or Klinefelter‘s syndrome (Hultborn *et al.*, 1997). There are definitive familial tendencies, with an increased incidence seen in men who have a number of female relatives with breast cancer. An increased risk of male breast cancer has been reported in families in which the BRCA2 mutation on chromosome 13q has been identified (Wooster *et al.*, 1995; Thorlacius *et al.*, 1995).

All of the types of breast cancer seen in women can occur in men, although some are quite rare. The pathology is similar to that of female breast cancer, with infiltrating ductal cancer the most common tumour type (Harris *et al.*, 1997), though intraductal cancer has also been described. Inflammatory carcinoma and Paget‘s disease of the nipple have also been seen in men, but lobular carcinoma *in situ* has not (Harris *et al.*, 1997). This is due to the fact that lobules are normally absent from the male breast.

The same procedures used to diagnose breast cancer in women can be used to diagnose breast cancer in men. These include medical history, physical examination, mammography and thermography. Even though the small male breast should facilitate early diagnosis, breast cancer in men has often spread before it can be diagnosed. Lacking the bulk of the typical female breast, even a small carcinoma in a male lies close to the skin above it and the tissues of the chest wall beneath it. Consequently, the cancer can more readily invade these nearby structures (Crichlow, 1972; Crichlow, 1977).

The treatment of male breast cancer is generally similar to the treatment of female breast cancer. The basic therapy for primary cancer that shows no signs of distal spread is surgery. In advanced disease, it is hormonal therapy, chemotherapy or a combination of both or with radiotherapy.

1.6 Cancer Gene Therapy

Cancer results from a series of genetic alterations that confer a loss of growth control plus the development of invasion, angiogenesis, and metastatic capability. Several methods such as surgery, radiation, and chemotherapy have been used to treat cancer, but they are still insufficient. Developments in gene technology coupled with the knowledge gained into how genes function (*e.g.* in their tissue specificity, cell cycle dependency etc.) is creating a promise of treating tumours by gene therapy. The range of different cancers encountered and the mutations they carry have led to a variety of strategies for cancer gene therapy; immunomodulatory, oncogene inactivation gene therapy, tumour suppressor gene replacement therapy, suicide gene therapy and drug resistance genes.

1.6.1 Immunomodulatory Gene Therapy

The aim of immunotherapy is to enhance the response of the immune system to cancer, thereby leading to its destruction. Active and passive approaches to immunotherapy have both benefited from these advances.

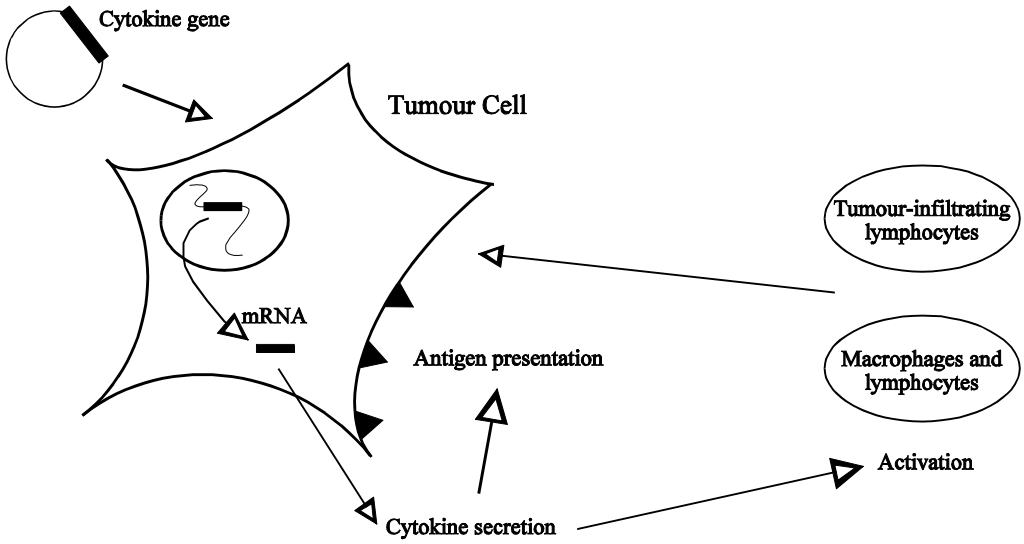

Figure 1.21: Cytokine Gene Addition Therapy
Genetic immunomodulation can reduce tumorigenicity or metastatic potential, or both, convert a weakly immunogenic tumour to strongly immunogenic, and enhance cytotoxic lymphocytes.

Passive immunotherapy aims to increase the pre-existing immune response to the cancer whilst active immunotherapy initiates an immune response against an unrecognised or poorly antigenic tumour (Barnes *et al.*, 1997). Passive immunotherapy usually involves harvesting tumour infiltrating lymphocytes and treating them to express increased levels of cytokines [*e.g.* IL-2 and TNF-alpha (Yanelli *et al.*, 1993)]. The cell population is then expanded *in vitro* and returned to the patient. Tumour cells are used for active immunotherapy, genetically modifying them to increase expression of antigen presenting molecules/costimulatory molecules (*e.g.* B7; Chen *et*

al., 1992), local concentration of cytokines (IL-2; Leming *et al.*, 1996) or tumour antigens (erbB2 oncoprotein; Disis *et al.*, 1994). These cells are then irradiated prior to being returned to the patient, preventing the reintroduction of viable tumour cells. These approaches have also been termed as a cancer vaccination.

1.6.2 Oncogene inactivation (Anti-sense oligonucleotide therapy)

The oncogene may be targeted at the level of the DNA, RNA transcription or protein product. An alternative approach is to use antisense oligonucleotides (Fig. 1.22). These molecules are nucleic acids whose nucleotide sequence is complementary to a DNA or RNA sequence corresponding to a gene whose expression is thought to be deleterious to the cell. Oligodeoxynucleotides are designed in a sequence specific manner to target the promoter regions of oncogenes (Ebbinghouse *et al.*, 1993). Antisense techniques prevent transport at the RNA level and translation of oncogene mRNA by providing oligoribonucleotides with a cleavage action, will also reduce the stability of oncogene mRNA (Synder *et al.*, 1993). Transport of the oncogene product to the cell surface can be prevented by a single chain antibody with specificity for the oncogene product and a localisation signal for the endoplasmic reticulum (Beerli *et al.*, 1994).
The main limitation of this approach is to ensure that every single tumour cell gets transduced by the therapeutic antigen, which is currently not technically feasible.

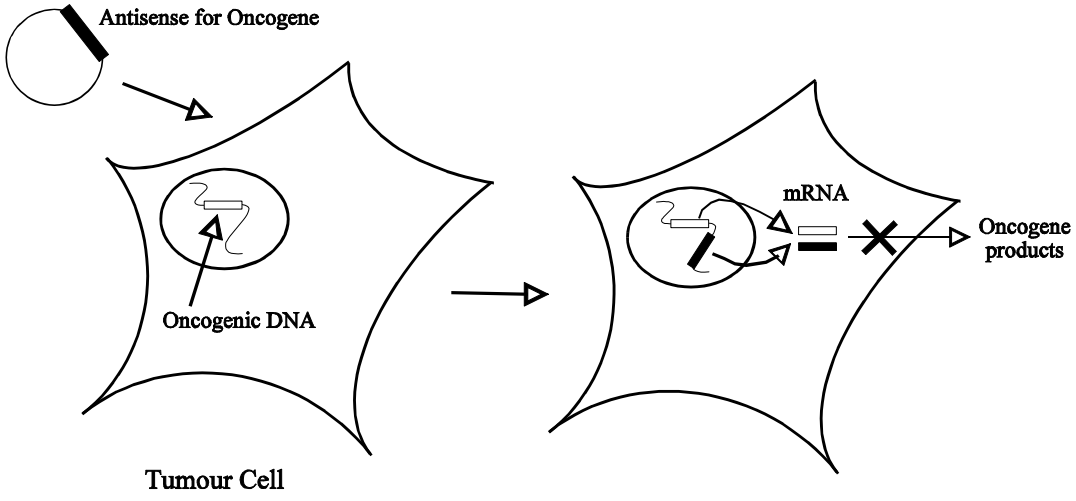

Figure 1.22. Anti-oncogene Therapy
Vector mediated transfer of an antisense construct complementary to oncogene messenger RNA sequences is capable of modulating oncogene expression in a target-specific manner.

1.6.3 Tumour Supressor Gene Replacement

The aim of tumour suppressor gene replacement therapy (TSGR-Therapy) is cell death and changes in growth, behaviour, invasiveness and metastatic ability of the cell. p53 is the most common mutated gene in cancer and influences transcription, cell cycle movement, apoptosis and angiogenesis. It is therefore a prime target for gene replacement. In several model systems, transduction of cancer cells with p53 has been demonstrated to inhibit growth, angiogenesis and apoptosis. Expression of p53 is synergistic with chemotherapeutic drugs such as cisplastin and adjacent tumour cells that have not been transduced are killed, in what is termed the bystander effect (Roth *et al.*, 1996). How this occurs is not yet well understood. This effect may be a result of cell-cell contact, immune mediated response or achieved through other local actions. Other tumour supressor genes may also be suitable for TSGR-Therapy *e.g.* BRCA1sv (Tait *et al.*, 1997).

The main limitation of tumour suppressor gene therapy is the limited number of target genes known to induce or maintain malignancy and the difficulty in transducing enough cancer cells to result in a cure.

1.6.4 Suicide Gene Therapy

Suicide gene therapy is the transduction of a gene that transforms a non-toxic "prodrug" into a toxic substance (Fig. 1.23). There are several systems available for use in gene therapy (Tab. 1.5). The most widely used system involves the thymidine kinase gene of the Herpes simplex virus (HSVtk). The HSVtk gene confers sensitivity to the anti-herpes drug ganciclovir (GCV), by phosphorylating GCV to a monophosphate form (GCV-MP). Phosphorylation of the triphosphate form (GCV-TP) by cellular kinases results in the inhibition of DNA polymerase, leading to cell death. The affected cell is supported via the gap junction of adjacent cells (Wygoda *et al.*, 1997) until the toxin burden is too great, killing both the affected cell and its neighbours (Bystander Effect).

GCV metabolites transfer via gap junctional intracellular communication (GJIC) and this accounts for the effect in several cell lines. However, it has been suggested that multiple pathways may be responsible for the bystander effect (Princen *et al.*, 1999; Grignet-Debrus *et al.*, 2000). Princen *et al.*, describe the presence of a bystander effect in a rat colon adenocarcinoma (DHD/K12) and a rat gliosarcoma cells (9L) in experiments where no cellular contacts were present. Similarly, non-communicating MDA-MB-435 cells have been shown to exhibit a bystander effect both *in vivo* and *in vitro* (Grignet-Debrus *et al.*, 2000)

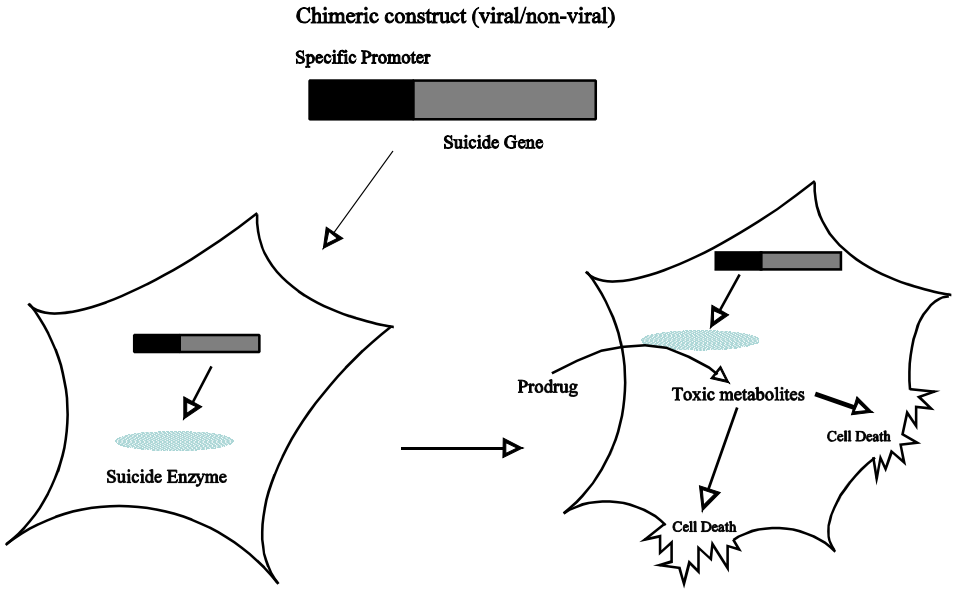

Figure 1.23: The Mechanism of Prodrug Activation Therapy
A chimeric construct consisting of a gene encoding a prodrug metabolising enzyme (suicide gene) is introduced into tumour cells. The prodrug is administered to all the cells. Only the cells producing the enzyme can convert the prodrug to its highly toxic metabolite. The toxic metabolite causes the death of the tumour cells, leaving the normal cells unaffected.

The other common enzyme prodrug system is *Escherichia coli* cytosine deaminase (CD; Blaese *et al.*, 1994; Conners 1995) together with 5-fluorocytosine. CD converts the non-toxic anti-fungal drug 5-fluorocytosine into the cytotoxic 5-fluorouracil (Culver *et al.*, 1992). This combination produces a bystander effect and has been demonstrated to have some success in animals with hepatic metastasis of gastro-intestinal tumours. The bystander effect of CD on the cells can spread from gene modified cells to adjacent tumour cells that do not express the CD gene due to its capability to freely diffuse across cell membranes (Huber *et al.*, 1994; Wei *et al.*, 1995; Manome *et al.*, 1996).

Enzyme	Prodrug	Activated Prodrug	Mechanism	Bystander Effect
HSV Thymidine Kinase (HSV tk)	Ganciclovir (GCV)	GCV-triphosphate	Termination of replication	via Gap Junction
Cytosine Deaminase (CD)	5-Fluorocytosine	5-Fluorouracil	DNA alkylation	via Gap Junction
E. coli nitroreductase	CB1954	5- aziridine-1-yl-4-hydroxamino-2-nitrobenzamide	DNA alkylation	diffusible metabolites
Cytochrome P450 CYP 2B1	Ifosfamide, Cyclophosphamide	Phosphoramide mustard, Acrolein	DNA alkylation	diffusible metabolites

Table 1.5: Most Common Enzyme Prodrug Systems

The nitroreductase/CB1954 is another alternative gene directed enzyme pro-drug therapy system. The pro-drug CB1954 or 5-aziridinyl-2,4-dinitro-benzamide is a monofunctional alkylating agent originally found to be highly active against a transplantable rat tumour (Walker tumour, Cobb *et al*., 1969). The difference in this system is that the active drug introduces poorly repaired interstrand DNA crosslinks, kills dividing and non-dividing cells by induction of apoptosis and is diffusible and membrane permeable resulting in a bystander effect.

This general strategy is extendable to conventional anticancer drugs, in particular those non-toxic prodrugs that are subject to enzymatic activation by endogenous drug-metabolising enzymes (Leblanc *et al.*, 1989). The cancer chemotherapeutic agent cyclophosphamide (CPA) and its isomer ifosfamide (IFA) are alkylating agent prodrugs that require metabolism by liver cytochrome P450 (P450) enzymes for anti-tumour activity. P450s are a multigene family of constitutive and inducible enzymes that have a central role in oxidative metabolic activation and detoxification. The majority of cytochrome P450s are primarily expressed in the liver (Wrighton, and Stevens, 1992; Nedelcheva and Gut, 1994). There are several isoforms of P450, like CYP4B1 (from rabbit; Rainov *et al.*, 1998) or CYP2B1 (from rat; Wei *et al.*, 1994; Chen *et al.*, 1996), which are being used in several gene therapy protocols. The DNA alkylating metabolites of CPA and IFA kill tumour cells, in a cell cycle-independent manner, by inducing DNA cross-links whose cytotoxic potential manifests itself at the timepoint when the cells begin to replicate.

The problem in this type of therapy is often the failure to control metastatic disease and systemic delivery of vectors containing genes may not be sufficiently specific or efficient to achieve the desired aim. Concerns of specificity could possibly be addressed via delivering such genes with tissue-specific promoter/enhancer elements.

1.6.5 Multi Drug Resistence Gene Therapy (MDR-1)

Protection of haematopoietic stem cells (HSCs) from the toxic effects of chemotherapy by using the gene that confers multi drug resistance type 1 (MDR-1; Sorrentino *et al.*, 1992) is another possible strategy for cancer therapy.

MDR-1 is a gene encoding the multidrug transporter protein (P-glycoprotein). This transmembrane protein is capable of pumping a wide variety of chemotherapeutic agents (*e.g.*,VP16, VM26, actinomycin-D, adriamycin, taxol etc.) and other drugs out of cells, thus protecting them from the agents toxic effects (Gottesman *et al.*, 1994). Precursor bone marrow cells that have been transduced with the MDR gene, coding for the drug efflux protein p170, can enable a population of resistant cells to be cultured. This enables much higher doses of chemotherapy to be used after returning these cells to the patient (Deisseroth *et al.*, 1994). Although gene transfer into marrow stem cells leads to transgene expression in only a few percent of haematopoietic cells, successive cycles of chemotherapy can be used to enrich for transduced marrow cells. The danger that accompanies this strategy is that tumour cells may become transduced and subsequently become drug resistant. The inclusion of sequences that are either capable of reducing oncogene expression or restore tumour suppressor gene function may be a method of circumventing this problem (McIvor, 1999).

1.7 The Aim of the Work

Breast cancer, the most frequent cancer in women, is often discovered after it has already metastasised, making conventional treatments difficult or impossible. A future treatment could utilise retroviral vectors to target the carcinoma in the context of an *in vivo* gene therapy protocol. When developing such an *in vivo* approach several factors should be incorporated. These should include the utilisation of tissue specifically expressed promoters to control expression of therapeutic genes, in this case using breast/breast tumour specific promoters.

A number of breast/breast tumour specific promoters are available, such as those of the DF3/MUC 1 (Wen *et al.*, 1993), whey acidic protein (WAP; Kolb *et al.*, 1994) genes or from Mouse Mammary Tumour Virus (MMTV; Günzburg and Salmons, 1992).

Large amounts of proteins are secreted by the mammary gland into milk during lactation. One of these proteins is whey acidic protein (WAP). WAP is present in the milk of pigs (Simpson *et al.*, 1998), camels (Beg *et al.*, 1984; Beg *et al.*, 1986), mice (Hennighausen and Sippel, 1982), rats (Hobbs *et al.*, 1982; Hennighausen *et al.*, 1982) and rabbits (Devinoy *et al.*, 1988) and is expressed almost exclusively in the mammary gland during late pregnancy and lactation (Mckenzie and Larson, 1978; Hobbs *et al.*, 1982). It has no human homologue. Expression of milk protein genes is dependent upon the interaction between tissue-specific and developmentally and hormonally induced regulatory factors (Hobbs *et al.*, 1982). The promoter sequence of the WAP gene and its binding proteins have been studied in detail (Campbell *et al.*, 1984; Lubon and Hennighausen, 1987). Previous studies have shown that a negative regulatory element (NRE) is present in the WAP promoter that limits its expression (Kolb *et al.,* 1994) and a factor binding to the NRE has also been characterised (Kolb *et al.*, 1994; Kolb *et al.*, 1995). It is not known whether this promoter is activated in mammary tumours, although other milk genes are often activated in such tumours.

MMTV is a type B retrovirus responsible for the majority of mammary carcinomas appearing in mice in the first year of life as a result of insertional activation of a host proto-oncogene (Bentvelzen and Hilgers, 1980; Nusse and Varmus, 1982). The MMTV long terminal repeat (MMTV LTR) is mainly active in the mammary gland of infected mice as well as in mammary tumours. Its expression is controlled by a number of factors, including glucocorticoid hormones (Günzburg and Salmons, 1992).

The coupling of such a breast/breast tumour specific promoter to a suicide gene, such as the HSV-tk gene or cytochrome P450 (CYP2B1), and their subsequent introduction into retroviral vectors might be a feasible approach for the targeted and controlled expression of toxic gene products specifically within the breast tumour or any metastases present.

To achieve this aim, tissue specific retroviral vectors, based upon the widely used MLV retroviral systems, should be constructed. The ProCon system, which was developed in the lab of Günzburg and Salmons, should be used. Here the U3 from the 3' LTR is replaced with either the WAP NRE or the U3 from MMTV. After infection and reverse transcription, expression of the gene (in this case a reporter gene) will be controlled by either the WAP NRE or the MMTV U3 region (Fig. 1.13).

These vectors should be first tested in an *in vitro* system to investigate if the replacement of the U3 from the 3' LTR has an effect on the virus production and, if after infection of target cells, whether or not promoter conversion has taken place. In order to test tissue specificity of the vectors both *in vitro* and *in vivo* system should be used.

In the *in vitro* system suitable cell culture condition should be determined for the maintenance of both primary tumour cells as well as established tumour cell lines. Studies of the hormonal requirements for WAP gene regulation performed in various explant and cell culture systems have indicated that WAP induction is dependent upon the synergistic action of glucocorticoids, insulin and prolactin (PRL; Hobbs *et al*., 1982; Pittius *et al*., 1988; Schoenenberger *et al.*, 1990; Doppler *et al*., 1991), but also requires some features of cell-cell interactions including extracellular matrix interactions occurring within the mammary gland (Hansen and Bissell, 2000).

Transgenic mice were also made in order to examine the tissue specificity of both the WAP NRE and MMTV promoter in the context of a recloned provirus *in vivo*. In order to determine the *in vivo* activity of the WAP NRE and MMTV in human breast cancer cells in the context of a retroviral vector, the established SCID mouse model should be used carrying either breast tumours or other types of human tumour implants.

If either the WAP NRE or the MMTV promoter shows enough specificity, they could potentially be use to drive therapeutic gene expression in the context of a retroviral vector for *in vivo* gene therapy strategies directed against breast cancer.

2. MATERIALS AND METHODS

2.1 CHEMICALS

The following chemicals were not self-made:

Agarose	Gibco Europa (Munich)
Ammonium persulfate (APS)	Sigma (Munich)
Ampicillin	Sigma (Munich)
Bisbenzimide	Boehringer Mannheim (Roche)
Bromophenol blue	Sigma (Munich)
Bovine serum albumin (BSA)	Sigma (Munich)
BSA fraction V	Sigma (Vienna)
Caesium chloride	Gibco Europa (Vienna)
Cholera Toxin	Sigma (Munich)
Chloroform	Sigma (Munich)
Collagenase	Worthing Biochemical Corporation
Collagene	Becton Dickinson
Cortisol	Sigma (Munich)
Denhardt	Sigma (Munich)
Deoxynucleosidetriphoshate (dNTPs)	Perkin Elmer (Munich)
Dexamethasone	Sigma (Munich)
Dextran sulfate	Sigma (Vienna)
Dithiothreitol (DTT)	Sigma (Munich)
Dimethylsulfoxide (DMSO)	Sigma (Munich)
Dulbecco's modified eagle medium (DMEM)	Gibco Europa (Munich)
Dulbecco's MEM with Glutamax-1	Gibco Europa (Munich)
Epidermal growth factor (EGF)	Sigma (Munich)
17-ß-Estradiol	Innovative Research of America (Florida)
Ethidium bromide	Sigma (Munich)
Ethylenediaminetetraacetic acid (EDTA)	Sigma (Munich)
Fetal calf serum (FCS)	Gibco Europa (Munich)
Ficoll	Sigma (Munich)
Formaldehyde	Sigma (Munich)
Formamide	Sigma (Munich)
Fungizone	Sigma (Munich)
ß-Galactosidase	Gibco Europa (Munich)
Geneticin (G418)	Gibco Europa (Munich)
Glycerine	Sigma (Munich)
L-Glutamine	Gibco Europa (Munich)
Guanidium isothiocyanate	Sigma (Munich)
Hyaluronidase	Gibco Europa (Munich)
Isoamylalcohol	Sigma (Vienna)
Isopropanol	Sigma (Vienna)
Insulin	Gibco Europa (Munich)
Kanamycin	Sigma (Munich)
Lipofectin	Gibco Europa (Munich)

2-Mercaptoethanol	Sigma (Munich)
PCR Buffer (10X)	Perkin Elmer (Vienna)
Penicillin	Gibco Europa (Munich)
Phenol	Sigma (Munich)
Prolactin	Sigma (Munich)
Polybren	Sigma (Munich)
Sarcosyl (N-lauryl sarcosine)	Sigma (Munich)
Streptomycin	Gibco Europa (Munich)
Sodium dodecyl sulfate (SDS)	Sigma (Munich)
N,N,N',N'tetramethylethylenediamine (TEMED)	Biorad (Munich)
Trypsin/EDTA	Gibco Europa (Munich)

Molecular Biological Products:

Alkaline phosphatase (CIP)	Boehringer Mannheim
DNase, RNase free	Boehringer Mannheim
Klenow polymerase	Boehringer Mannheim
Nuclease S1	Boehringer Mannheim
Proteinase K	Boehringer Mannheim
Restriction enzymes and buffers	Boehringer Mannheim, Promega, New England
RNase, DNase free	Boehringer Mannheim
Rnasin	Promega (Munich)
T4 Ligase and 5 x Ligation Buffer	Gibco Europa (Munich)
T4-Polynucleotide kinase	Boehringer Mannheim
Taq Polymerase	Perkin Elmer (Munich)

DNA Ladders:

1 kb Ladder	Gibco Europa (Munich)
DNA Molecular weight marker IV	Boehringer Mannheim

RNA Ladder:

0.24-9.5 kb Ladder	Gibco Europa (Vienna)

Radioactivity:

α^{32}P-dCTP	Amersham Buchler GmbH (Braunschweig)
γ^{32}P-ATP	Amersham Buchler GmbH (Braunschweig)
X-ray films	Amersham Buchler GmbH (Braunschweig)

Plasticware:

Plasticware (petri dishes, tubes, tips, pipettes etc.)	Greiner, Sarstedt, Nunc (Munich)
Cell culture flasks (25 cm^2, 80 cm^2, 175 cm^2),	Geriner, Sarstedt, Nunc (Munich)
Cell culture plates (⌀3 cm, 6 cm, 10 cm)	Geriner, Sarstedt, Nunc (Munich)

2.2 Molecular Biological Techniques

2.2.1 Nucleic Acid Extraction Techniques

2.2.1.1 Isolation of Genomic DNA

2.6.1.1.1 Isolation of Genomic DNA from Cell Culture

One confluent 175 cm^2 cell culture flask was used for the preparation of genomic DNA. Cells were washed twice with PBS, trypsinised and pelleted in a centrifuge in a tabletop centrifuge (2000 x g, for 10 minutes). The pellet was washed again with PBS and recentrifuged. This pellet was then resuspended in a lysis buffer (50 mM Tris-HCl, pH 8.0; 100 mM EDTA; 0.125% SDS; Proteinase K 0.8-1 mg/ml ; H_2O dest). The lysate was then incubated for 4 hours at 50°C, until the solution became clear. Subsequently, the lysate was transferred into a 6 ml SST tube (serum separation tube, 6 ml Vacutainer, Becton Dickinson) and 1 ml phenol/chloroform/isoamylalcohol (25:24:1) was added. After centrifugation (Heraeus 400 R, 200 x g , for 10 min) the organic phase was seen to be clearly separated from the aqueous phase by the gel present in the SST tube. An additional 1ml phenol/chloroform/isoamylalcohol was added, mixed and once again centrifuged. To eliminate the rest of the phenol, an equal volume of chloroform was added after this centrifugation step and centrifuged once more. The aqueous phase was then transferred to a new 2 ml eppendorf tube. 2.5 volumes of cold ethanol (96%) and 100 µl sodium acetate (3 M, pH 6.0) were added and mixed by inverting gently, flicking the tube to produce a gentle swirling that wound the DNA strands. The DNA was fished out with a pasteur pipette (that had previously been heated using a bunsen burner so that the end formed a hook) and washed in a tube of 70% ice cold ethanol. The DNA was then air-dried and resuspended in 200 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). The resuspended DNA was rehydrated at 4°C overnight and subsequently dissolved at 50-60°C for 1-2 hours. The amount and quality of DNA was measured by OD (OD 260/280, Pharmacia GeneQuant II RNA/DNA calculator) or using agarose gel electrophoresis (see 2.2.2.1).

2.2.2.1.2 Isolation of Genomic DNA from Organs

Organs (1 g to 5 g of tissue) were directly homogenized using homogeniser (IKA-T25 basic) in 1-1.5 ml lysis solution (see 2.6.1.1.1) and Proteinase K was added. The protocol is then identical to the one for cells.

2.2.2.1.3 Isolation of Genomic DNA from Mouse Tails (Qiagen-kit)

Mouse tails (0.5-1 cm) were added to an eppendorf tube containing 0.5 ml of the Qiagen protease stock solution (20 mg/ml) in buffer G2 (800 mM HCl, 30 mM EDTA, 30 mM Tris-HCl, 5% Tween-20, 0.5% Triton X-100, pH 8.0). The tube was then vortexed and incubated at 50°C for 2 hours (or until the lysate was clear). This avoids clogging of the Qiagen genomic tip. The lysate was centrifuged in a tabletop centrifuge (5000 x g, 5 min). A Qiagen genomic tip was equilibrated using buffer QBT (750 mM NaCl, 50 mM Mops, 15% Ethanol, 0.15% Triton X-100, pH 7.0) allowing the tip to empty by gravity flow. The supernatant was applied and centrifuged for 10 seconds at maximum speed in a tabletop centrifuge. The Qiagen genomic tip was washed 3 times with 500 µl buffer QC (1 M NaCl, 50 mM MOPS, 15% Ethanol, pH 7.0).

The genomic DNA was eluted using buffer QF (1.25 M NaCl, 50 mM Tris-HCl, pH 8.0). Subsequently, 0.7 volumes of isopropanol were added and the DNA precipitated by inverting the tube 10-20 times. After precipitation, the solution was centrifuged in a tabletop centrifuge (5000 x g, 15 min at 4°C) and the supernatant carefully removed. The DNA was washed with 1ml ethanol (70%) and then centrifuged again (5000 x g, 1 minute) and then air-dried for 10 minutes. The DNA was resuspended in 200 µl TE buffer and shaken overnight at 4°C to dissolve it. The amount and quality of DNA was measured by OD (OD 260/280, Pharmacia GeneQuant II RNA/DNA calculator) or agarose gel electrophoresis (see 2.2.2.1).

2.2.2.2 Isolation of Total RNA

2.2.2.2.1 Isolation of Total RNA From Cells (CsCl Method, Chirgwin *et al.*, 1979)

All solutions used for RNA analysis, including glassware and pipettes (with the exception of guanidium isothiocyanate), were treated with diethyl pyrocarbonate (DEPC) before use. DEPC was added to the solutions at a concentration of 0.1%. The solutions were than stored at room temperature overnight in the dark (DEPC is light sensitive) and autoclaved the next day. DEPC is a potent oxidizing agent that reacts with virtually all biological macromolecules and is able to irreversibly inactivate all enzymes including RNases.

2-5x10^7 cells (a confluent 225 cm^2 cell culture flask) were used for the preparation of RNA samples. Cells were washed twice with ice cold PBS and then scraped in 7 ml lysis buffer [6 M guanidium isothiocyanate (GITC), 5 mM sodium citrate, 0.1 M ß-mercaptoethanol, 0.5% N-lauryl sarcosyl]. GITC is a powerful denaturing agent and rapidly terminates any enzymatic activity. Thus, RNases are prevented from degrading the cellular RNA. The cell extracts were layered onto a 3 ml CsCl cushion (5.7 M CsCl, 0.1 M EDTA; sterile filtered) and ultracentrifuged in a SW41 Beckman rotor (at 150000 x g) at 20°C for 12 hours. During this centrifugation step the RNA migrates through the caesium chloride cushion and is pelleted at the bottom of the tube, whereas the denatured cellular proteins and the viscous DNA do not enter the cushion. From this point onward all steps were performed on ice. The supernatant containing proteins and DNA was removed; the RNA pellet was resuspended in 1 ml ice cold 10 mM Tris-HCl, pH 7.5 and precipitated by adding 67 µl of 3 M NaAc, pH 5 and 2.5 ml ice cold ethanol (96%). The samples were incubated at -20°C overnight. The RNA was pelleted by centrifugation in an HB4 swing-out rotor in a Sorvall centrifuge or Beckmann JS13.1 rotor (10000 x g) at 4°C for 40 minutes. The pellet was subsequently dried and resuspended in 400 µl DEPC treated water. The quality and the amount of RNA was checked using either a sodium phosphate or formaldehyde gel. The OD was measured (OD 260/280, Pharmacia GeneQuant II RNA/DNA calculator).

2.2.2.2.2 Isolation of Total RNA From Organs (Single step method, Chomczynski & Sacchi 1987)

Preparation of the buffers was identical to the RNA extraction from cells. Organs (1 g to 5 g) were homogenised in 1-2 ml lysis buffer (4 M guanidium isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% N-lauryl sarcosyl, 0.1 M ß-mercaptoethanol).The lysate was forced through a needle (23-gauge) fitted to a syringe into a 2 ml eppendorf tube. This step removes insoluble material and reduces the viscosity of the lysate by disrupting gelatinous material. 1/10 volume

of 2 M NaOAc (pH 4.6) was added and vortexed for 30 seconds. Additionally, 1 volume of aqua phenol and 1/5 volume of chloroform: isoamyl alcohol (49:1) was added. After 30 seconds vortexing, the mixture was incubated on ice for 15 minutes. After centrifugation (Beckman, rotor JX13.1, 10000 x g, 30 minutes) the organic and aqueous phases separate. The supernatant (aqueous phase) was carefully removed into a new eppendorf tube and 2.5 volumes of ice-cold ethanol (96%) added, as well as 1/10 volume of lithium chloride (8 M). After briefly vortexing, the samples were incubated at -20°C overnight or -80°C for 2 hours. The RNA was pelleted by centrifugation in a JS13.1 rotor in a Beckmann centrifuge (10000 x g 20 minutes, 4°C). The pellet was then dried and resuspended in either 100 or 200 µl DEPC treated water. The quality and the amount of RNA was checked on either a sodium phosphate or formaldehyde gel. The OD was also measured (OD 260/280, Pharmacia GeneQuant II).

2.2.2 Gel Electrophoresis Techniques

2.2.2.1 Agarose Gel Electrophoresis

The DNA was separated electrophoretically in horizontal agarose gels. The gels contained 0.8% agarose in a 1 x TAE buffer (40 mM Tris-base, 10 mM sodium acetate, 2.5 mM EDTA at pH 8.1).

The mixture was cooked in a microwave and after cooling sufficiently was poured into a prepared gel chamber. After the polymerisation of the gel the samples were pipetted into the wells. The gels were run in 1 x TAE buffer and depending on the amount of gel running time, at 100 volts for 2 hours or 16 volts overnight.

The DNA fragments were visualised by staining with ethidium bromide (1 µg/ml) for 15 minutes. Ethidium bromide (3,5-diamino-6-ethyl-5-phenylphenathridium-bromide, $C_2H_2ON_3Br$) intercalates into the DNA and fluoresces in UV light (at a wavelength of 320 nm). A video camera with video printer (Mitsubishi Video Copy Processor) was used to document the gels.

2.2.2.2 Polyacrylamide Gel Electrophoresis

This electrophoresis system was used for S1 analysis (see 2.2.5.2.1). The samples were resolved in a vertical 6% polyacrylamide gel in a Bio-Rad apparatus (Sequi-Gen GT). The glass plates of the apparatus were cleaned first with detergent, than hot water and finally ethanol (70%) and subsequently coated with a fine layer of a non-stick cooking fat spray (PAM). The glass plates were then assembled and the bottom of the gel sealed with acrylamide in a casting tray [10 ml of INSTA gel solution (6% INSTA gel solution is 6% acrylamide 19:1 monomer: bis, 8 M urea in 1 x TBE pH 8.3), 175 µl 10% ammonium persulfate (APS), 50 µl TEMED]. The sealing was allowed to polymerise for 15 minutes. Subsequently the gel was poured after mixing 60 ml 6% INSTA gel solution, 430 µl 10% APS and 30 µl TEMED together and allowed to polymerise for 1 hour. Gels were run in a Tris-borate-EDTA [1 x TBE (89 mM Tris base, 89 mM boric acid, 3.2 mM EDTA, pH 8.3)] buffer system, and depending on the expected size of the fragments, for 2 to 4 hours at 2000 volts (at 50°C). At the end of the electrophoresis the plates were taken out the apparatus dismantled and the gel attached to a Whatman paper. It was then dried on a gel vacuum dryer for 120 minutes at 80°C and exposed to phosphor imager plates (Fuji).

2.2.2.3 RNA Gel Electrophoresis

2.2.2.3.1 Non-denaturing Gel Electrophoresis

The separation of RNA under nondenaturing conditions, where the secondary structure of the molecules is left intact during electrophoresis, was made to check the integrity and quality of the RNA immediately after extraction. As with all RNA work, solutions and plasticware were treated with DEPC and autoclaved, the glassware and pipettes being baked at 180°C.

2 x loading buffer (10 mM Na_2HPO_4/NaH_2PO_4, pH 7.0, 50% glycerine, 0.25% bromophenol blue, 0.25% xylene cyanol) was added to 3 µg of total RNA and loaded onto a 1% nondenaturing agarose gel. A neutral phosphate buffer (10 mM Na_2HPO_4/NaH_2PO_4, pH 7.0) was used as both gel and electrophoresis buffer. The electrophoresis system requires constant mixing of the electrolyte as a pH gradient quickly builds up because of the low buffer strength (10 mM). A pump was therefore used to recirculate the buffer throughout the electrophoresis run. The gel was run at a voltage of 100 volts until the bromophenol blue marker had migrated about two-thirds through the gel. The gel was then stained in ethidium bromide (1 µg/ml) for 30 minutes and then photographed under UV light (at a wavelength of 320 nm, Mitsubishi Video Copy Processor). The 18S and 28S rRNA are normally clearly resolved and this allows any degradation or DNA contamination to easily be seen.

2.2.2.3.2 Denaturing Gel Electrophoresis

The separation of RNA under denaturing conditions, where the secondary structure of the molecules is disturbed during electrophoresis, was used when making for northern blot analysis.

For the separation of RNA a 1% formaldehyde agarose gel was used. 3 g of agarose in 220 ml DEPC treated autoclaved water was cooked and subsequently cooled to 60°C. 30 ml of fresh 10 x gel buffer [0.2 M 3-(N-morpholino) propane sulfonic acid (MOPS), 0.05 M sodium acetate, 0.01 M EDTA, pH 7.0] and formaldehyde (37%) were added until its final concentration was 6.6%. Subsequently, the cooled gel was poured into an RNase free gel tray. The gel was set allowed to for one hour at room temperature and was prerun for 5 minutes at 5 volts before the RNA samples were loaded.

20 µg of total RNA containing loading buffer (50% glycerol,10 x gel buffer, 37% formaldehyde, deionised formamide 10 mg/ml ethidium bromide, 0.25% bromphenol blue, $water_{DEPC}$) were denatured for 15 minutes at 65°C and then chilled on ice. The samples were centrifuged for 5 seconds to deposit all of the fluid in the bottom of the tubes before loading. The gel was run in 1 x gel buffer (1 x MOPS) at a voltage of 100 volts until the bromophenol blue marker had migrated about two-thirds through the gel. An RNA ladder was used (0.24-9.5 kb RNA ladder, Life Technologies) to allow the size of the RNA to be calculated, and was treated identically to the RNA samples. The gel was run at 100 volts until the bromophenol blue marker had migrated about two-thirds through the gel. The gel was then stained in ethidium bromide (1 µg/ml) for 30 minutes and then photographed under UV light (at a wavelength of 320 nm, Mitsubishi Video Copy Processor). The 18S and 28S rRNA are normally clearly resolved and this allows any degradation or DNA contamination to easily be seen.

2.2.3 Determination of DNA/RNA Concentration

2.2.3.1 Photometric Determination

The DNA, RNA or oligonucleotide was diluted 1:100 in milliQ water. The absorption of this solution was determined at wavelengths of 260 nm and 280 nm using a Pharmacia GeneQuant II RNA/DNA calculator. DNA displays a maximal absorbance at 260 nm, with proteins having a maximum absorbance at 280 nm. The ratio between the OD values at 260 nm and at 280 nm reveals possible contamination of the DNA or RNA with proteins or phenol. The expected 260/280 ratio for a pure DNA and RNA preparations is 1.8- 2.0. An OD_{260} of 1.00 is obtained with a DNA (double-stranded DNA) concentration of 55 µg/ml, with a RNA or single stranded DNA 40 µg/ml, and with oligonucleotides ~20 µg/ml.

OD_{260} x dilution factor x 50= µg/ml

OD_{260} x dilution factor x 40= µg/ml

OD_{260} x dilution factor x 20= µg/ml

2.2.3.2 Quantification on Agarose Gel

The concentration of plasmid DNA can also be determined by comparison of the plasmid DNA with different dilutions of a standard DNA or a molecular weight marker (1 kb ladder), which has a known concentration on agarose gel or one that has been stained after running with ethidium bromide.

2.2.4 Recombinant DNA Techniques

2.2.4.1 Restriction Digestion of Plasmid

In order to analyse the structure of the plasmid DNA 1or 0.5 µg was used whereas for a preparative digest, depending on the expected size of the DNA fragments of interest, between 10 and 20 µg were used. The digest contained 2.5 U/µg DNA restriction enzyme (New England Biolabs or Promega), and a tenth of 10 x concentrated reaction buffer. One unit is is defined as the amount of enzyme needed to completely digest one µg of DNA in one hour at the appropriate temperature. The reaction mix was incubated for 1-3 hours at 37°C [except *BssHI*I (50°C), *Age*I (25°C)]. Subsequently, a fifth of 5 x sample buffer (10% Ficoll, 10 mM EDTA, 0.1% SDS, 0.02% Bromphenol blue) was added and the fragment separated on an agarose gel.

2.2.2.4.2 Cleaning of DNA Fragments (QIAquick nucleotide removal kit , Qiagen)

This protocol was used to clean up DNA fragments after enzyme treatment. 10 volumes of buffer PN was added to 1 volume of the reaction sample and in the QIAquick column that was placed in a collecting tube. The QIAquick column was then centrifuged for 1 minute in a tabletop centrifuge (at 4000 x g). Thus nucleic acids (>10 nucleotide) were bound to the column matrix. The QIAquick column was washed twice with 500 µl PE buffer and centrifuged each time for 1 minute (4000 x g) and finally for 1 minute (at 10000 x g), to remove any rest ethanol.

50 µl of Milli-Q water was added to QIAquick column, and the DNA eluted after centrifugation for 1 minute (at 10000 x g).

2.2.2.4.3 Isolation of DNA Fragments from Agarose Gel (Qiaex extraction kit, Qiagen)

The DNA fragments were visualised under UV light, the bands of interest removed with a scalpel and transferred into an eppendorf tube. 300 µl buffer QX1 (3 M sodium iodide, 4 M sodium perchlorate, 250 mM mannitol, 5 mM Tris-HCl pH 7.5, 0.1% sodium sulphite) was added to 100 mg of gel and incubated for 10 minutes at 50°C. To help dissolve the gel the mixture was shaken in a eppendorf shaker. If the expected fragment was >4 kb or <500bp 100 µl isopropanol was added (per 100 mg agarose gel slice). Subsequently, the mixture was loaded on to a QIAquick spin column in a 2 ml collection tube and centrifuged for 1 minute (at 10000 x g). The QIAquick spin column was then placed in a fresh collection tube and 500 µl of buffer QX1 was added and centrifuged for 1 minute (at 10000 x g). To wash the QIAquick column 750 µl PE buffer was added and centrifuged for 2 minutes (at 10000 x g). The QIAquick column was then placed in a 1.5 ml eppendorf tube, 50 µl milli-Q water was added, and the DNA eluted by centrifuging for 1 minute (at 10000 x g). The amount and quality of DNA was measured using agarose gel electrophoresis (see 2.2.2.1).

2.2.4.4 Modification of DNA Fragments

2.2.4.4.1 Generation of Blunt Ends with Klenow Enzyme

If one of the ends generated by restriction digestion is not compatible with the respective end in the vector or insert, the Klenow fragment of bacterial DNA polymerase I (*E.coli*) can be used to blunt the fragment. The 5'-3' polymerase activity of Klenow enzyme can be used to fill in a fragment with a 5' overhang whereas a 3' overhang can be removed via the 3'-5' exonuclease activity of this enzyme. Both reactions create blunt ends on the DNA fragment.
The DNA was mixed together with all four deoxyribonucleotides (dNTP, 1.25 mM), one unit enzyme and was then incubated for 30 minutes at 30°C. The enzyme was inactivated by incubating at 70°C for 30 minutes. The conditions for 3'→5' exonuclease activity was a 45 minute incubation at 30°C in half the dNTP concentration. To remove any unincorporated nucleotides and the inactivated enzyme, the DNA was subsequently purified using the QIAquick nucleotide removal kit (Qiagen) or by phenol-chloroform extraction.

To phenol-chloroform extract, the volume of the reaction was increased to 100 µl with water (milli-Q) and 100 µl of a phenol was added. The sample was vortexed and centrifuged in a tabletop centrifuge for 2 minutes at room temperature. The upper aqueous phase was transferred to a new tube and 100 µl chloroform/isoamylalcohol (24:1) mix was added and centrifuged as above. The upper aqueous phase was again transferred to a new tube and precipitated by the addition of 6.5 µl sodium-acetate (3 M, pH 4.8) and 300 µl ethanol (96%). The mixture was then centrifuged (10000 x g) for 20 minutes at room temperature. The pellet was washed with 500 µl ethanol (70%) centrifuged once more, dried in a vacuum dryer and resuspended in water (milli-Q).

2.2.4.4.2 Dephosphorylation of Plasmid DNA

Dephosphorylation is necessary both to prevent linearised plasmids that should take up an insert for ligation from religating to themselves and to prepare DNA fragments for subsequent radioactive labelling with polynucleotide kinase.

The dephosphorylation reaction was made with 1 µl alkaline phosphatase (0.01 U/pmol calf intestine alkaline phosphatase, CIP, Roche) and 3 µl 10x dephosphorylation buffer (50 mM Tris pH 8.0, 0.1 mM EDTA) in a total volume of 30 µl. The reaction was incubated for 30 minutes at 37°C and then inactivated for 60 minutes at 65°C.

2.2.4.5 DNA Ligation

A total of approximately 50 ng of DNA fragments were used in the ratio 1:2 vector: insert for the ligation. The reaction was performed in a total volume of 10 µl, using 1 µl T4 DNA ligase (Life-Technologies 1 U/µl) and 2 µl of 5 x (or 1µl of 10 x) ligation buffer (1 mM ATP, 1 mM $MgCl_2$, 3% PEG8000). The ligation reaction was incubated for 12 hours at 16°C for blunt ends and for 1 hour at room temperature for compatible ends. The inactivation of the ligation reaction was made by incubating for 10 minutes at 65°C. A control reaction containing the same amount of vector but no insert DNA was also made in order to see frequency of self-ligation of the vector.

2.2.4.6 n-Butanol Precipitation

Before electroporation, ligation products were precipitated with n-butanol. This reduces the salt concentration. Lower salt concentrations raise the efficiency of the electroporation. The total volume of the reaction was increased to 50 µl with water and 500 µl n-butanol was added. The reaction mix was briefly vortexed and then centrifuged (13000 x g) for 20 minutes at room temperature. The supernatant was then removed and the pellet dried in a vacuum dryer. It was then resuspended in 30 µl water (milli-Q), 15 µl being used for the electroporation.

2.2.5 Analysis of Nucleic Acids

2.2.5.1 Analysis of DNA

2.2.5.1.1 Polymerase Chain Reaction (PCR, Saiki *et al*., 1988)

PCR is an *in vitro* method for enzymatically synthesising a defined sequence of DNA. The reaction uses two oligonucleotide primers that hybridise to opposite DNA strands and flank the target sequence that is to be amplified. The elongation of the primers is catalysed by Taq DNA polymerase (from *Thermus aquaticus*). A repetitive series of cycles involving template denaturation, primer annealing and extension of the annealed primers by Taq DNA polymerase results in an exponentialamplification of a specific DNA fragments.

All PCR reactions were made using the Ampligene PCR kit (Perkin Elmer), which contained all reactions components. As a template either 1 µg genomic DNA or 1ng plasmid DNA was used and the primer concentration was 40 µM. 1 U Taq DNA polymerase and 10 mM dNTPs were

added per PCR reaction. The PCR reactions were performed with the following thermal cycling programe [in a Gene Amp PCR system 9600 or 2400 (Perkin Elmer)] 35 cycles; denaturating step, 30 seconds at 94°C; annealing: 30 seconds (primer dependent temperature); elongation: 90 seconds at 72°C. The annealing temperatures are usually between 50°C and 60°C and were calculated using computer software (Primer designer, version 2.0-3.0 from Scientific & Educational Software).

2.2.5.1.2 DNA Transfer to Nylon Membrane (Medveczky *et al*., 1987**)**

The agarose gel with separated DNA was transferred on to a nylon membrane (Zeta-Probe, Bio-Rad), to immobilize it for hybridisation. The Zeta-Probe is an amine derived nylon membrane which has unique binding and handling properties. The transfer of the DNA was made using a vacuum blot apparatus (Pharmacia). The apparatus was put together as follows: a Whatman filter paper was cut to the size of the gel to be blotted and placed on a porous plastic sheet, ensuring no air bubbles were present. The membrane was placed on top of the whatman paper and then covered with a plastic mask to seal the apparatus. The gel was then placed over the mask, making sure that the mask was covered by the gel and no spaces were left (Fig. 2.1).

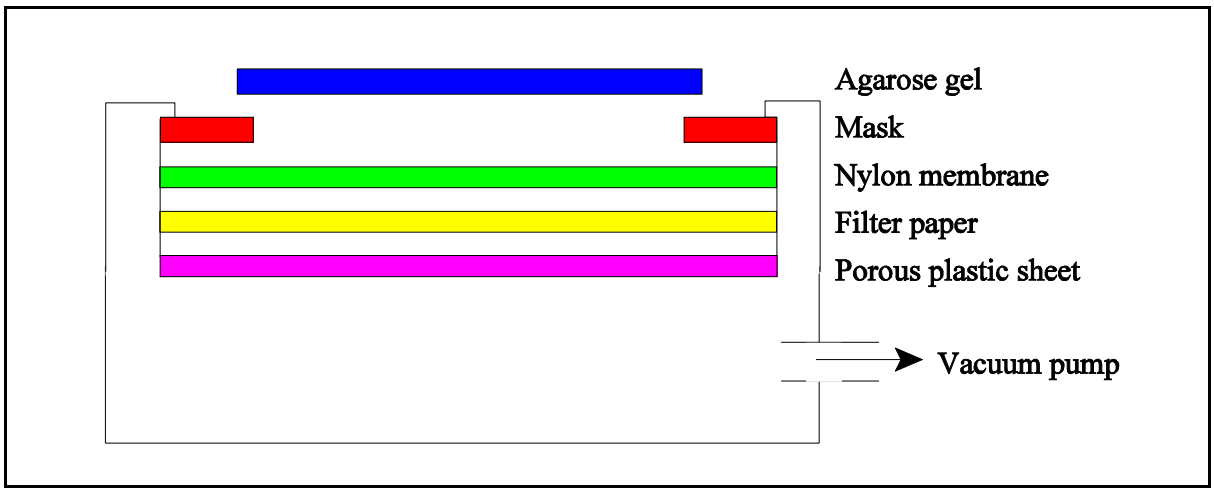

Figure 2. 1: Diagram of Vacuum Blot Apparatus

To depurinate the DNA 0.25 M HCl was added to the gel and the vacuum set to 60 millibar. After 20 minutes, the HCI was soaked into the gel. The excess HCl on the gel was removed with tissue paper and 0.4 M NaOH was added. The gel was blotted for a further 2 hours. During denaturating the DNA fragments move from the gel into the membrane. The gel was then removed and stained again with ethidium bromide to check the DNA transfer efficiency. The filter was then washed three times for 10 minutes with 2 x SSC (3 M NaCl, 0.3 M Tri-sodium citrate x $2H_2O$, pH 7.5-8.0). The filter was air dried and fixed to the membrane by UV cross linking (Pharmacia Crosslinker, 700 joule for 10sec).

2.2.5.1.3 DNA Sequencing (ABI PRISM® Dye terminator Cycle Sequencing Ready Reaction Kit, PE)

DNA sequence analysis was performed with the ABI PRISM® Dye kit in a Perkin Elmer analyser (PE 373). 0.3-0.5 µg of plasmid or 50-200 ng of PCR product containing the region of

interest was mixed with 3 µM of an appropriate sequencing primer in 4 µl of premix (ABI PRISM® Dye kit, PE) and water added to a final volume of 10 µl. Subsequently, a sequencing PCR was run for 25 cycles (Gene Amp PCR system 9600 or 2400, PE): denaturating: 30 seconds at 96°C; annealing: 5 seconds at 50°; elongation: 4 minutes at 60°C). The sequencing reaction product was then removed into an eppendorf tube and 50 µl 96% ethanol added, 3 µl Na-acetate (3 M, pH 5), and after vortexing, centrifuged in a tabletop centrifuge (10000 x g) for 20 minutes. The supernatant was removed, 250 µl 70% ethanol added, and centrifuged again. The pellet was air dried and resuspended in 20 µl TSR buffer. The DNA was denaturated for 3 minutes at 95°C before loading the gel.
Separation of the DNA fragments was performed on a sequencing gel using the Perkin Elmer (PE) sequencing apparatus (PE 373) or a capillary PE sequencing apparatus (PE 310).

2.2.5.2 Analysis of RNA

2.2.5.2.1 S1 Analysis

S1 nuclease analysis was initially described as a method for the determination of transcription initiation sites (Berk and Sharp, 1977). Total cellular RNA or mRNA is hybridised to a γ-^{32}P-ATP 5' end-labelled DNA probe that overlaps the transcriptional start site. The DNA-RNA hybrids are then digested with S1 nuclease (from *Aspergillus oryzae*), which digests single stranded nucleic acids. Thus, only the regions of homology where the RNA forms a stable double strand with the DNA probe are protected from nuclease digestion. The nucleic acids are then precipitated, denatured and separated on a polyacrylamide gel. The size of the DNA probe detected after digestion corresponds to location of the transcription initiation site.

For S1 analysis, 40 µg of total RNA were ethanol precipitated to obtain an equal concentration of RNA for all samples. The RNA was resuspended in 10 µl of hybridisation buffer (80% formamide, 0.4 M NaCl, 40 mM Pipes pH 6.4, 5 mM EDTA). Subsequently, 1 µl of a γ-^{32}P-ATP end-labelled DNA probe was added. If the site of transcriptional initiation is to be determined, the DNA probe must be labelled at the 5' terminus, since a radioactive label at the 3' end would be lost from the DNA during S1 digestion.

```
mRNA                          5'--------------------------3'
Labelled DNA    3'----------------------5'*

S1 nuclease digestion         ↓

mRNA                          5'--------------------3'
Labelled DNA                  3'------------------5'*
```

The hybridisation mix was incubated at 70°C for 30 minutes to resolve secondary structures in the RNA. The mix was then immediately transferred to a water bath set to 50°C and incubated overnight. The homologous DNA and RNA molecules hybridise at a temperature between 45°C and 50°C depending on the length of the region of homology between the nucleic acids. The samples were then chilled on ice and 100 µl of a S1 digestion mix [containing S1-buffer (1.25 M NaCl; 0.15 M NaAc, pH 4.5; 50 mM $ZnSO_4$), 10 µg/µl small denaturated salmon sperm carrier DNA and 60 U nuclease S1] was added. The samples were then incubated at 30°C for 1

hour. The samples were cleaned by extracting once with phenol/chloroform and once with chloroform. The nucleic acids were than precipitated by adding 1 µl tRNA (1 mg/ml), 250 µl of ice cold ethanol (96%) and incubated for 1 hour at -80°C. The precipitated mixture was centrifuged (750 x g, 10 minutes at 4°C). The supernatent was discarted, 1 ml 70% ethanol added and centrifuged once again (750 x g, 10 minutes at 4°C). This step was repeated once more. The supernatant was again discarded and the pellet dried in vacuum dryer. The pellet was resuspended in 16 µl sample buffer (USB). After denaturing the samples in a heating block at 95°C for 5 minutes, the nucleic acids were separated on a 6% denaturing polyacrylamide gel (Fig. 2.2). The gels were run at between 2000 volts and 2500 volts at a temperature of 50°C depending on the expected size of the labelled fragments. At the end of the electrophoresis the plates were taken out the apparatus dismantled and the gel attached to a Whatman paper. It was then dried on a gel vacuum dryer for 120 minutes at 80°C and exposed to phosphor imager plates (Fuji).

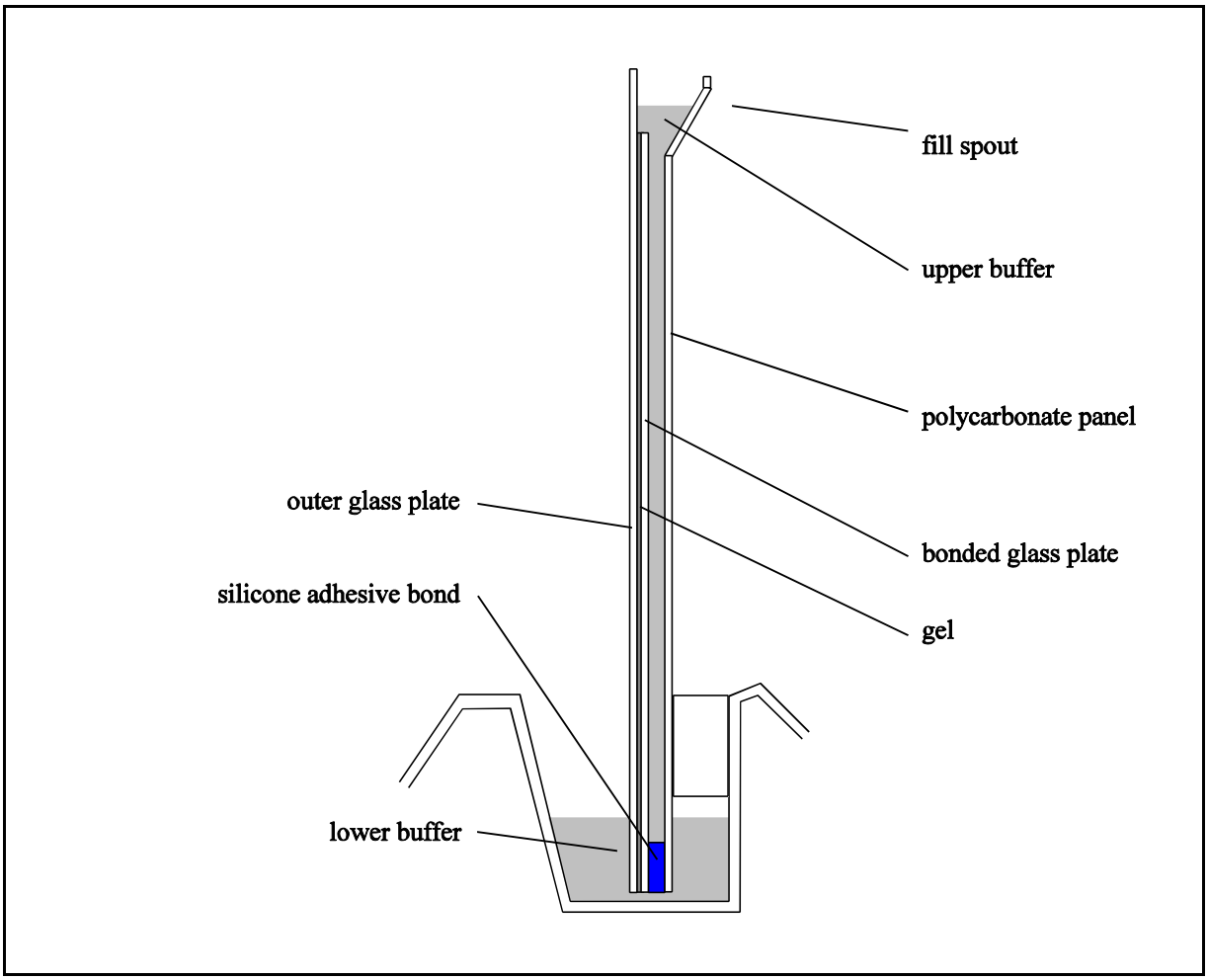

Figure 2.2: S1 Gel Apparatus

2.2.5.2.2 Reverse Transcription-PCR (RT-PCR, Titan™ One Tube RT-PCR System)

RT-PCR was used as a sensitive technique to quantify the level of marker gene expression. RT-PCR was performed using a one step technique. In a one step RT-PCR, both the cDNA synthesis reaction as well as the PCR reaction are performed with an optimised buffer (5 x, 7.5 mM $MgCl_2$, DMSO) and enzyme (AMV RT and Expand™ High Fidelity enzyme mix) without requiring the addition of reagents between cDNA synthesis and the PCR. The one step reaction system uses the AMV RT for first strand cDNA synthesis and the Expand™ High Fidelity enzyme blend (which consist of Taq DNA polymerase and Pwo DNA polymerase) for the PCR part. This technique allows amplification of fragments up to 2 kb with decreased error rates.

0.5 µg of DNase treated RNA was used as a template and the primer concentration was 20 µM. 1 µl of enzyme mix, 1 µl RNase inhibitor (40 U/µl), 2.5 µl DTT-solution (100 mM), 10 µl 5 x RT-PCR buffer and 0.2 mM dNTPs were added per PCR reaction. The PCR reactions were performed with the following thermal cycling program [in a Gene Amp PCR system 9600 or 2400 (Perkin Elmer)]: incubation step for 30 minutes at 50°C, denature template at 94°C for 2 minutes; 10 x cycles: denaturation step: 30 seconds at 94°C; annealing: for 30 seconds (primer depending temperature); elongation: 45 seconds-4 minutes (depending on the size of the expected fragment) at 68°C (Tab. 2.1). The annealing temperatures are usually between 50-60°C and were calculated with computer software (Primer designer, version 2.0-3.0 from Scientific & Educational Software). 25 x cycles (depending on the abundance of the respective mRNA): denaturating step for 30 seconds at 94°C; annealing: 30 seconds (primer depending temperature); elongation: 48 seconds-4 minutes (depending on the size of the expected fragment) at 68°C, and one single cycle of prolonged elongation (up to 7 minutes) at 68°C.

PCR Fragment length (kb)	<1	1.5	3	4.5	6
Elongation time	45 secs	1 min	2 mins	3 mins	4 mins

Table 2.1: RT- PCR Elongation Times

2.2.5.2.3 Real Time RT-PCR

Real Time RT-PCR was used to accurately quantify expression of hGH in WAP3hGH transgenic mice.

An accurate quantification using normal RT-PCR is not possible as here the amplification efficiency cannot be measured after each cycle, which would allow the samples to be compared with each other during the duration of the PCR amplification step. However, in the real time RT-PCR this is possible. The principle can be seen in figure 2.3.

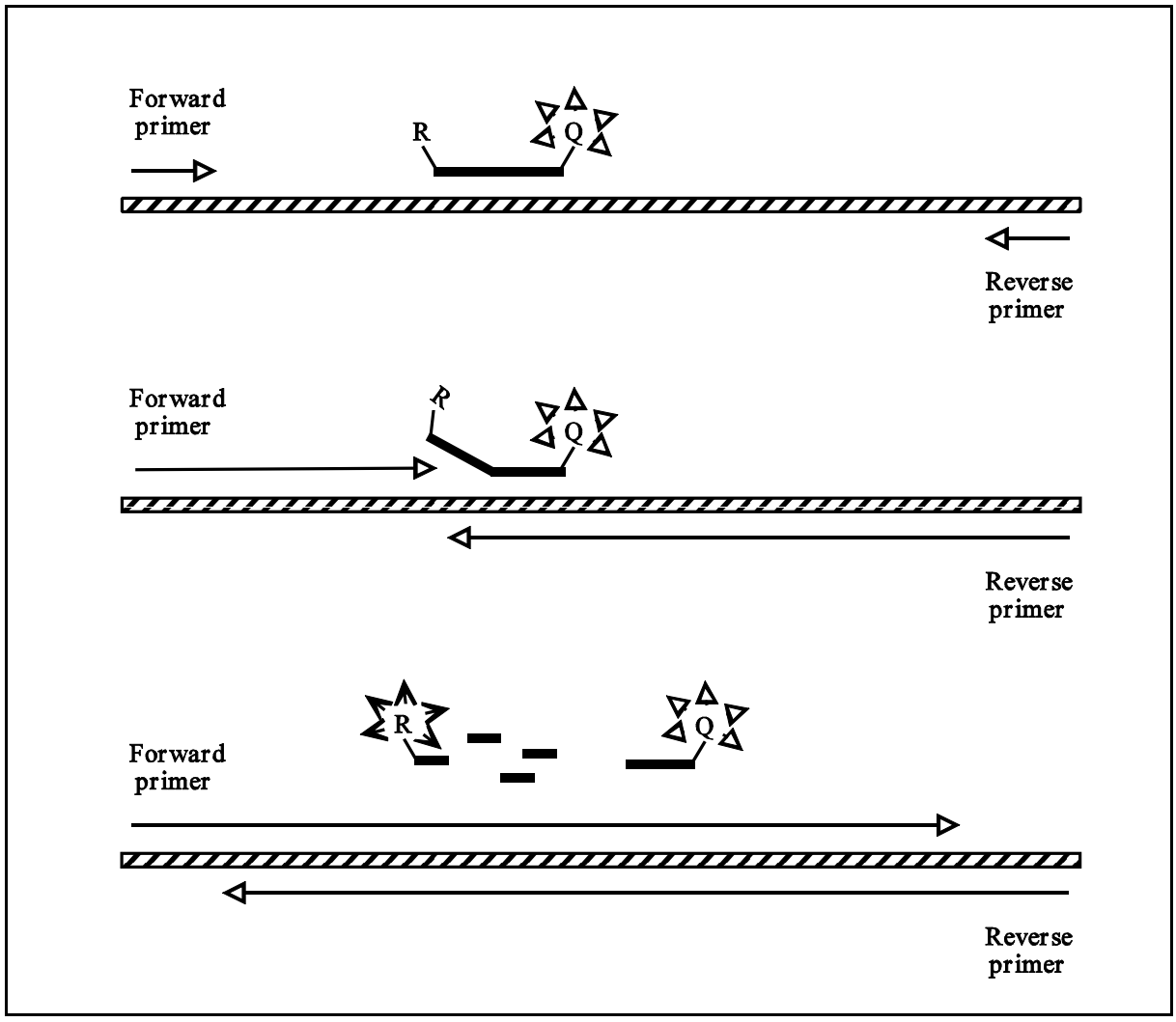

Figure 2.3: Principle of Real Time PCR

In addition to the two primers normally present in the RT-PCR reaction, a probe that is labelled with two different fluorescent marker and that is specific to a sequence in between the binding sites of the two primers is also added to the reaction. When both markers are close to each other (i.e. the probe is intact) and the reaction is excited with an argon laser energy is transferred from the reporter (R) to the quencher (Q) which then emits light. However, during the PCR reaction both primers are extended through the action of Taq polymerase until they meet the probe. The probe is then displaced from the DNA and, via the 5' nuclease activity of the Taq, degraded. This means that the two markers are no longer next to each other and the energy transfer from the reporter to the quencher can no longer take place. Now both fluorescent markers can emit light.

The strength of the emission from the markers is proportional to the amount of DNA that has been amplified in each reaction. The fluorescence is measured once every 7 seconds allowing the PCR reaction to be accurately followed and, after comparison with a known standard, the amount of RNA/DNA in the sample to be calculated. As the quantification is made during the PCR run, there is no need for the samples to be loaded on to a gel and this, in turn, excludes a

source of sample contamination from the experiment.

The following ingredients were pipetted together for each reaction: 45 µl master mix (contains reverse transcriptase and Taq polymerase, RT/PCR buffer, nucleotides, Mg^{2+}, the 2 primers and the fluorescently marked probe) and 5 µl of an RNA sample (or water as a negative control). The following PCR program was then run: reverse transcription for 45 mins at 48°C, inactivation of the RT activity and denaturing of the template for 2 mins at 94°C, 40 PCR cycles (15 secs 94°C, 1 min 60°C) then hold at 25°C.

2.2.5.2.4 Northern Blot (Lehrach *et al*., 1977)

Northern blot analysis is used to determine the size of transcription products. 20 µg of total RNA was mixed with a 2 x loading buffer (Sigma), denatured for 15 minutes at 65°C and loaded on to a 1% formaldehyde agarose gel. RNA was then transferred immediately to a nylon membrane (Zeta-Probe, Bio-Rad) by capillary elution (Fig. 2.4).

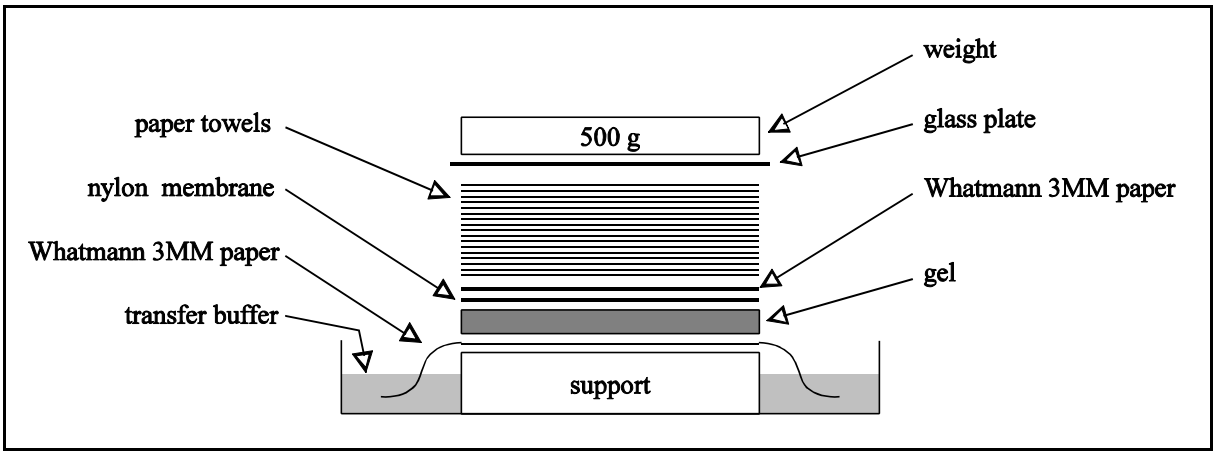

Figure 2.4: Northern Blot Apparatus

One long piece of Whatman 3 MM paper was soaked in 20 x SSC and laid on a glass plate to form a support. Any air bubbles between the glass plate and the Whatman 3 MM paper were smoothed out with a glass rod. Whilst the gel was soaking in 10 x SSC (for 20 minutes at room temperature), the nylon membrane was washed in $water_{DEPC}$ and then soaked in 10 x SSC. The gel was placed in an inverted position on the Whatman 3 MM paper. The nylon membrane was cut exactly to the size of the gel and was placed on top of the gel. Any air bubbles between gel and nylon membrane were smoothed out again. Three more pieces of Whatman 3 MM paper were cut out to the size of the gel and soaked in 20 x SSC before being placed on top of the nylon membrane. Finally, a stack of paper towels was mounted on top of the Whatman 3 MM papers. A glass plate was placed on the top and weighted down with a 500 g weight. The capillary transfer was left to proceed overnight in the presence of 20 x SSC. After the transfer the filter was washed once for 10 minutes in 6 x SSC and cross-linked. The gel was stained in ethidium bromide to evaluate the efficiency of the RNA transfer. The membrane was then prehybridised for 4 hours at 42°C and subsequently hybridised overnight at 42°C.

2.2.6 Radioactive Labelling of DNA Fragments

2.2.6.1 Labelling by Random Priming (Ready-To-Go DNA labelling kit, Pharmacia)

50 ng DNA was used for radioactive labelling. The DNA was denatured in 45 µl H_2O for 5 minutes at 95°C and immediately transferred to ice, to prevent the DNA from renaturing. The DNA was then pipetted into a tube containing a lyophilised reaction mix (dATP, dGTP, dTTP, Klenow fragment of DNA polymerase, random nonadeoxyribonucleotides [$d(N)_9$], and reaction buffer) and resuspended. 50 µCi α^{32}P-dCTP was added to the reaction mix and then incubated in a water bath for 15 minutes at 37°C (Fig. 2.5).

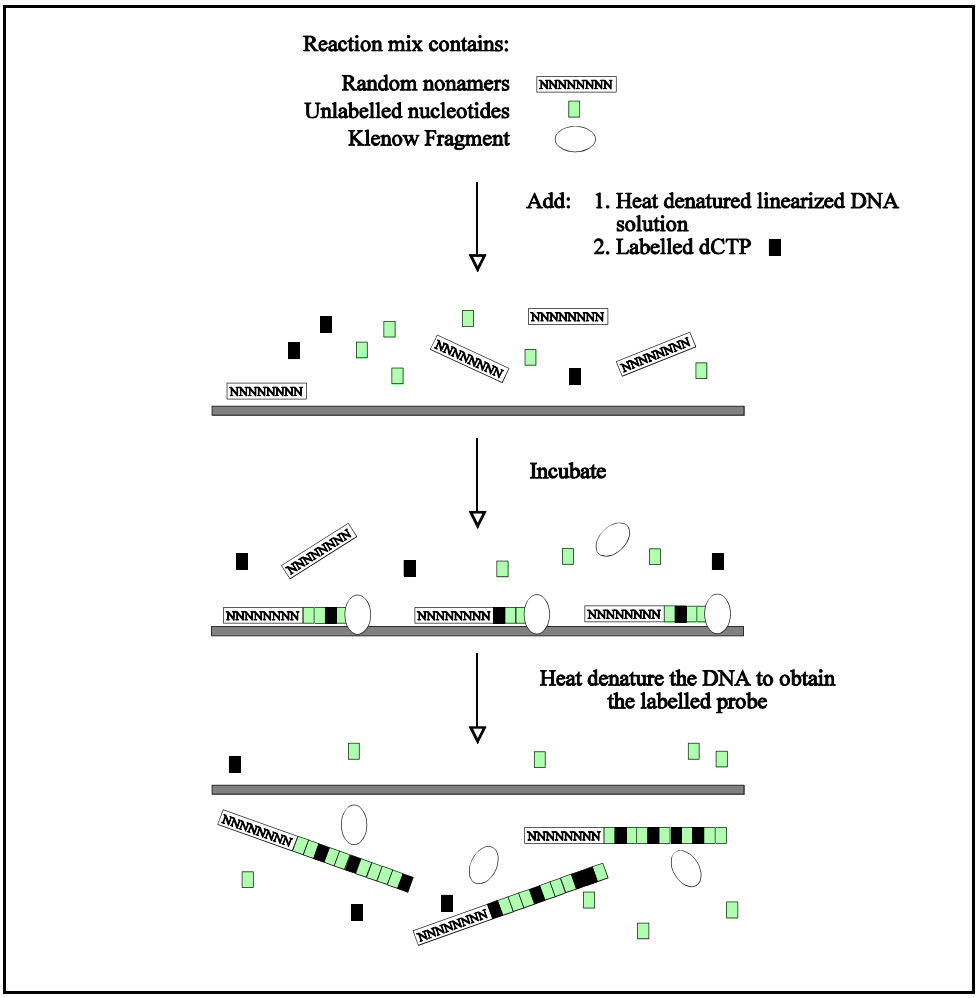

Figure 2.5: Principle of Labeling Using Random Priming

The reaction was stopped by adding 50 µl TNE (10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA). The efficiency of labelling was checked with Trichloroacetic acid precipitation. 2 x 1 µl the from reaction mix was pipetted on to two a GF/C (Whatman) filters. The rest of the reaction mix (98 µl) was filtered through a column (Bio-spin 30, Bio-Rad) and centrifuged for 3 minutes (700 x g). This step removes any unincorporated radioactive nucleotides. During centrifugation both free nucleotides and fragments less than 30 bp long stay bound to the column. After filtering 2 x 1 µl from the filtered reaction mix was pipetted on to two other GF/C filters, to

check the efficiency of the column filtration. During the TCA precipitation the filters were washed once with 10 ml 15% TCA, then twice with 10 ml 5% TCA and once with 10 ml ethanol (96%) before drying. The incorporated radioactivity on the filters before and after filtration was measured using a scintillation counter (Beckman LS6500). The comparison of the filters allows the rate of incorporation and specific activity of the probe to be calculated. For hybridisation a minimum activity of $4x10^8$ cpm/µg DNA is required. The labelled DNA was denatured for 5 minutes at 95°C before it was added to the hybridisation buffer.

2.2.7 Hybridisation Techniques

2.2.7.1 Southern Blot Hybridisation

The hybridisation took place under stringent conditions with both prehybridising and hybridising temperature of 68°C. This temperature means that the minimum homology between probe and DNA required for hybridisation to take place must be 81% (during washing this increases to 94%) (Howley *et al*., 1979).

In the first stage of hybridisation, the membrane was incubated in a prehybridisation solution containing reagents that block nonspecific DNA binding (Denharts solution, salmon sperm), thereby reducing background hybridisation. The membrane was then placed in a hybridisation tube (DNA side up) and ~1 ml hybridisation solution (5 x SSC, 5 x Denharts solution, 0.1% SDS, 50 µg/ml salmon sperm DNA) per 10 cm^2 of membrane was added. The incubation was made in a hybridisation oven rotating for 3 hours at 68°C. In the second stage, the prehybridisation solution was replaced by a pre-warmed (68°C) hybridisation solution containing the labelled probe, and was incubated in the hybridisation oven (Biometra OV 5) with rotation overnight at 68°C. Overnight incubation is carried out to allow the probe to bind to target sequences in the immobilized DNA. In the final stage of the hybridisation, the membrane was washed with a series of wash solutions. The first wash solution (2 x SSC, 0.1% SDS) was added to the tube and rotated for 10 minutes at room temperature. The first wash solution was then replaced then with second wash solution (0.2 x SSC, 0.1% SDS) and rotated for 15 minutes at 42°C. If a high stringency wash was necessary, the membrane was also washed with prewarmed (68°C) wash solution (0.1 x SSC, 0.1% SDS) for 15 minutes at 68°C. The membrane was then wrapped incling film before exposing it to a phosphorimager plate (Fuji).

2.2.7.2 Northern Blot Hybridisation

The steps of the hybridisation are the same as in the southern blot (see 2.2.7.1.) The difference occurs in the solutions for prehybridisation (10 x SSC, 50% deionised formamide, 1% sarcosyl, 0.1% SDS, 100 µg/ml salmon sperm DNA) and hybridisation (10 x SSC, 50% deionised formamide, 1% sarcosyl, 0.1% SDS, 100 µg/ml salmon sperm, 15 mg/ml dextran sulphate, 2 x Denharts) and the temperature at which these steps take place. The prehybridisation took place for 6 hours at 42°C and the hybridisation took place for 24 hours at 42°C. The prehybridisation solution and hybridisation solution both contained deionised formamide. The formamide is a helix destabiliser this allowing the annealing temperature to be lowered. The lower hybridisation temperature means that less target nucleic acids are washed from the membrane during the incubation steps.

2.3 Bacterial Techniques

2.3.1 Electroporation

Electrocompetent bacteria (DH10B, Life technologies) were used for the electroporation. A 100 µl aliquot of frozen bacteria (from -80°C) was thawed on ice for 10 minutes and 25 µl added to each ligation reaction (1 ng plasmid DNA) in a volume of 15 µl and mixed. The bacteria-DNA mix was then transferred to a precooled electroporation cuvette (Bio-Rad, electrode gap 0.1 cm) and electroporated (1.8 kV, 2. Ω, 25 µF):(Fig. 2.6). The time constant was normally between 4.5 and 5.5 ms. The bacteria were then immediately resuspended in 1ml SOC medium (2% bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM glucose in sterile milli-Q water pH 7.5) and transferred to a 15 ml glass test tube. The transformation mix was incubated in a shaker at 220 rpm (MultitronR, AJ110/111) for 1 hour at 37°C. 50-100 µl of the transformation mix was then plated out on agar plates (1% bactotryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% bacto agar in sterile milli-Q water) containing the appropriate antibiotic (50 µg/ml ampicillin, 30 µg/ml kanamycin or 25 µg/ml tetracyline) and incubated at 37°C overnight.

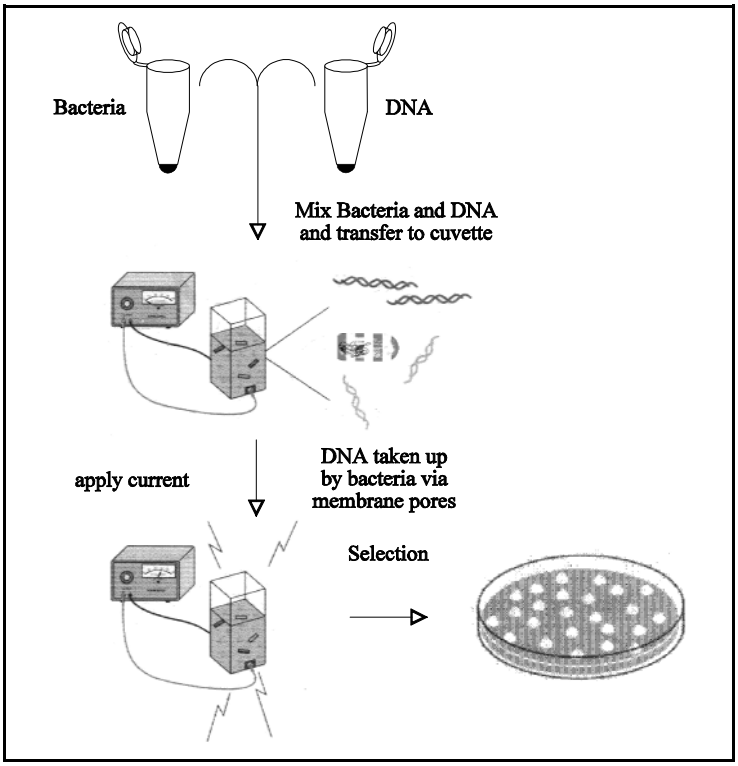

Figure 2.6: Principle of Electroporation

2.3.2 Preparation of Plasmid DNA

2.3.2.1 Small Scale Preparation of Plasmid DNA (QIAscreen)

In order to analyse a bacterial colony growing on an agar plate after electroporation, plasmid DNA was isolated by the QIAscreen method (Qiagen). 2-10 µg plasmid DNA per colony picked could be isolated using this technique, depending on the copy number of the plasmid (pBR322 derivative 15-200 copies/cell, pUC derivative 500-700 copies/cell). This amount is usually enough to perform restriction digests to check the DNA isolated.

2ml of Luria-Bertani (LB)-Broth (1% bactotryptone, 1% NaCl, 0.5% yeast extract) containing antibiotic in a 15 ml tube were inoculated with a single bacterial colony and incubated in a shaker (220 rpm) at 37°C overnight. A 1.5 ml aliquot of the bacterial suspension was then transferred to a 1.5 ml eppendorf tube and the bacteria pelleted in a tabletop centrifuge (6000 x g) for 5 minutes at room temperature. The pellet was resuspended in 300 µl buffer P1 (50 mM Tris/HCl, 10 mM EDTA, 100 µg/ml RNase A). 300 µl buffer P2 (200 mM NaOH, 1% SDS) was added and the suspension mixed by gentle inversion of the reaction tube. After an incubation period of 5 minutes at room temperature in which the bacterial membranes were lysed by buffer 2, 300 µl buffer P3 (3 M KAc pH 5.5) was added to precipitate the bacterial genomic DNA and proteins. The reaction tubes were inverted several times and the mixture centrifuged in a tabletop centrifuge (10000 x g) for 20 minutes. The supernatant, containing the plasmid DNA, was transferred into a new reaction tube, the DNA precipitated with 0.8 volumes of isopropanol and centrifuged (10000 x g) at room temperature for 20 minutes. The DNA pellet was then washed once with 500 µl ethanol (70%), centrifuged once more for 20 minutes (10000 x g) and dried in a vacuum dryer. The dried pellet was resuspended in 40 µl H_2O and DNA analysed by restriction digestion. For a digest 8 µl of plasmid solution was normally used in a total volume of 10 µl.

2.3.2.2 Large Scale Preparation of Plasmid DNA (Qiagen maxi prep)

A large scale plasmid DNA preparation was made by inoculating 200 ml of LB-broth including antibiotic with 100 µl of bacterial suspension from a small scale plasmid preparation. The bacteria were incubated at 37°C in a shaker (220 rpm) overnight. 10 ml of the bacterial suspension was taken for freezing (see 2.3.3), and the rest of the suspension (190 ml) was centrifuged for 10 minutes (Beckman, JLA-16.250, 5524 x g) at 4°C. The supernatant was discarded and the pellet resuspended in 10 ml ice cold buffer P1 (50 mM Tris/HCl, 10 mM EDTA, 100 µg/ml RNase A, pH 8) and transferred to a 40 ml tube. To lyse the bacterial membranes, 10 ml of buffer P2 (200 mM NaOH, 1% SDS) was then added and the components carefully mixed by inversion of the tube. After incubation for 5 minutes at room temperature, 10 ml of buffer P3 (3 M KAc pH 5.5) was added and gently mixed again. The mixture was incubated for 20 minutes on ice, and was then centrifuged (Beckmann, JA-25.50, 30966 x g) for 30 minutes at 4°C. The supernatant was decanted into a new 40 ml tube and centrifuged again for 15 minutes to separate residual bacterial proteins and precipitated SDS from the plasmid DNA. The supernatant was then applied to an ion exchange column (Qiagen- tip 500) that had been previously equilibrated with 10 ml of buffer QBT (750 mM NaCl, 50 mM MOPS, 15% ethanol, 0.15% Triton X-100). The detergent present (Triton X-100) in this buffer lowers the surface tension of the buffer so that it enters the column by gravity flow. After the supernatant had passed through the column, it was washed two times with 25 ml of buffer QC (1 M NaCl, 50

mM MOPS pH 7.0, 15% ethanol). Under these conditions (1 M NaCl) RNA and proteins are washed from the column whereas the plasmid DNA remains bound. The DNA was then eluted (because of high salt conditions) into a new 40 ml tube with 15 ml of buffer QF (1.25 M NaCl, 50 mM MOPS pH 8.5, 15% ethanol). The plasmid DNA was then precipitated by the addition of 0.8 volume of isopropanol and incubated at room temperature for 5 minutes before being centrifuged (Beckman, JS.13.1, 26688 x g) for 30 minutes at 4°C. The pellet was washed with 5 ml ethanol (70%) and after centrifuging once more for 15 minutes, dried in a vacuum dryer. The pellet was resuspended in 400 µl of 1 mM EDTA and left to dissolve overnight at 4°C. The concentration was then determined (as described in 2.2.3) and the DNA examined by digestion with restrictions enzymes.

2.3.3 Freezing of Transformed Bacteria

For each large scale preparation, 10 ml of the overnight culture was transferred to a 40 ml tube and centrifuged (Beckman, JA 25.50, 4355 x g) for 5 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 1.8 ml of a freezing medium (95% LB-broth, 5% DMSO). The bacterial suspension was then stored at -20°C. Addition of the DMSO inhibits formation of ice crystals during the freezing and thawing process.

2.4 Cell Culture Methods

2.4.1 Cell Lines

Transfection and infection experiments were made using cell lines as well as primary breast carcinoma cells. The cell lines were obtained from American Type Culture Collection (ATCC, Rockville, Maryland).

2.4.1.1 Rat-2 Cells (Topp, 1981)

The Rat-2 cells were derived from rat fibroblasts. Rat-2 cells lack appreciable levels of nuclear thymidine kinase, and are highly transfectable with DNA.

2.4.1.2 NIH-3T3 Cells (Jaichill *et al*., 1969)

The NIH-3T3 cell line is highly contact-inhibited and was established from mouse embryo cultures. It is highly sensitive to sarcoma virus focus formation and leukaemia virus propagation and has proven to be very useful in DNA transfection studies.

2.4.1.3 CrFK Cells (Crandell Feline Kidney)

CrFK cells were isolated from the cortical portion of the kidneys of a 10 to 12 week old normal female domestic cat (*Felis catus*) (Crandell *et al*., 1973). These cells have a epithelial morphology and they are permissive for infection with Mouse Mammary Tumour Virus (MMTV, Lasfargues *et al*., 1974, Howard *et al*., 1977). The viral structure proteins are correctly processed (Massey and Schochetman, 1979; Salmons *et al*., 1985) in this cell line.

2.4.1.4 EJ (T-24) Cells (Bladder carcinoma, Bubenik *et al.*, 1970)

This cell line was developed from a primary tumour of urinary bladder carcinoma in 1970.

2.4.1.5 MCF7 Cells (Human breast carcinoma, Brooks *et al*., 1973)

These cells retain several characteristics of differentiated mammary epithelium including the ability to process estradiol via cytoplasmic estrogen receptors and the capability of forming domes.

2.4.1.6 T47-D Cells (Human ductal breast carcinoma)

The T47-D line was isolated from an infiltrating ductal carcinoma of the breast. These differentiated epithelial cells were found to contain cytoplasmic junctions and receptors for beta estradiol, other steroids (Freake *et al*., 1981; Sher *et al*., 1981) and calcitonin (Lamp *et al*., 1981). It forms colonies in soft agar.

2.4.1.7 ZR-75-1 Cells (Human breast carcinoma, Engel *et al*., 1978)

The ZR-75-1 has a epithelial like structure. This cell line contains estradiol and other steroid receptors.

2.4.1.8 PANC-1 Cells (Human epithelioid pancreas carcinoma, Lieber *et al*., 1975)

PANC-1 is a permanent cell line initiated from a pancreatic carcinoma of ductal origin.

2.4.1.9 GR Cells (Ringold *et al*., 1975)

Mouse mammary tumour cell line, which produce MMTV virus particles

2.4.1.10 MDA-MB-435S Cells

MDA-MB-435S is a spindle shaped strain that evolved from the parent line (435) as isolated in 1976 (Cailleau *et al*., 1976) from the pleural effusion of a 31 years old female with metastatic ductal adenocarcinoma of the breast.

2.4.2. Packaging Cell Lines

2.4.2.1 PA317 Cells (Miller and Buttimore, 1986)

This packaging cell line was established by transfection of an MLV retroviral construct (pPAM3) and the gene for herpes simplex virus thymidine kinase (HSV-TK) into NIH 3T3 tk negative cells. After transfection with a retroviral vector, amphotropic virus particles are produced.

2.4.3 Cultivation of Cells

2.4.3.1 General Conditions

Cells were cultivated in an incubator at 37°C in 95% air with 5% CO_2. Humidity was kept at 95%. Cells were routinely cultivated in dulbecco's modified eagle's medium (DMEM) (Dulbecco and Freeman, 1959). The medium was supplemented with 10% fetal calf serum (FCS) and 50 µg Gentamycin. Cells were normally grown in a 75 cm^2 or 25 cm^2 T-flask (Nunc) and passaged every 3 days. The old medium was removed and cells washed with phosphate buffered saline (PBS) (0.82% NaCl; 0.04% KCl; 0.17% Na_2PO_4 x $2H_2O$; 0.03% KH_2PO_4) to remove trypsin-inhibitors from the culture flask. Subsequently, a trypsin/EDTA solution (8 mg/ml NaCl; 0.4 mg/ml KCl; 0.09 mg/ml Na_2PO_4 x $7H_2O$; 0.06 mg/ml KH_2PO_4; 0.2 mg/ml EDTA; 0.5 mg/ml trypsin; 0.35 mg/ml $NaHCO_3$; 0.01 mg/ml phenol red) was added and the cells incubated for 5 minutes. When the cells were no longer attached to the surface of the cell culture flask, they were pipetted up and down several times to produce a single cell suspension. A tenth of the suspension was then transferred to a new cell culture flask and fresh medium added, which in turn inactivated the trypsin.

2.4.3.2 Special Conditions for Breast Tumour Cell Lines

2.4.3.2.1 Monolayer

The culture conditions for these cells are the same as stated above. The differences occur in the medium and supplements.

ZR-75-1 cells are cultivated in RPMI-1640 medium supplemented with 10% FCS, 1 mM Na-pyruvate and 2 mM L-glutamine.

MCF-7 cells are cultivated in minimum essential eagle medium (MEM) (Soule *et al*., 1973) with non-essential amino acids supplemented with 10% FCS, 10 µg/ml bovine insulin and 1 mM Na-pyruvate.

T47-D cells are cultivated in RPMI-1640 medium supplemented with 10% FCS and 0.2 IU bovine insulin/ml.

2.4.3.2.2 In Collagen

Rat-tail collagen (Becton Dickinson) was diluted (50 µg/ml) in acetic acid (0.02 N) and vortexed until dissolved. The mixture was layered on to culture dishes (1 ml/35 mm dishes) and incubated for 1 hour in a laminar flow hood. The remaining solution was removed and the plates washed with serum free medium. $5x10^4$-$5x10^5$ cells in cell culture medium were then seeded on to the collagen gel. The collagen gel enhances the expression of cell specific morphology and function.

2.4.3.2.3 In Matrigel®

Matrigel (basement membrane matrix, Becton Dickinson) was thawed at 4°C on ice overnight. The matrigel was mixed to ensure homogeneity before use. The tips and cell culture dishes were

kept on ice at all times. 150 µl of matrigel was mixed with the $5x10^4$-$1x10^5$ cells and placed in cell culture plates (12 wells dishes). The cell culture dishes were then incubated for 30 minutes at 37°C after which cell culture medium was added.

Dispase was used to recover cells cultured in matrigel. Dispase yields a single cell suspension and cleaves fibronectin, collagen IV and minimally collagen I, but does not cleave collagen V and laminin. The medium was removed from the matrigel and a dipase solution was added (0.2 ml/cm^2). After incubation (2 hours at 37°C) the cell suspension was mixed up and down using a pipette, and transferred to a sterile tube. The action of dipase was stopped by adding PBS. The cell suspension was centrifuged (5 minutes at 200 x g) and was washed once more with PBS. Cells were either resuspended in fresh medium or prepared for further analysis.

2.4.4 Primary Tissue Culture

2.4.4.1 Organoid Preparation (Stampfer *et al*., 1985)

All material was received fresh immediately after operation and put into falcon tubes that contained PBS. After transportation on ice the tumour was either prepared immediately or stored (for a maximum of 3-4 hours) at 4°C. The tumour tissue was washed with fresh cold PBS and transferred into a large sterile tissue culture dish. Epithelial areas appear as white strands embedded in the more yellow stromal matrix. These areas were gently dissected, scraping away the fatty material. The epithelial tissue was made smaller using opposing scalpels. The minced epithelial tissue was placed into a conical centrifuge tube (50 ml). The tube was then filled up, leaving only a small air space to allow for mixing during rotation, using a tissue digestion mixture (DMEM; 5% FCS; 100 U/ml penicillin/streptomycin; 200 U/ml collagenase; 100 U/ml hyaluronidase). The tubes were then placed on a shaker and shaken overnight at 37°C. The next day the tubes were centrifuged at 200 x g for 10 minutes. The supernatant, fat and medium were then discarded. The completion of digestion was checked by diluting a small aliquot of the pellet in medium and subsequently microscopically examining the digested material. The digest was said to be complete when tumour tissue that only showed unstructured clumps of epithelial cells was present. When digestion was complete, the pellet was resuspended in modified Joklik Medium (10 µg/ml insulin; 10% FCS; 100 ng/ml cholera-toxin; 0.5 µM cortisol; 20 ng/ml epidermal growth factor; 2 M glutamine; 100 U/ml penicillin/streptomycin; 5 µg/ml fungizone). To eliminate fibroblasts and collect the organoids, multiple filtration steps were made. Firstly, a 200 µm nylon filter was used. The organoids that were collected on the filter were then washed with medium into a small glass beaker. Next, a 53 µm nylon filter was used to filter the medium from the first step. After this filtration the medium contains mainly single cells (stromal fibroblast and some epithelial cells). The 200 µm organoids and any 53 µm organoids collected were then put together into one or more 50 ml tubes filled with medium and were centrifuged at 200 x g for 10 minutes. The supernatant was then removed, the pellet was resuspended in Joklik medium and seeded into either 10 cm or 6-well cell culture dishes.

2.4.5 Freezing and Thawing of Tissue Culture Cells

Prior to freezing, the cells were washed with PBS, trypsinised and centrifuged (200 x g, 5 minutes). The pellet was washed again with PBS and recentrifuged. This pellet was resuspended in an appropriate amount of freezing medium [75% DMEM, 20% fetal calf serum, 5% DMSO

(to prevent the formation of ice crystals during freezing and thus protects the cell membranes from damage)], transferred to a polystyrol tube and incubated on ice for 30 minutes (a cell pellet from a 175 cm^2 flask was resuspended in 3 ml of freezing medium). The tubes were then transferred to a polystyrene holder and incubated for 24 hours at -80°C. Subsequently the cells were moved to a storage box and stored in liquid nitrogen or at -80°C.

Cells were thawed as quickly as possible, transferred into 13 ml falcon tubes containing 10ml DMEM (10% FCS) medium and centrifuged (200 x g, 5 minutes). The supernatant was then removed, the cells resuspended in fresh medium, and centrifuged again. This was done to remove the DMSO that is present in the freezing medium. After thawing, DMSO is toxic to the cellular metabolism and kills the cells. It is therefore important to remove all traces of DMSO as soon as possible after thawing the cells. After centrifugation the pellet was resuspended in fresh medium and seeded into a cell culture flask (25 cm^2 or 75 cm^2).

2.4.6 Selection Methods

2.4.6.1 G418-Selection (Colbere-Garapin *et al*., 1981)

After transfection of constructs or infection of cells G418 (400 µg/ml or 800 µg/ml Geneticin) was added to select the cells based upon the expression of the neomycin gene. Normally eucaryotic cells are sensitive to G418 as this antibiotic inhibits the activity of 80S ribosomes and blocks protein synthesis (Davies and Jimenez, 1980). The death of the cells correlates with their rate of growth. This means that cells that grow faster will also die faster as a result of G418 selection (Southern und Berg, 1982). The concentration of G418 required is different from cell line to cell line. For example, for Rat-2 or PA317 cells the concentration is 400 µg/ml whereas for EJ or GR cells it is 800 µg/ml. The selection should normally be completed in two weeks.

2.4.7 DNA-Transfer Methods

A variety of methods can be used to introduced foreign DNA into cells. The most common DNA transfer method is calcium mediated transfection (Graham and van der Ebb, 1973; Wigler *et al*., 1978). DNA and viruses can also be introduced into cells with diethylaminoethyl-dextran [DEAE (Pagano *et al*., 1967; McCutchan and Pagano, 1968)]. A third method used to introduce DNA into cells is lipofection. (Felgner *et al*., 1987).

When foreign DNA is introduced into tissue culture cells it is taken up into the nucleus and is not integrated into the cellular genome for 48 to 72 hours (Cullen, 1987). The so-called “transgenome” is transiently expressed in the cell until, after several cell cycles, the DNA is either degraded or incorporated into the genome. During the first two or three days after the transfection, the expression of the introduced DNA is not influenced by neighbouring sequences in the host genome, but is dependent on the strength of the promoter of the introduced construct. After this period, the expression of the relatively small amount of DNA that has become integrated into the cellular genome may be influenced by silencing or enhancing effects of the surrounding DNA sequences.

2.4.7.1 Calcium-Phosphate Mediated Transfection (CellPhect, Pharmacia)

The negatively charged DNA is loaded with calcium ions in a $CaCl_2$ solution and coprecipitated by the addition of phosphate. This DNA calcium phosphate precipitate is taken up by many tissue culture cells.

24 hours before the beginning of the experiment $5x10^5$ cells were plated in 6-wells cell culture dishes (diameter of 3 cm), to have approximately 70% confluent cells for transfection. 3 hours before transfection the medium of the cells was changed. 5 to 10 µg of plasmid DNA were diluted to a final volume of 240 µl in sterile tissue culture water (Gibco) in a 10 ml polystyrol tube. 120 µl of buffer A (CellPhect transfection kit, Pharmacia) was added and the components gently mixed. After an incubation at room temperature for 10 minutes, 480 µl of buffer B (CellPhect transfection-kit, Pharmacia) was added. The components were mixed by vortexing for a few seconds. In every experiment, one mock transfection (without DNA) was made and acted as the negative control. After incubating at room temperature for a further 15 minutes, the total mix of 600 µl was added dropwise to the cell culture medium. The cells were incubated in the presence of the DNA calcium phosphate precipitate for 18 hours. The old medium was then removed and the cells were washed twice with PBS. Subsequently, either fresh medium was added or the cells were washed, trypsinised and transferred to a culture dish depending on what the transfected cells were to be used for. 24 hours later G418 was added.

2.4.7.2 Lipofection (Lipofectamine, Gibco-BRL)

Lipofection was used successfully only for stable transfections. The principle of lipofection is based on the formation of a DNA-liposome complex between the strongly positively charged lipofectamine (Gibco-BRL) and the negatively charged DNA. Subsequent fusion with a cell membrane leads to the uptake of the DNA into the cell. The liposome is not degraded and moves to the nuclear membrane, where it passes into the nucleus, degrades and sets the DNA free.

24 hours before the beginning of the experiment $5x10^5$ cells were plated in 6-well cell culture dishes (diameter of 3 cm), to have approximately 70% confluent cells for lipofection. The cells were washed three times in serum free medium to remove all traces of serum that would decrease the efficiency of the transfection. 2 µg of plasmid DNA was mixed with 100 µl serum free medium. In parallel, 15 µl lipofectamine was mixed with 100 µl serum free medium. The two solutions were then mixed and incubated for 45 minutes at room temperature. In every experiment, one mock lipofection (without DNA) was made and acted as the negative control. Subsequently, 800 µl serum free medium was added and the resultant 1 ml applied to the cells. The cells were incubated in the presence of the liposome DNA mixture for 5 hours. 1 ml of normal medium (DMEM) containing 20% of fetal calf serum was then added to the cells. 24 hours later the cells were trypsinised, 1:10 diluted and transferred into cell culture dishes (10 cm). The cells were left to attach to the surface in normal medium for 24 hours. The old medium was then removed and selection medium, supplemented with G418, was added.

2.4.7.3 Infection

2.4.7.3.1 Infection of Cell Lines

24 hours before the beginning of the infection $5x10^5$ virus producing cells were plated in 6-well cell culture dishes (diameter of 3 cm) in 3 ml normal medium. At the same time $5x10^5$ target cells were plated in 10 cm cell culture dishes. The next day, medium from virus producing cells was removed and filtered using a 45 µm filter (Nalgene, Millipore) to eliminate any contamination from virus producing cells. 8 µg/ml polybrene®(hexadimethrine bromide) was added to the filtered medium and mixed (1 µl polybrene®/ml supernatant). Polybrene® is a positively charged macromolecule and functions as an adapter between the negatively charged cell membrane and the similarly negatively charged virus envelope in order to facilitate the adhesion of one to the other (Hornsby and Salmons, 1993). 2 ml of the mix was added to the cells. After 5 hours, 6ml normal medium was added to reduce the polybrene concentration to 2 µg/ml as high concentrations of polybrene are toxic to cells. The following day the cells were trypsinised and diluted (1:10 - 1:100 depending on cell type). 24 hours later selection medium was added. The titre could be calculated as soon as the mock infected control cells were dead.

2.4.7.3.2 Infection of Primary Tumour Cells

Primary tumour cells were infected in an identical manner to cell lines (see 2.4.7.3.1). The only difference is in the treatment after infection. 24 hours after infection the cells were washed and fresh medium was added containing different hormones (10^{-6} M dexamethasone, 3 µg/ml insulin, 3 µg/ml prolactin). After 24 or 48 hours hormone stimulation the cells were washed and prepared for either x-gal staining or a ß-galactosidase assay.

2.4.8 Titre Calculation

The titre could be calculated using the following equation:

$$\frac{\text{Colony number/plate x dilution factor}}{\text{Total volume of the infectious medium}} = \text{colony forming units/ml (cfu/ml)}$$

2.4.9 Cell Clone Isolation

After selection had taken place and it could be shown that the cells in the negative control were dead, clones could be isolated from the infected/transfected cells. The cells were washed twice with PBS and individual colonies isolated using stainless steel cloning cylinders (diameter 1 cm, bore 0.5 cm) coated on the underside with silicone grease. The cylinders were placed onto the colonies and then filled with a trypsin/EDTA solution (Fig. 4). After 5 minutes the cells were resuspended using a pasteur pipette and transferred to either a 24 or 6 well dish. The colonies were then expanded.

2.5 Protein Techniques

2.5.1 Isolation of Cellular Proteins

For the isolation of nuclear cell proteins, 25 cm^2 cell culture flasks or 6-well plates (in the case of primary cells) that had been grown to confluence were trypsinised and centrifuged at room temperature (600 x g, 10 minutes). The supernatant was discarded and the cell pellet washed twice with PBS. The washed cells were resuspended in 200 µl lysis buffer [100 mM K_2PO_4, pH 7.8; 0.2% triton X-100; 1 mM dithiothreitol (DTT)] and centrifuged (15000 x g, 2 minutes). The supernatant was transferred into a new tube and either used directly or frozen and stored at -80°C.

2.5.2 Determination of Protein Concentration

The quantification of protein was performed using a protein detection kit from Bio-Rad (Lowry, 1951). A bovine serum albumin (BSA) solution standard was made (0.2 mg/ml; 0.4 mg/ml; 0.8 mg/ml; 1.2 mg/ml; 1.6 mg/ml; 2 mg/ml) using the lysis buffer. A standard curve was made for every experiment. 5 µl of the standards and samples were pipetted, in duplicate, into the wells of a 96 well microplate. Additionally, 25 µl of reagent A (alkaline copper tartate solution) and 200 µl of reagent B (a dilute folin reagent) were added. Before the reagent A was added, 20 µl of reagent S was added per ml of reagent A used. After 15 minutes incubation at room temperature (RT) the absorbance was measured in a microplate reader (λ750 nm).

2.5.3 Detection of Reporter Gene Products

2.5.3.1 Chemoluminescent ß-galactosidase Activity (Tropix-100 Galactolight Kit, PE)

5-10 µg (15-20 µl) of a total protein extract (2.2.3) was pipetted into a reaction tube (Sarstedt). 70-100 µl of the reaction buffer (100 mM Na_2PO_4, pH 8; 1 mM $MgCl_2$, 1 x Galacton^R substrate: 0.035 mM AMPGD chemiluminescent substrate) was added and incubated for 1 hour at room temperature, to keep the signals within the linear range. Some cells were incubated at 48°C for 1 hour, before the reaction buffer was added. At this temperature the endogenous ß-galactosidase activity is reduced. For tissue extracts, protease inhibitors (PMSF, Sigma, Leupeptin, Sigma) were used. Measurements were made by injecting 100 µl of accelerator solution (light emission accelerator, 10% emerald luminescence amplifier, 0.2 N NaOH, Tropix), and then counting for 10 seconds in a luminometer (Berthold Autolumat LB953) (Fig. 2.7).

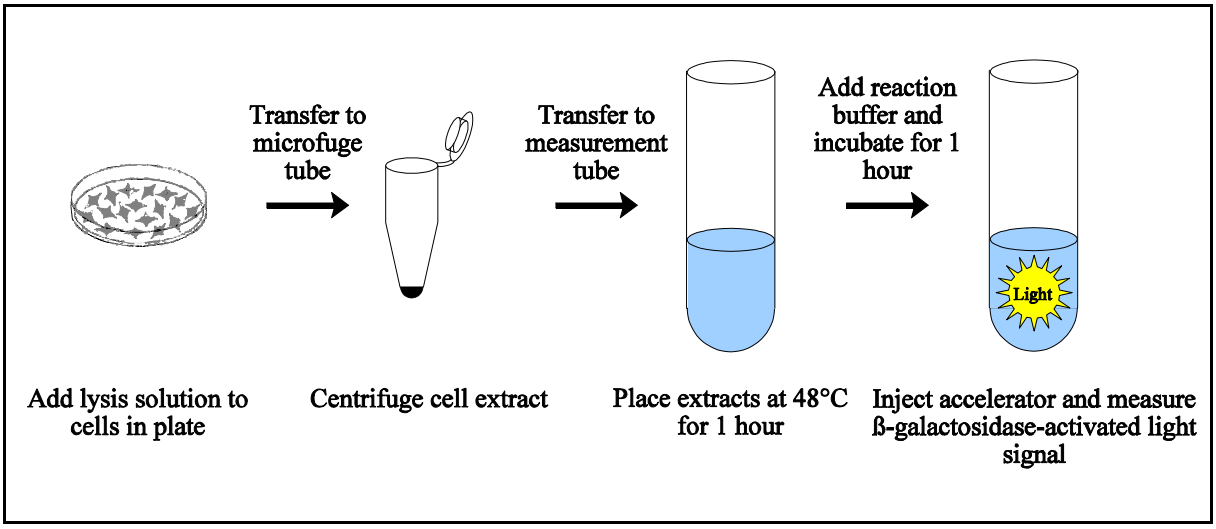

Figure 2.7: Principle of ß-galactosidase Assay

2.5.3.2 *In situ* ß-galactosidase

2.5.3.2.1 X-gal Staining of Cultured Cells (Cepko, 1989)

Cultured cells were washed twice with cold PBS and fixed for 20 minutes using 2% paraformaldehyde in PBS. The cells were then washed twice in PBS and were incubated in PBS for 10 minutes, to remove excess paraformaldehyde. Subsequently the cells were washed one more time in PBS and staining buffer added (20 mM $K_3Fe(CN)_6$, 20 mM $K_4Fe(CN)_6x3H_2O$, 2 mM $MgCl_2$) containing 1 mg/ml of X-gal (5-brom-4-chlor-3-indolyl ß-D-galactopyranoside). The cells were incubated at 37°C overnight. After staining, the cells were examined using light microscopy.

2.5.3.2.2 X-gal Staining of Mouse Tissues

Either normal or tumour tissue as disected from from a mouse and washed one time with PBS. An eppendorf tube was filled up with 4% paraformaldehyde in PBS and the tissue placed into it for fixation. This took between 1 and 4 hours at 4°C. The tissue was then removed into a rinsing buffer (100 mM sodium phophate pH. 7.5, 2 mm MgCl2, 0.01% sodium deoyxcholate, 0.02% NP-40) or PBS for 30 minutes. The buffer was then replaced and the tissue rinsed for a further 30 minute. This step was repeated one more time. The rinse buffer or PBS was then removed and X-gal solution was added. This staining took between 4 and 48 hours at 3°C. About 90% of the total staining will occur in the fist 24 hours. The stained tissue was the either photographed or was fixed in a paraffin-block and cut.

2.6 Animals

The first generation of SCID mice were obtained from Harlan Winkelmann, BRD and subsequently bred further in our animal facility. The mice were housed in sterile cages under laminar flow hoods in a temperature controlled room and were fed autoclaved food and water. All animals were handled in accordance with institutional guidelines for the care and use of experimental animals.

2.6.1 Implantation of Cancer Cells into the Mammary Fatpad of Severe Combined Immune Deficient (SCID) Mice

2.6.1.1 Preparing of the SCID Mice and Cells for Transplantation

To generate tumours infected and selected (G418) human breast cancer cell lines (MCF7, T47-D, ZR-75-1) and some other cancer cell lines (GR, EJ, Panc-1) were prepared for mammary fat pad (mfp) injection in female SCID mice. Four SCID mice, 3-4 weeks old and 17-19 g in weight, were used per construct for all cell lines. One day before injection, an estrogen pellet (Innovative Research of America, Florida) was implanted subcutaneously into the SCID mice. The estrogen supplementation increase the growth rate of tumours (Rae-Venter *et al*., 1980, Osborne *et al*., 1981), especially breast tumours.

The cells were not kept in medium containing G418 before injection as it inhibits tumorigenesis (Holt *et al*., 1997). Cells were washed with PBS, trypsinised and centrifuged (200 x g, 5 minutes). The cell pellet was washed twice with PBS and $1x10^7$ cells were mixed with matrigel and diluted 1:1 in serum free medium containing antibiotics (streptomycin/penicillin). These steps were made on ice. The matrigel can also increase the incidence of tumours and enhance the tumour growth rate (Friedman *et al*., 1990). A syringe was used to inject a volume of 400 µl into each of the four mice (per construct) in the mfp. The mice were anaesthetised using chloroform and then the implantation was made. Animals were examined once a week for tumour growth.

3. RESULTS

3.1 Heterologous Promoters

The most frequently used promoters are of viral origin, often being derived from viruses different to the vector backbone i.e. cytomegalovirus promoters have been used in all almost vector systems. Viral promoters have the advantage of being smaller, generally more active and better understood than human promoter sequences.

A range of cellular promoters have been developed for specific tissues including the liver (albumin; Miyatake *et al*., 1997), muscle (myosin light chain 1; Shi *et al*., 1997), endothelial cells (von Willebrandt; Ozaki *et al*., 1996, smooth muscle 22a; Kim *et al*., 1997). Tumour specific promoters are also being used in deve78loping cancer therapies, including tyrosinase for B16 melanoma (Diaz *et al*., 1998), DF3/MUC1 for certain breast cancers (Chen *et al*., 1995) and alpha-fetoprotein for hepatomas (Wen *et al*., 1993).

3.1.1 Heterologous Viral Promoters (MMTV U3 Region)

The U3 region of mouse mammary tumour virus (MMTV) was chosen as the heterologous viral promoter to be investigated. The regulation of MMTV expression is controlled by mammary specific factors, pregnancy hormones (Ringold, 1975) and steroid hormones (Groner *et al*., 1984; Rousseau, 1984). Sequences conferring mammary cell specific expression are located in an enhancer element in the extreme 5' end of the long terminal repeat (LTR) region of the MMTV provirus (Lefebvre *et al*., 1991; Mink *et al*., 1990; Yanagawa *et al*., 1991).

Retroviral vectors in which the MMTV LTR (or U3 region) controls transgene expression after reverse transcription could be useful for preferential, inducible expression of transferred genes in organs such as the mammary gland or in mammary tumours. However, MMTV based vector systems have been constructed (Günzburg *et al*., 1986; Shackleford *et al*., 1988; Morris *et al*., 1989; Salmons *et al*., 1989,) and unfortunately have relatively low titres. This is probably due to the complex regulation mechanisms of MMTV (Acha-Orbea *et al*., 1995; Günzburg *et al*., 1995).

Insertion of part of the MMTV U3 region (containing the glucocorticoid response element) into the 3' LTR U3 region of MLV at the expense of the MLV enhancer has been previously shown to be incompatible with recombinant virus production (Overhauser *et al.*, 1985). Only when part of the MMTV U3 region was additionally inserted into the 3' MLV U3 region could recombinant virus be obtained. However, this virus showed a serious reduction in infectivity and was not stable, undergoing frequent rearrangements. To circumvent these problems, Overhauser and Fan had to insert MMTV U3 fragments into both the 3' and 5' MLV LTRs. Again only constructs with additional MMTV sequences in both LTRs gave functional virus and no virus could be recovered when the MLV enhancer sequences were replaced by MMTV sequences. This instability may have been due to the inability of this virus to undergo more than one round of infection.

In this study, the MLV vector U3 sequences except for the inverted repeat (IR) located at the 5' end of the U3 region, which is required for vector provirus integration (Goff *et al*., 1992), were

replaced with those of MMTV.

3.1.2 Construction of Modified Retroviral Vectors

The retroviral vectors BAG (Price *et al*., 1987) and LXSN (Miller and Rosman, 1989) were used as the basis for construction of the hybrid retroviral vectors. These vectors are both modified murine leukæmia viruses where the structural genes *gag*, *pol* and *env* have been replaced with a marker gene driven by a heterologous promoter.

The BAG vector carries the bacterial lacZ gene under the transcriptional control of the MLV promoter in the 5' LTR (Fig. 3.1). The expression of the lacZ gene can be measured in either a qualitative [staining of cells or tissues (Cepko *et al*., 1989)] or quantitative manner [enzymatic assay from cell extracts (Jain and Margrath, 1991)]. The expression of this gene can be determined as quickly as 48 hours after infection and such detection methods can be used to rapidly show that infection events have taken place.

This vector also carries a second marker gene, conferring neomycin resistance. The neomycin resistance gene from the TN5 transposon (Eglitis, 1991) is driven by an internal heterologous simian virus 40 (SV40) promoter. The presence of this cassette allows the selection of stably infected eukaryotic cells using neomycin sulphate. A bacterial origin of replication (ori) allows the plasmid to multiply in bacteria facilitating amplification of large amounts of vector DNA for cloning and permits recloning of an integrated provirus from the DNA of infected cells.

The vector LXSNegfp (Klein *et al*., 1997) is a modified version of the MLV based vector LXSN (Fig.3.1). LXSN contains the 5' LTR of the murine sarcoma virus (MuSV), a hybrid packaging signal from both MuSV and MoMLV and the 3' LTR of MoMLV. Similarly to BAG, it carries the SV40-neo^r cassette to allow selection in eukaryotic cells. The gene encoding enhanced green fluorescent protein (EGFP) has been cloned in between the packaging signal and the SV40-neo^r cassette. EGFP is a modified version of the green fluorescent protein (GFP) gene found in *Aequoria victoria* (Chalfie *et al*., 1994) in which two mutations in the chromophore region shift the emission spectrum of the protein into the visible region (Cormack *et al*., 1996). The expression of this modified version can thus be detected in living cells using both fluorescence microscopy and flow cytometry without the need for special reagents or enzymatic substrates. It is possible to detect expression of this gene only 24 hours after infection (Kandel *et al*.,1997; Klein *et al*., 1997).

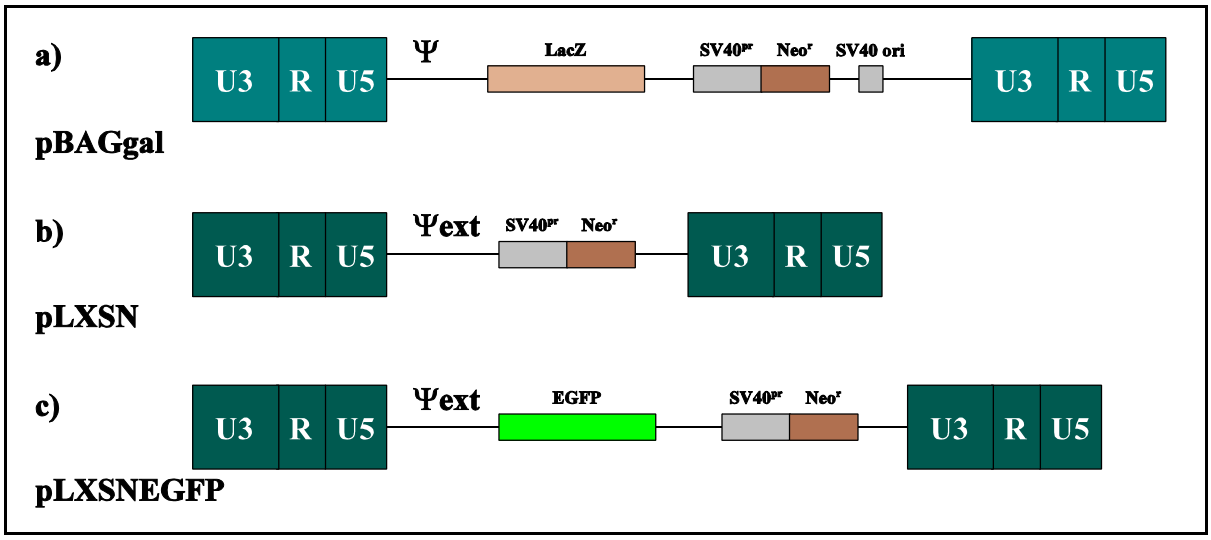

Figure 3.1: Retroviral Vectors
These vectors are based upon two variants of murine leukaemia virus (MLV). The structural genes (*gag*, *pol* & *env*) have been replaced with marker genes. Essential sequences necessary for viral packaging and integration [LTR, primer binding site (PBS) & packaging signal Ψ or an extended packaging signal Ψext] are present in all three vectors. The presence of a "p" in front of the vector name indicates that the sequence for the retroviral vector is flanked by the necessary elements to enable replication in bacteria (such as ori & amp^r).

a) BAG (Price *et al*., 1987): The BAG vector is based upon moloney murine leukaemia virus (MoMLV). The LTRs and packaging signal Ψ originate from MoMLV. It contains both the ß-galactosidase (lacZ) gene under the control of the 5'LTR, and the neomycin resistance (neo^r) gene from the TN5 transposon (Eglitis, 1991), which is under the control of SV40 promoter (SV40pr). The polyadenylation signal is present at the end of the R region of the 3' LTR. As a shuttle vector BAG contains a prokaryotic origin of replication (ori, from pBR322) allowing the plasmid to multiply in bacteria facilitating amplification of large amounts of vector DNA for cloning and permits recloning of an integrated provirus from the DNA of infected cells.

b) LXSN (Miller and Rosman, 1989): LXSN contains the 5' LTR of the murine sarcoma virus (MuSV), a hybrid packaging signal from both MuSV and MoMLV and the 3' LTR of MoMLV. The vector carries a resistance marker gene (neomycin), which is driven by a simian virus 40 promoter (SV40pr). Like BAG, the polyadenylation signal is at the end of the R region of the 3' LTR.

c) LXSNEGFP (Klein *et al*., 1997): This vector was developed from LXSN. It contains as a reporter gene EGFP (Cormack *et al*., 1996). The EGFP gene is under the control of the MLV promoter in the 5' LTR and the polyadenylation signal is at the end of the R region of the 3' LTR.

3.1.2.1 Construction of MMTV-BAGgal Vector

pCon6 was used as the source plasmid for the cloning of pMMTV-BAGgal. It is a derivate of pBAG, where the 3' U3 region of the 3' LTR has been replaced by a polylinker (Saller, 1994). The inverted repeat (IR) remains intact, as it is necessary for integration. This polylinker facilitates the incorporation of heterologous promoters into the U3 region of the 3' LTR. The strategy used ensured that the inverted repeat at the 5' border of the U3 region was left intact and also that unique *Sac*II and *Mlu*I sites were inserted to facilitate the introduction of heterologous sequences such as the MMTV U3 region. The complete 1211 bp MMTV U3 region was isolated by PCR using specific primers carrying *Sac*II or *Mlu*I restriction enzyme cleavage sites and inserted into the pCON6 plasmid (Fig. 3.2). The resulting plasmid was named pMMTV-

BAGgal. In the majority of the following experiments pMMTV-BAGgal was used as the presence of a prokaryotic origin of replication in the vector allows recloning of integrated proviruses for later molecular characterisation.

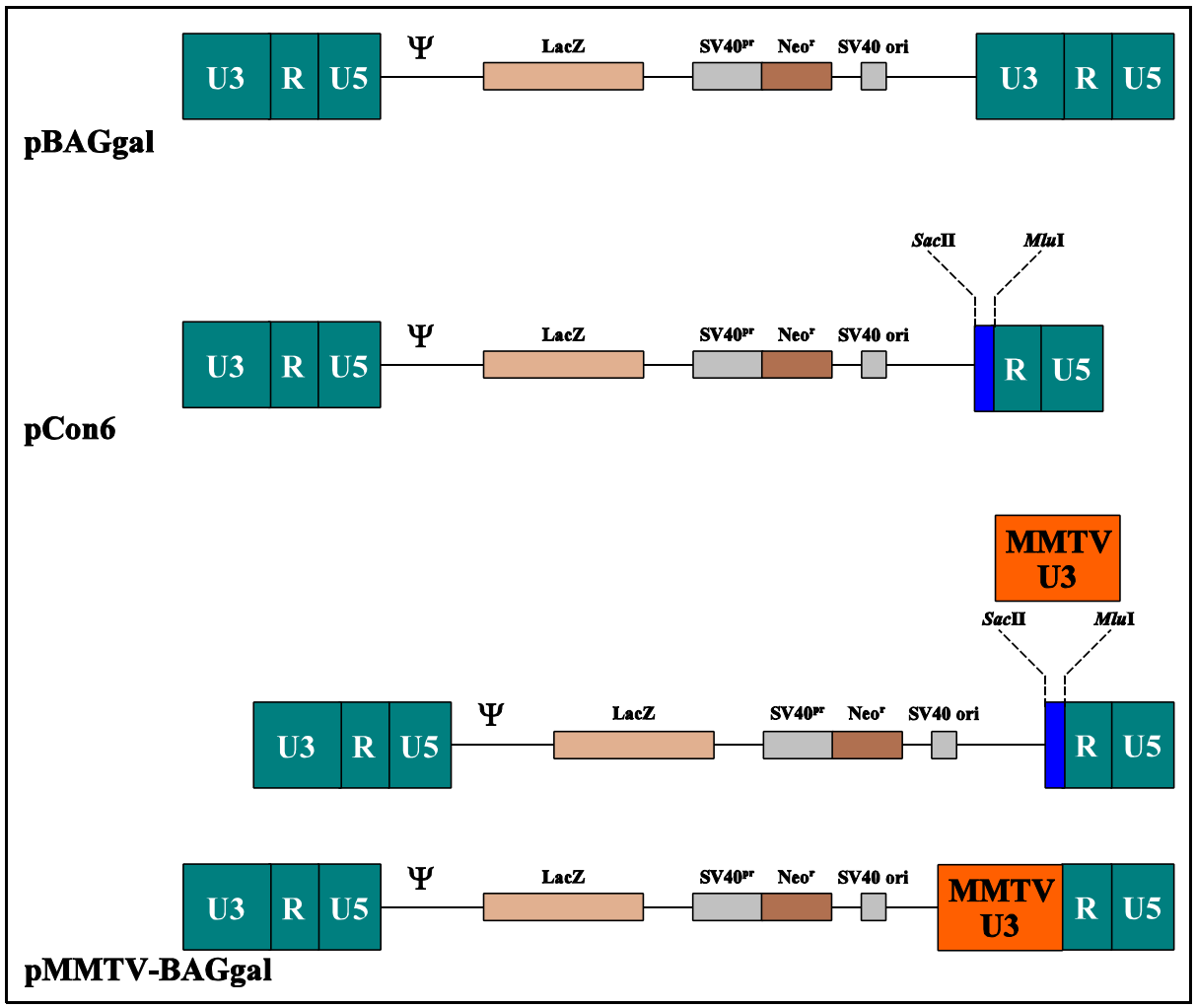

Figure 3.2: Construction of pMMTV-BAGgal
The complete U3 region except the inverted repeat (IR) of the 3' LTR within the BAG vector was deleted by a PCR mediated approach. This gave the plasmid pCON6 (Saller, 1994). The U3 region of MMTV was amplified by PCR using the plasmid pBG102 (Jaggiet *et al*., 1986; a plasmid containing the 3' LTR from mtv2) with the primers carrying either an *Mlu*I or *Sac*II extension. The product was digested with *Sac*II and *Mlu*I and ligated to the *Sac*II/*Mlu*I digested pCON6 to give the plasmid pMMTV-BAGgal (8377 bp), in which the ß-galactosidase (lacZ) gene is under the transcriptional control of the MLV promoter after transfection.

In order to construct the plasmid pLXPCMTVEGFP, pLXSN and p125.6 were used. p125.6 is a modified MMTV-BAGgal vector without the reporter gene ß-galactosidase. The 5' LTR and Ψ region from pLXSN was brought together with the 3' LTR, which carries the MMTV promoter in its' U3 region, and the rest of the plasmid backbone of p125.6. The EGFP gene was taken from the plasmid pEGFP-1 and inserted in to give pLXPCMTVEFGP (Fig. 3.3). This plasmid was used to for infection experiments in which the quantification of expression was not required.

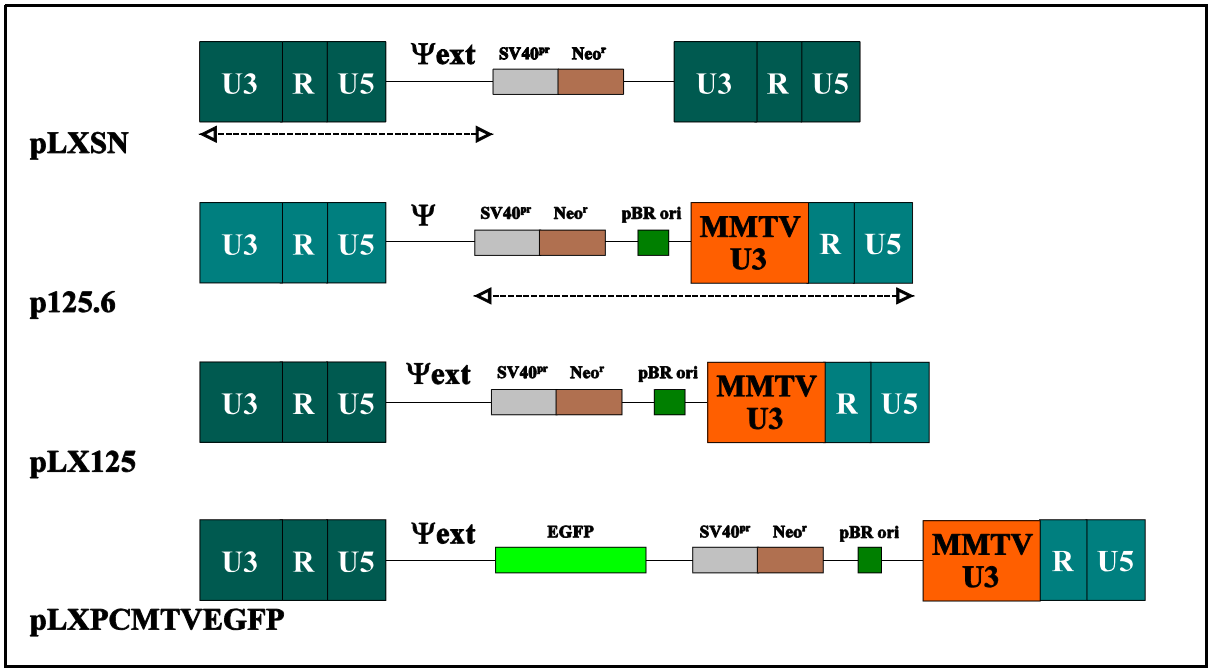

Figure 3.3: Construction of pLXPCMMTVEGFP

The plasmids pLXSN and p125.6 (Saller *et al*., 1994) were used. pLXSN was digested with the enzymes *Afl*III and *Bam*HI and a 3545 bp fragment, containing the 5' LTR and Ψ region, was ligated to a 4263 bp *Afl*II/*Bam*HI fragment from p125.6. This fragment contains the 3' LTR carrying the U3 of MMTV. The resulting 7812 bp long plasmid was named pLX125 and after a further cloning step, to remove an unwanted SacII site, was renamed pLXPCMTV. The EGFP gene was isolated as a 862 bp *Hpa*I/*Sma*I fragment from the plasmid pEGFP-1. pLXPCMMTV was linearised with the enzyme *Hpa*I and ligated to the EGFP fragment. The new 8672 bp plasmid was named pLXPCMTVEGFP.

3.1.3 Production of Recombinant Retroviral Particles

To produce recombinant virus particles, either the hybrid pMMTV-BAGgal, pBAGgal, pLXSNEGFP or the hybrid pLXPCMTVEGFP vector was introduced by lipofection into the amphotropic packaging cell line PA317 (Miller and Buttimore, 1986) (Fig. 3.4). The PA317 packaging cell line carries a provirus with a deletion in the Ψ region and that lacks part of the 5' LTR and the 3'LTR has been replaced with the polyadenylation signal from SV40. Transfected PA317 cells were selected in medium containing neomycin (Neo^r). When the non-transfected, negative control had died, both clones and populations of stable, virus-producing cells could be obtained. After transfection, the viral RNA is under the control of the MLV promoter in the 5' LTR. The supernatant from these stable virus-producing cells could then be used for the infection of several cell lines. The transfection efficiencies can be seen in Table 3.1, with no significant difference being observed between both vectors.

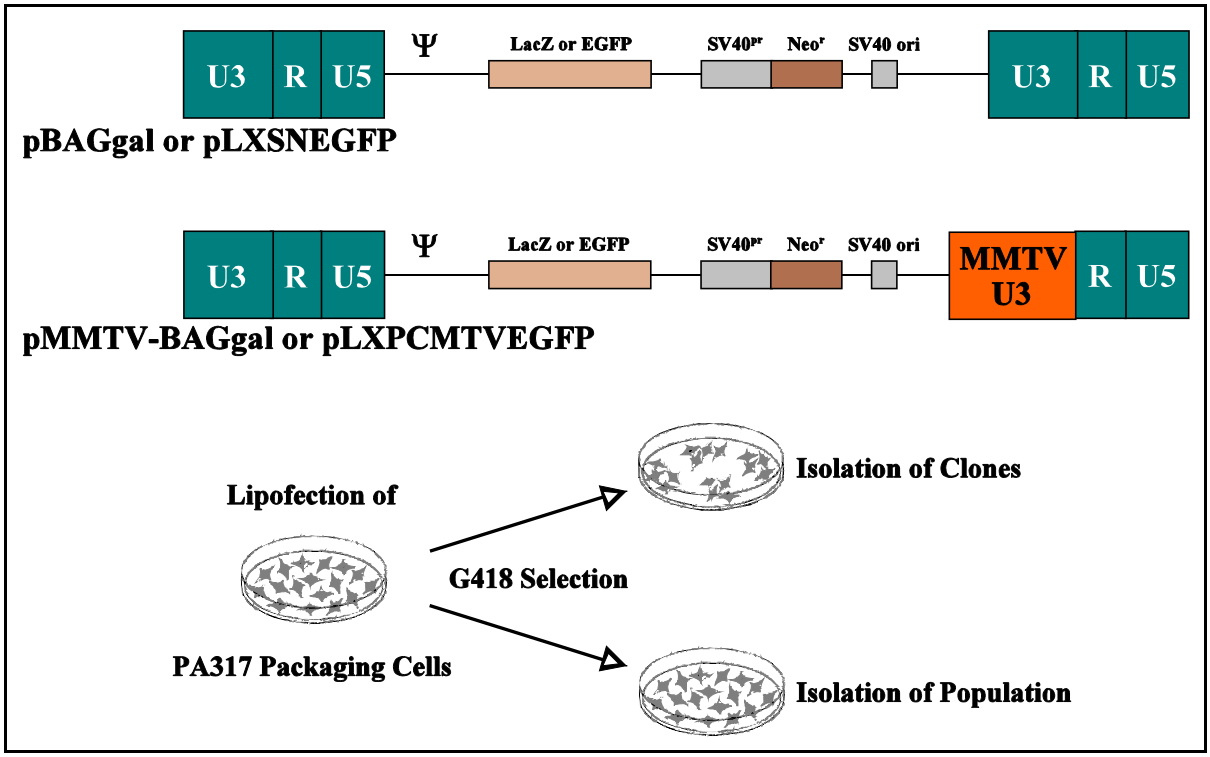

Figure 3.4: Production of Recombinant Retroviral Particles
pBAGgal, pMMTV-BAGgal, pLXSNEGFP and pLXPCMTVEGFP were introduced separately by lipofection into the PA317 packaging cell line. Af8383ter neomycin selection (Neo^r) stable virus-producing cells were established. The transcription of the viral RNA is under the control of MLVU3 region in the 5' LTR. The virus present in the supernatant of thes83e cells is capable of infecting all types of mammalian cells.

Construct	Transfection Efficiency
pBAGgal	400
pMMTV-BAGgal	266
pLXSNEGFP	380
pLXPCMTVEGFP	260

Table 3.1: The Efficiency of Lipofection
2 µg of plasmid DNA were used for lipofection of $5x10^5$ PA317 cells. Transfected cells were selected on neomycin (G418, 400 µg/µl). After two weeks the negative control (untransfected cells) was observed to be dead. Colonies could be counted in the transfected cells and the transfection efficiency calculated. The efficiency of lipofection is shown as colonies per µg transfected plasmid DNA.

3.1.4 Effect of U3 Region Replacement on Viral Titre

After establishment of the infection system, it was important to investigate if there is any virus production and, if so, to determine whether the replacement of 3' U3 region of MLV with MMTV-U3 has any effect on virus production and infection efficiency. The infectivity of the virus and the titre were therefore examined.

Initially, viruses harvested from either a population of MMTV-BAGgal or BAGgal virus-producing cells were used to determine retroviral titre. The supernatant was assayed for the ability to confer G418 resistance to infected cells. The most commonly used target cell line is NIH 3T3 cells. In addition to NIH 3T3 cells other cell lines, such as Rat-2, EJ and CrFK were infected. This was done to show that the replacement of the U3 region of MLV, as expected, does not affect the infection spectrum of viral particles arising from the PA317 packaging cell line.

24 hours before infection the medium from virus-producing cells was changed. The supernatant was filtered (45 µm) and after addition of polybrene (8 µg/ml final concentration) was used to infect several cell lines. 24 hours after infection, the target cells were diluted into 10 cm plates and a further 24 hours later G418 was added to the medium. Approximately 14 days later, as soon as the negative control was seen to be dead, the number of resistant colonies was counted and the virus titre calculated as colony forming units per millilitre supernatant used. The virus titre was calculated from three independent experiments.

The titre of transducing particles obtained from a population of MMTV-BAGgal hybrid vector virus-producing cells was slightly reduced in comparison to that obtained from a population of the parental BAGgal vector producing cells (Fig. 3.5a) when assayed on Rat-2 or EJ cells but was similar as judged by infection of CrFK cells and NIH 3T3 cells.

The titre of several different virus-producing clones was also investigated. Similar results could be observed here as were seen in the populations, although clonal variation could, of course, be observed (Fig. 3.5b).

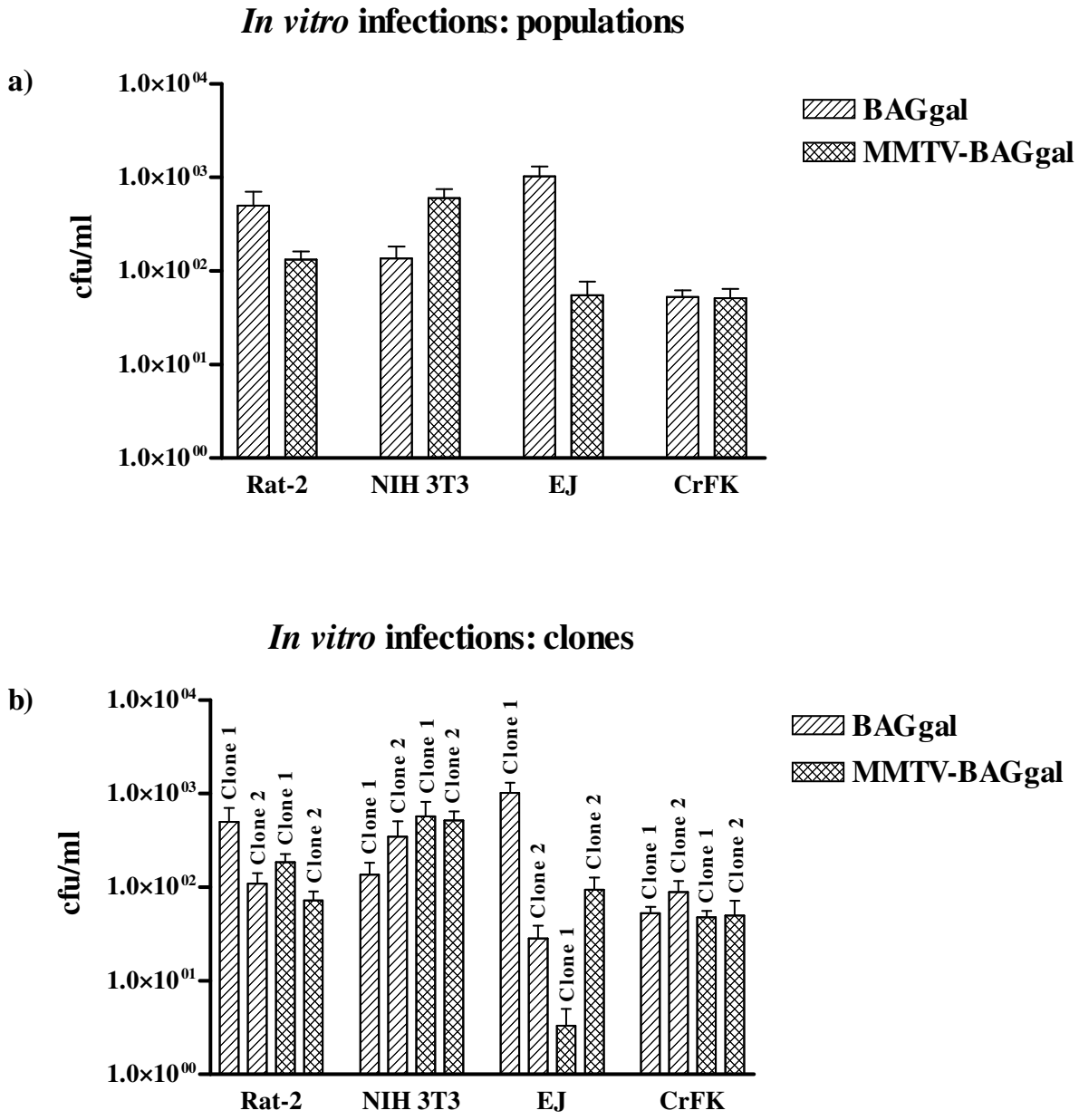

Figure 3.5: Infection of different cell lines with hybrid and parental retroviral vectors

a) Rat-2, NIH 3T3, EJ and CrFK cells were infected with supernatant from either the parental BAGgal retroviral particle producing cell populations or that from the hybrid MMTV-BAGgal population. 24 hours after infection, the target cells were diluted into 10 cm plates and a further 24 hours later G418 was added to the medium (Rat-2, CrFK & NIH 3T3, 400 µg/ml; EJ, 800 µg/ml). Approximately 14 days later, as soon as the negative control was dead, the surviving colonies were counted and the titre calculated as colony forming units per millilitre infectious medium used (cfu/ml).

b) Rat-2, NIH 3T3, EJ and CrFK cells were infected with supernatant from either two virus-producing clones of the parental BAGgal retroviral vector or that from two virus-producing clones from the hybrid MMTV-BAGgal vector. 24 hours after infection, the target cells were diluted into 10 cm plates and a further 24 hours later G418 was added to the medium (Rat-2, CrFK & NIH 3T3, 400 µg/ml; EJ, 800 µg/ml). Approximately 14 days later, as soon as the negative control was dead, the surviving colonies were counted and the titre calculated as colony forming units per millilitre infectious medium used (cfu/ml).

After it could be shown that the hybrid retroviral vectors were capable of producing infectious recombinant viral particles and that the replacement of the U3 region did not change the infection spectrum the second set of infection experiments, using pLXSNEGFP and pLXPCMTVEGFP were made on NIH 3T3 cells. In comparison to the BAG based vectors, retroviral particles arising from pLXSN carry a longer packaging signal and would therefore be expected to have a higher titre. This was actually the case, giving titres of between 1.2 x 10^4 cfu/ml compared to a titre of 4 x 10^4 from the parental vector (Fig. 3.6a).

The titre of several different virus-producing clones was also investigated. Similar results could be observed here as were seen in the populations, although clonal variation again could, of course, be observed (Fig. 3.6b). Titres from virus-producing clones of LXPCMTVEGFP were 1.5 x 10^4 and 1.8 x 10^4 cfu/ml compared to the LXSNEGFP clones, which showed titres of 5.4 x 10^4 and 6.7 x 10^4 cfu/ml.

Here it could be demonstrated that the modification of the U3 region of the 3' LTR had no drastic effect upon viral titre and that, as expected, the pLXSN-based vectors had a higher titre than those based upon pBAG.

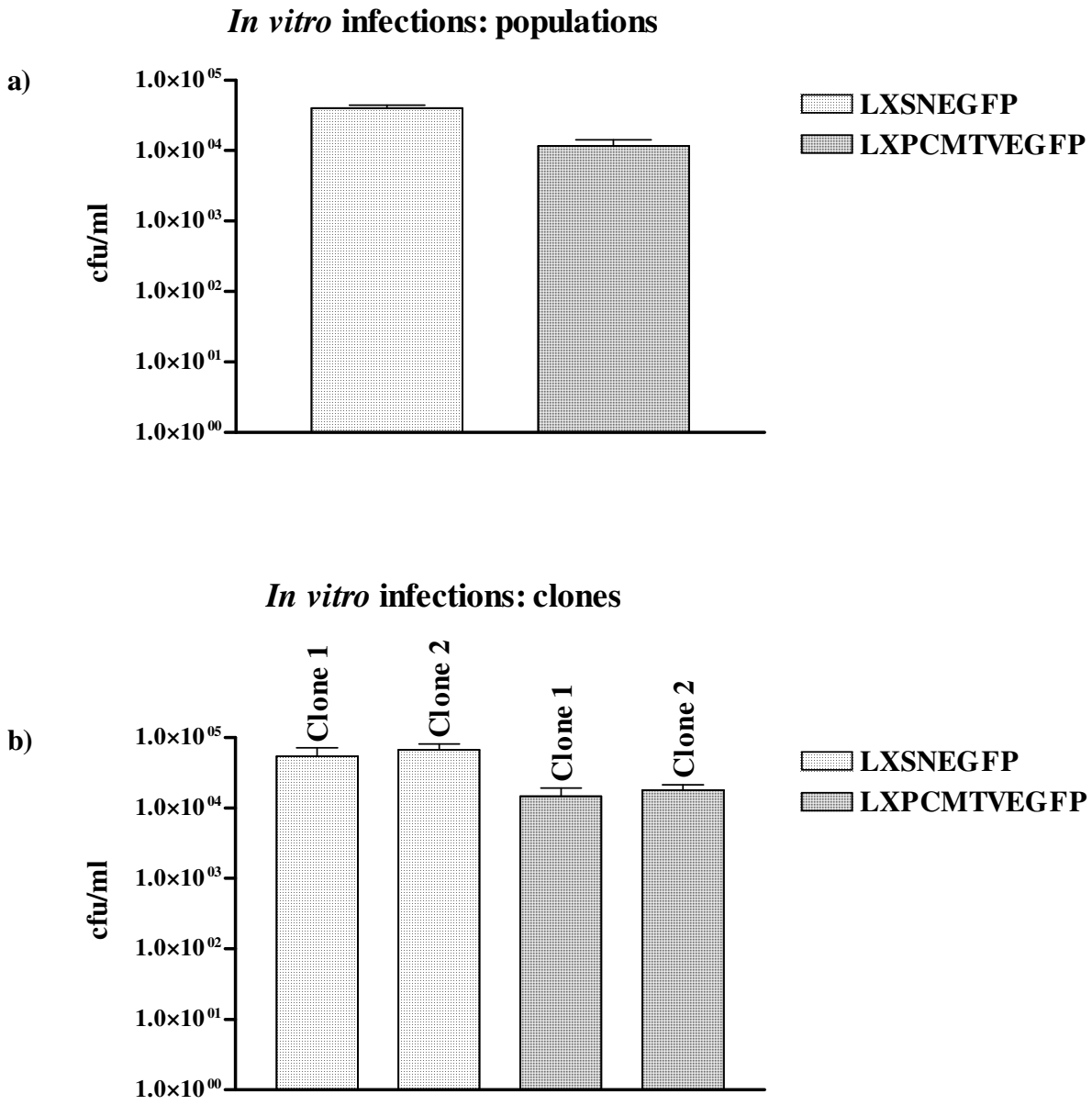

Figure 3.6: Infection of different cell lines with hybrid and parental retroviral vectors

a) NIH 3T3 cells were infected with supernatant from either the parental LXSNEGFP retroviral particle producing cell populations or that from the hybrid LXPCMTVEGFP population. 24 hours after infection, the target cells were diluted into 10 cm plates and a further 24 hours later G418 was added to the medium (400 µg/ml). Approximately 14 days later, as soon as the negative control was dead, the surviving colonies were counted and the titre calculated as colony forming units per millilitre infectious medium used (cfu/ml).

b) NIH 3T3 cells were infected with supernatant from either two virus-producing clones of the parental LXSNEGFP retroviral vector or that from two virus-producing clones from the hybrid LXPCMTVEGFP vector. 24 hours after infection, the target cells were diluted into 10 cm plates and a further 24 hours later G418 was added to the medium (400 µg/ml). Approximately 14 days later, as soon as the negative control was dead, the surviving colonies were counted and the titre calculated as colony forming units per millilitre infectious medium used (cfu/ml).

3.1.5 Improvement of Virus Titre

3.1.5.1 Pseudotyping with VSV-G

Retroviral vectors generally have a relatively restricted host range, based in part on the level of expression of the membrane protein that serves as a viral receptor and in part that the envelope is relatively fragile (Kavanaugh *et al*., 1994). Retroviral envelope pseudotyping can be employed to overcome the relatively low viral titres resulting from low levels of receptor expression, which would normally hinder the use of retroviruses in an *in vivo* gene therapy.

One strategy that has been proposed for expanding the host range of retroviral vectors is to take advantage of the long known capacity of the envelope proteins of certain viruses to become incorporated in the cell plasma membrane which then becomes the envelope of newly budded virions (Zavada, 1972; Weiss *et al*., 1977; Wilson *et al*., 1989). Such pseudotyped virions exhibit the host range and other properties of the virus from which the envelope protein is derived though the core and genome is from a different virus. Specifically, the G protein of vesicular stomatitis virus (VSV-G) (Rose and Gallione, 1981; Rose and Bergmann, 1983) efficiently forms pseudotyped virions with genome and core compenents derived from MuLV (Burns *et al*., 1993; Emi *et al*., 1991; Yee *et al*., 1994). The VSV-G pseudotyped vectors have a very broad host range and can reach titres of 10^5-10^6 cfu/ml (Yee *et al*., 1994), and by ultracentrifugation can be concentrated to titers of greater than 10^9 cfu/ml. The receptor for VSV-G is a phospholipid (Schlegel *et al*., 1983) that is widespread thus giving the virus a wide host range (Burns *et al*., 1993).

Unfortunately, the VSV-G protein is toxic to cells in which it is expressed. Thus pseudotyped vector particles had to be derived by transient expression of the VSV-G gene after transfection of an expression plasmid into virus-producing cells.

10 µg of the plasmid pHCMV.G was transfected into the virus-producing cells 48 hours prior to infection (Fig. 3.7). 24 hours later the cells were washed and fresh medium was added. The production of a functional VSV-G protein could be clearly observed as typical syncytia (multiple nuclei containing cells) due to multiple cell-cell-fusion events were seen under the microscope after transfection. After a further 24 hours, NIH 3T3 cells were infected and subsequently selected as in previous infection experiments. The results shown in figure 3.8 are taken from three independent experiments.

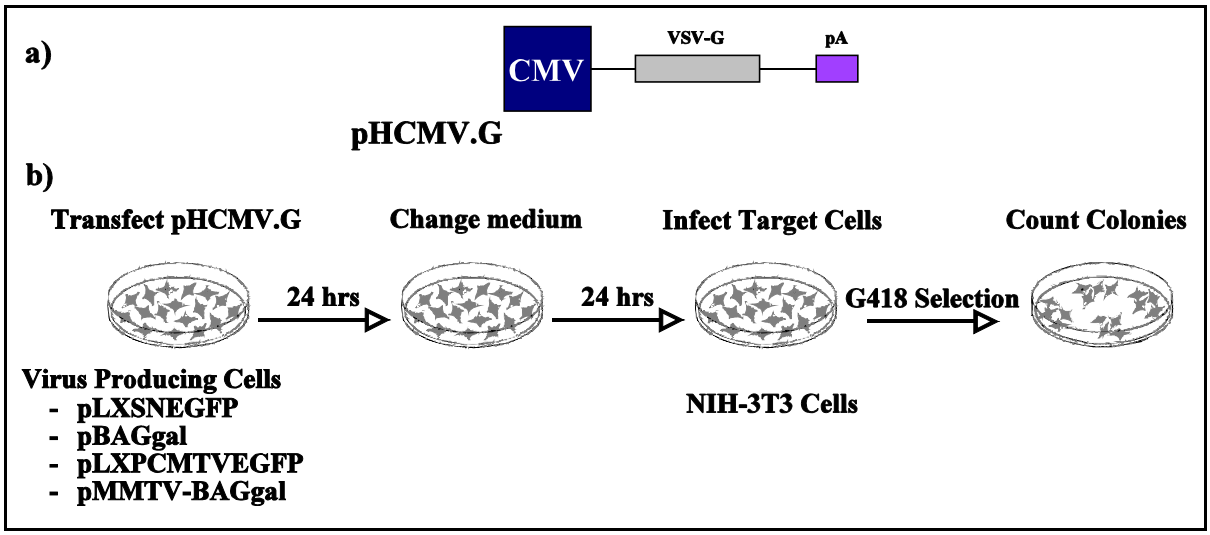

Figure 3.7: Pseudotyping with VSV-G

a) The plasmid pHCMV.G (courtesy J.C. Burns) carries the gene that codes for the G protein of the vesicular stomatitis virus (VSV) under the transcriptional control of the immediate early promoter of the cytomegalavirus (CMV). In order to select this plasmid in bacteria an ampicillin resistance gene is also present**.** No eukaryotic selection marker is necessary, as a stable cell line expressing VSV-G cannot be made due to the toxicity of the G protein.

b) Supernatant from virus-producing cells that had been transfected with 10 µg pHCMV.G were used 48 hours later to infect NIH 3T3 cells. A further 48 hours later, the infected cells were put into G418 selection (400 µg/ml). As soon as the negative control had died, the surviving colonies in the infected cells were counted and the titre calculated in colony forming units per millilitre (cfu/ml).

Here it can be seen that pseudotyping of recombinant retroviral particles with VSV-G leads to, as expected, an increase in titre. The BAGgal titre from a population was seen to increase from 6.6×10^3 to 1.9×10^4 cfu/ml after addition of VSV-G. Similarly, an increase from 3.7×10^3 to 1.4×10^4 cfu/ml could be observed in the MMTV-BAGgal population (Fig. 3.8a). Two virus-producing clones per construct were also analysed, the results being comparable to those seen in the virus-producing populations tested (Fig. 3.8b).

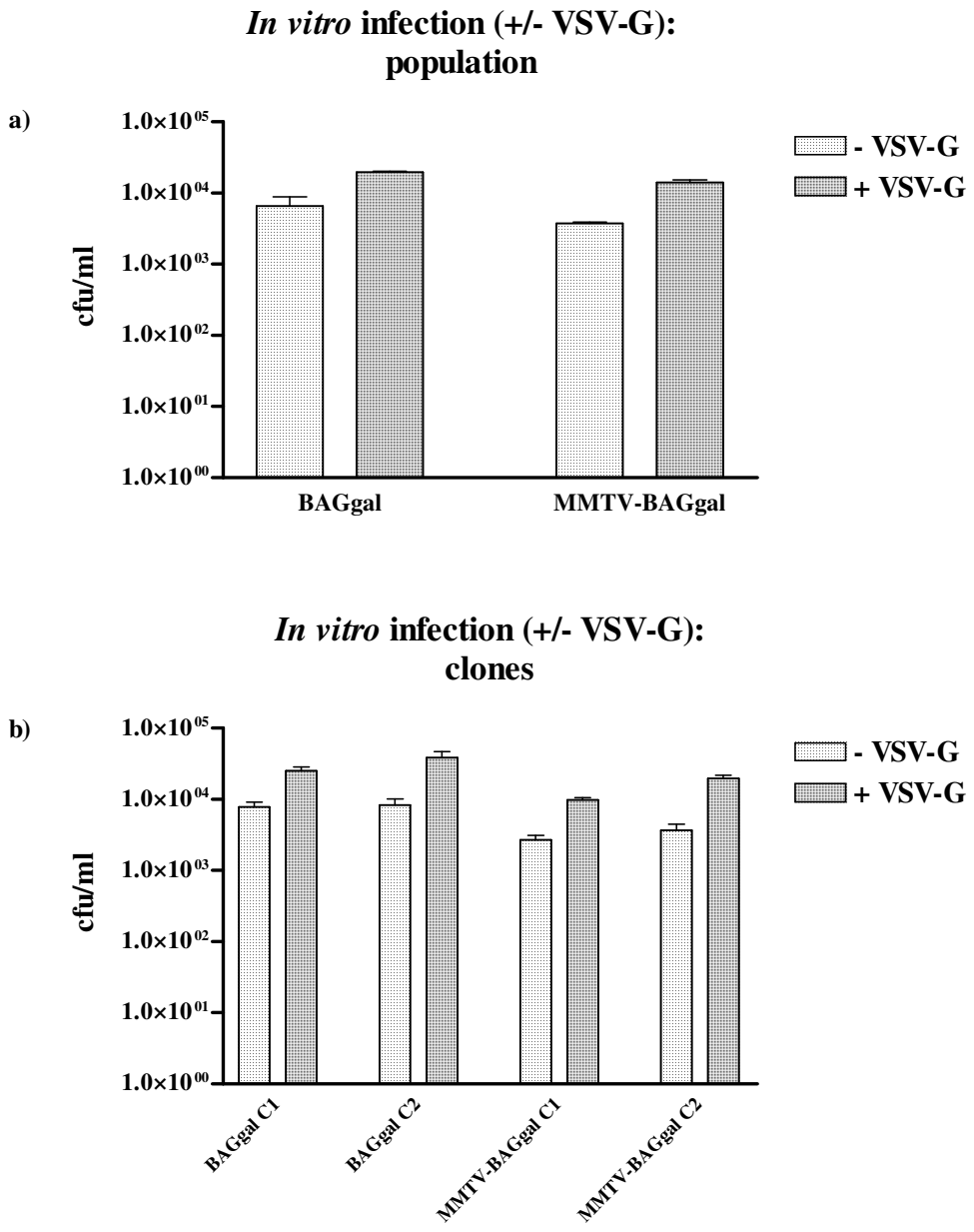

Figure 3.8: Infection of NIH 3T3 cell with VSV-G Pseudotyped Retroviral Vectors
Infection experiments were performed as described in figure 3.7.
a) Infection of NIH 3T3 cells with the supernatant from populations of either the parental vector BAGgal or the modified vector MMTV-BAGgal with or without the addition of the VSV-G protein
b) Infection of NIH 3T3 cells with the supernatant of two clones from either the parental vector BAGgal or the modified vector MMTV-BAGgal with or without the addition of the VSV-G protein

A second round of experiments was also performed, to investigate the virus production arising from the LXSN based MMTV vector pLXPCMTVEGFP and to compare its' titre with the parental pLXSNEGFP. The LXPCMTVEGFP titre from a population was seen to rise from 2 x 10^4 to 8.9 x 10^4 cfu/ml after addition of VSV-G whereas the titre of the parental pLXSNEGFP was seen to increase from 4 x 10^4 to 1.2 x 10^5 cfu/ml (Fig. 3.9a). Similar results could also be seen in virus-producing clones (Fig. 3.9b).

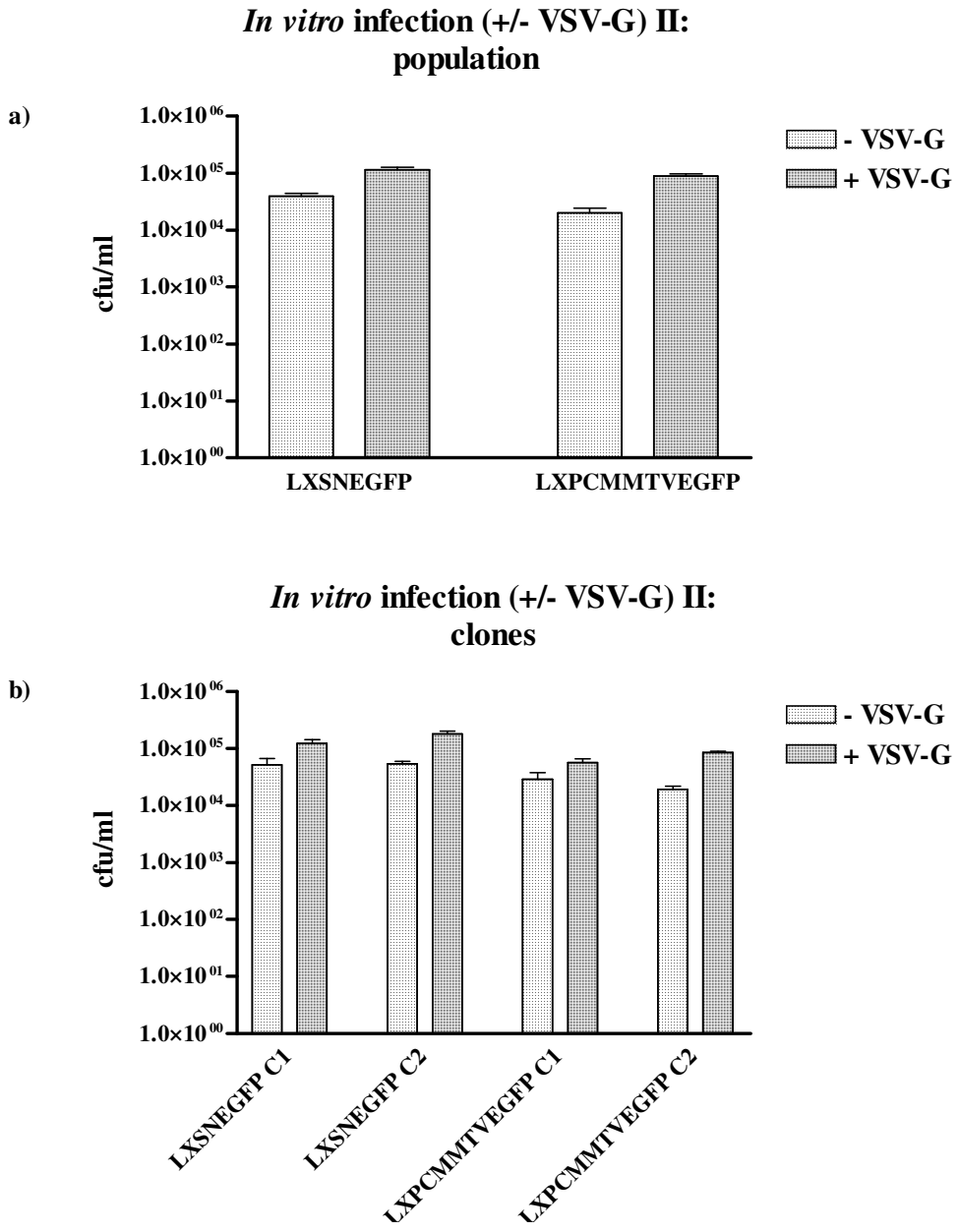

Figure 3.9: Infection of NIH 3T3 Cell with VSV-G Pseudotyped Retroviral Vectors
Infection experiments were made as described in figure 3.7.
a) Infection of NIH 3T3 cells with the supernatant from populations from either the parental vector LXSNEGFP or the modified vector LXPCMTVEGFP with or without the addition of the VSV-G protein.
b) Infection of NIH 3T3 cells with the supernatant of two clones from either the parental vector LXSNEGFP or the modified vector LXPCMTVEGFP with or without the addition of the VSV-G protein.

Here it can be seen that the hybrid MMTV vector, irrespective of whether it was BAG or pLXSN based, could be successfully pseudotyped with VSV-G and confirms the previous observation that the modification of the U3 region of the 3' LTR does not have a drastic effect upon virus titre after the incorporation of a heterologous viral promoter. It has also been shown that it is possible to concentrate VSV-G pseudotyped vectors and this may be important for a future *in vivo* application. However, in this study it was of primary interest to characterise the modified vectors.

3.1.6 Analysis of Promoter Conversion

In the retroviral life cycle, after infection and during reverse transcription, the U3 region of the 3' LTR is duplicated and one copy is translocated to the 5' LTR where it then controls expression of the structural genes. In the case of retroviral vectors it then controls the reporter gene expression, such as ß-galactosidase or EGFP. To confirm that the infected cells had acquired the vector constructs and to verify that the MMTV promoter was now present in the 5' LTR of the vector provirus, DNA was isolated from stably infected and non-infected populations of Rat-2, EJ & NIH 3T3 cells. The analysis of proviral DNA after the infection events was made using both PCR and Southern hybridisation.

Three different primer pairs were chosen for the PCR. Primers specific for either the MMTV U3 (P1 and P3) or MLV R (P4) sequences in combination with a second primer complementary to the packaging region (P2) or the ß-galactosidase gene (P5) present in all constructs were used for this analysis (Fig. 3.10)

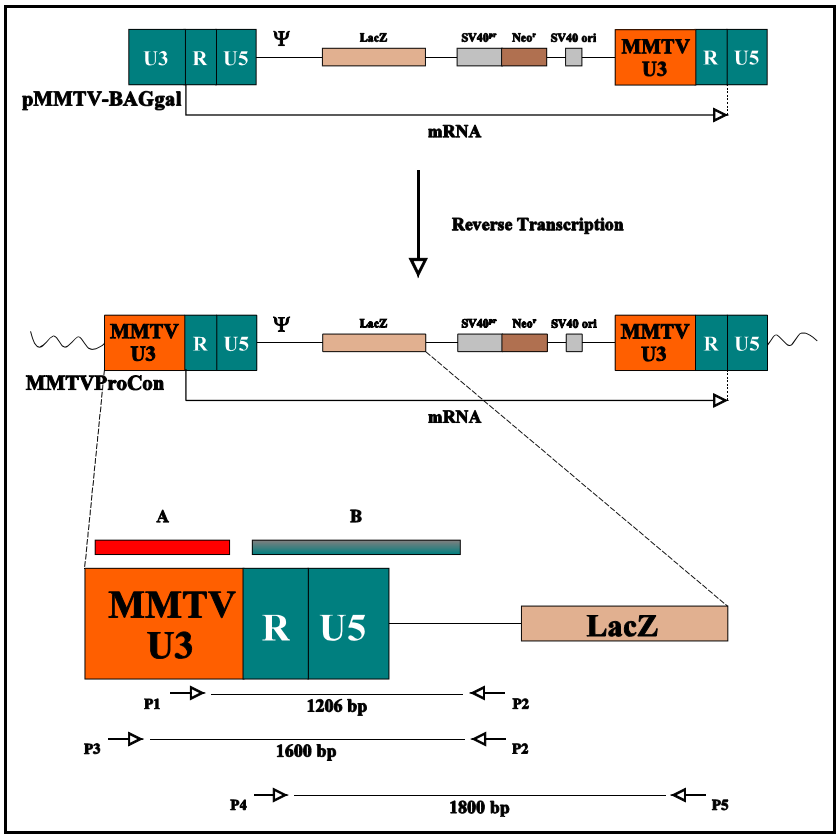

Figure 3.10:PCR Strategy
The position of primers specific for the MMTV U3 region (P1, 5'-GACCACAGCCAACTTCCTCTTAC -3'; P3, 5'-ATACCGCGGAAAAAGGGGGAAATGCC-3') or MLV R sequences (P4, 5'-GCGCCAGTCCTCCGATTGA-3') together with a primer specific for the MLV packaging region (P2 5'-GGTCCGCCAGATACAGAGCTAGTTA- 3') are shown, as are the expected sizes of the PCR products that can be obtained by using these primers and MMTV (labelled fragment A) and MLV (labelled fragment B) probes that can be used to detect them.

After electrophoresis of the PCR products they were transferred to a nylon membrane (see 2.2.5.1.2) and were then hybridised to either an MMTV U3 or an MLV specific probe (Fig.

3.10, labelled fragments A and B respectively).

DNA prepared from cells infected with the MMTV-BAGgal hybrid virus gave PCR products of 1.2 kb and 1.6 kb after amplification with the P1-P2 and P3-P2 primer pairs, respectively. These products hybridised, as expected, to both MMTV U3 specific (Fig.3.11 lanes 1, 3, 5, 7, 9, and 11) and MLV specific (Fig.3.11 lanes 1, 3, 5, 7, 9 and 11) hybridisation probes. In the non-infected cells (Fig.3.11 lanes, 2, 4, 6, 8, 10 and 12) neither PCR amplification nor hybridisation could be observed as expected.

In contrast, the primer pair P4-P5 gave a PCR product of 1.8 kb that hybridised to the MLV probe (Fig.3.11, lanes 13, 15, and 17) but not to the MMTV probe (Fig.3.11, lanes 13, 15, and 17). Here too, in the non-infected cells (Fig. 3.11 lanes 14, 16, and 18) neither PCR amplification nor hybridisation could be observed. These data are consistent with the occurrence of promoter conversion.

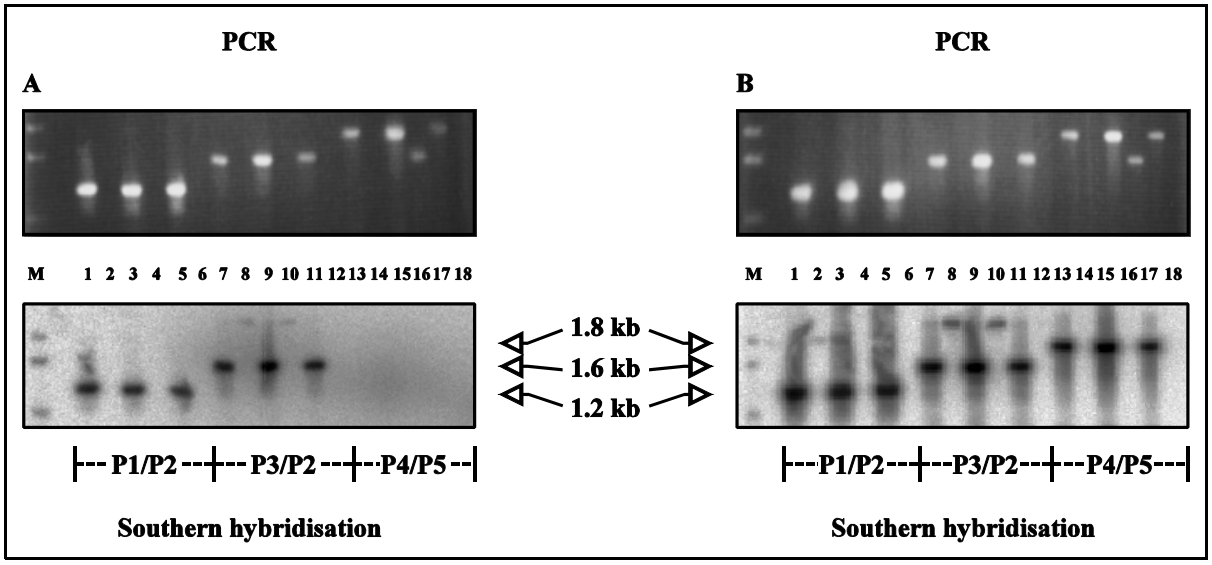

Figure 3.11: PCR Products and Hybridisation

PCR analysis of infected cell DNA for the presence of MMTV-BAGgal sequences. 1 µg of genomic DNA was amplified by PCR using 40 pmol of each primer specific for the MMTV U3 region or MLV R sequences and of a primer specific for the MLV packaging region or ß-galactosidase gene. The PCRs were performed under the following reaction conditions: denaturating for 1 min at 94°C, annealing for 2 mins at 55°C (P1-P2), 53°C (P3-P2) or 56°C (P4-P5), and elongation for 3 mins at 68°C for 35 cycles. 35 cycles were made. The primer pairs P1-P2 gave a 1.2 kb PCR product (lanes 1, 3 and 5); P3-P2 gave a 1.6 kb PCR product (lanes 7, 9 and 11); P4-P5 gave a 1.8 kb PCR product (lanes 13, 15 and 17). 10 µl of each PCR product was separated on a 0.8% agarose gel, transferred to a nylon membrane and hybridised to α^{32}P-labelled probes. Promoter conversion was detected by using two primers specific for the MMTV U3 region (P1 and P3) in combination with a primer specific for the MLV packaging region (P2). An MLV R region specific primer (P4) was also used in combination with a primer specific for the ß-galactosidase gene (P5). Nylon membranes were hybridised against a 0.9 kb MMTV U3 specific *Pst*I fragment (A) from the plasmid p0.9*Pst*I or an MLV specific 0.3 kb PCR fragment (B) (with was amplified with the primer pair, 125pos, 5'-GCGCCAGTCCTCCGATTGA-3' and 125posc2, 5'-GGTGGTCCGCCAGATACAGAGCTAGTTA-3'), as shown in lanes 1,7 and 13, infected Rat-2 cells; lanes 2, 8 and 14, non infected Rat-2 cells; lanes 3, 9 and 15, infected NIH 3T3 cells; lanes 4, 10 and 16, non infected NIH 3T3 cells; lanes 5, 11 and 17, infected EJ cells; lanes 6, 12 and 18 non infected EJ cells.

3.1.7 Recloning of the Integrated Provirus

To analyse the structure of the hybrid provirus (named MMTVProCon) three integrated proviruses were recloned from infected cells and one of them characterised by Southern blot, PCR or sequencing. The hybrid MMTV-BAG retroviral vector carries a prokaryotic origin of replication (Fig. 3.1), allowing the integrated provirus to be recloned from genomic DNA. Genomic DNA was isolated from a population of MMTV-BAGgal infected Rat-2 cells (Fig. 3.12). The genomic DNA was then digested with the restriction enzyme *Asp*700 I, which does not cleave within the vector provirus. The digested DNA was ligated and used to transform *Escherichia coli*. After selection on kanamycin containing medium a number of bacterial colonies containing plasmids, each carrying an integrated provirus, were isolated. Restriction enzyme mapping was then used to show that the promoter conversion had taken place correctly in the proviruses isolated and also to allow one to be chosen for further investigation.

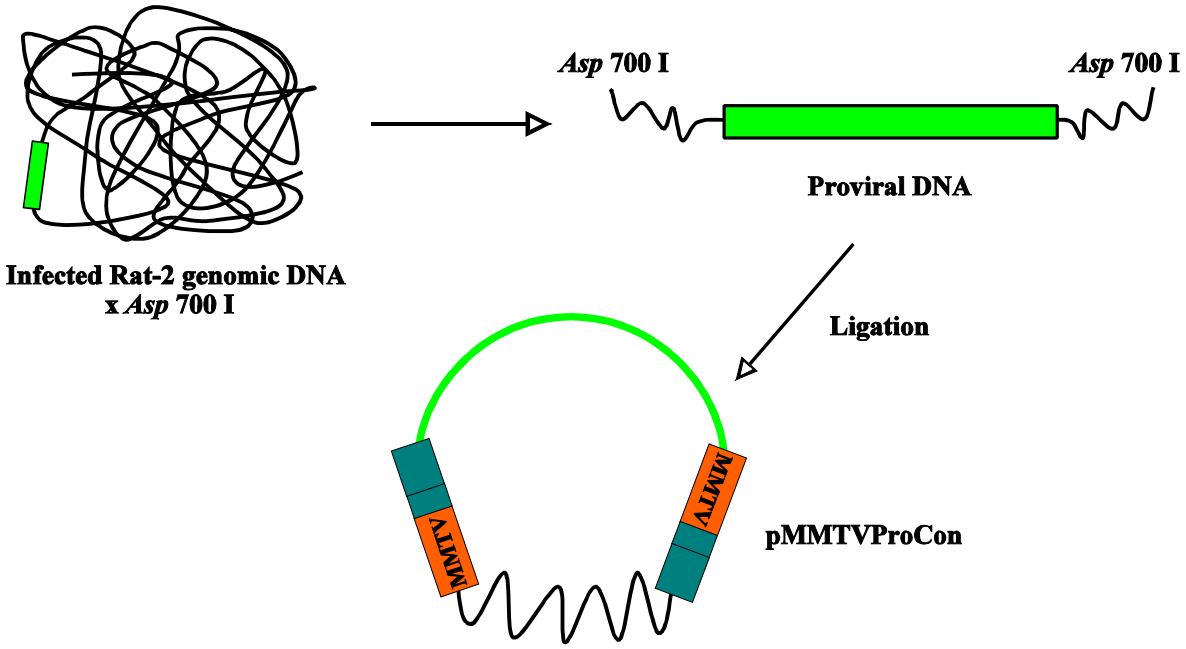

Figure 3.12: MMTV ProCon Recloning Strategy
Genomic DNA from Rat-2 cells that had previously been infected with the MMTV-BAGgal vector was digested with the restriction endonuclease *Asp*700 I which has no recognition site within the vector. It was then ligated and transformed into *E. coli*. After selection on kanamycin, colonies could be isolated which carried a promoter converted recloned provirus containing plasmid.

3.1.8 Investigation of the Recloned Vector

The recloned plasmid was analysed using many different molecular biological methods to show that a promoter converted provirus had been isolated and that the conversion had taken place correctly.

3.1.8.1 Southern Blot Analysis

The recloned plasmid was digested with the restriction enzyme *Kpn*I. pMMTV-BAGgal was

also digested with the same enzyme in parallel. The *Kpn*I recognition site is present in the R region of both LTRs. After digestion with *Kpn*I two fragments of 7.7 kb and approximately 3 kb could be seen in the recloned plasmid (Fig. 3.13, lane 1). In pMMTV-BAGgal bands of 7.7 kb und 0.7 kb (Fig. 3.13, lane 2) were expected. In the recloned plasmid both the 7.7 kb as well as the 3 kb fragment should contain the U3 region of MMTV if promoter conversion has taken place. In the plasmid pMMTV-BAGgal the U3 region of MMTV is only present in the 3' LTR and should only be detected in the 7.7 kb fragment. The gel was blotted and hybridised to an MMTV U3 specific probe. As expected both the 7.7 kb and the 3 kb band from the recloned plasmid hybridised to the probe giving a signal (lane 1), and only the 7.7 kb product in pMMTV-BAGgal gives a hybridisation signal.

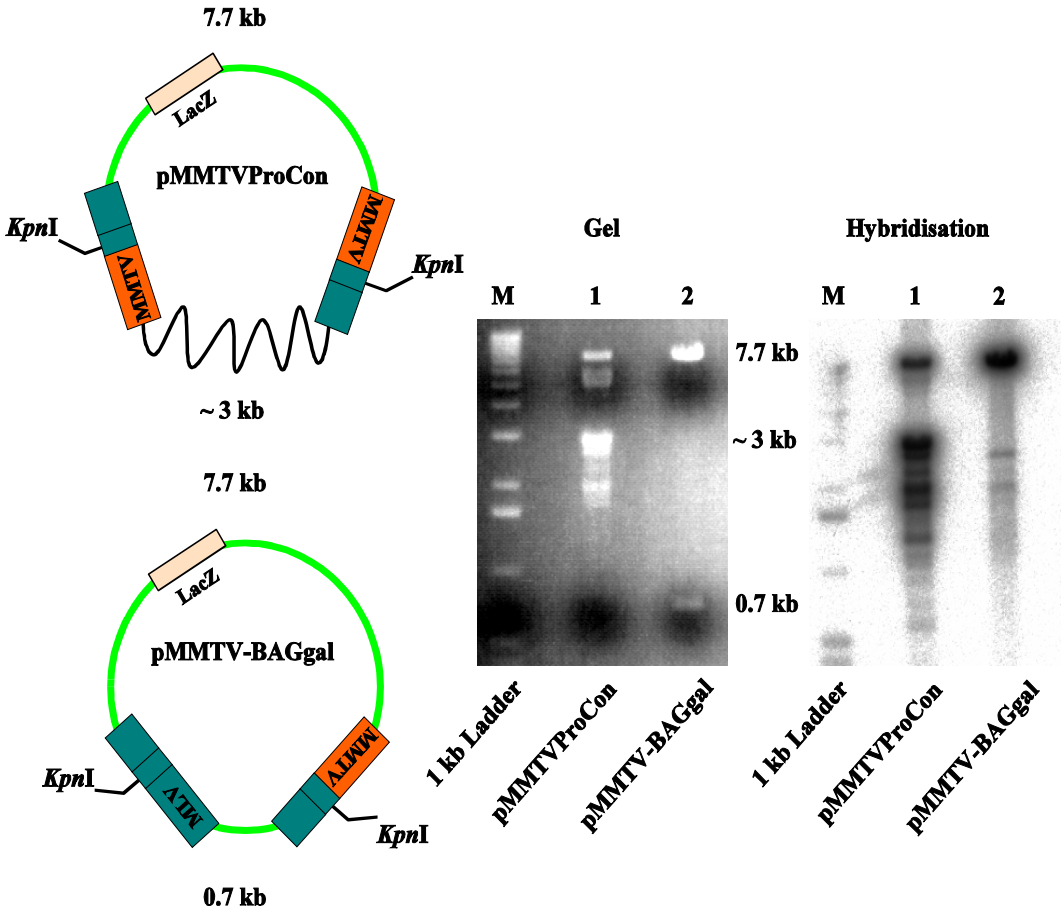

Figure 3.13: Southern Blot

a) 1 µg of plasmid DNA was digested with the restriction enzyme *Kpn*I. This enzyme was chosen as it could be used to show the presence of the U3 of MMTV in the 5' LTR after hybridisation.

b) The *Kpn*I digest shows a 7.7 kb fragment and an approximately 3 kb fragment in the recloned plasmid. Here it was expected that the U3 of MMTV would be present in both fragments after reverse transcription. The 3 kb fragment represents the remaining parts of the 5' and 3' LTRs as well as genomic DNA from the integration site. pMMTV-BAGgal gave fragments of 7.7 kb and 0.7 kb as expected. A molecular weight marker (1 kb Ladder, Life Technologies) is present in the lane marked M. The recloned plasmid is in lane 1 and pMMTV-BAGgal in lane 2. Some unspecific bands can also be seen. This is due to the star activity of *Kpn*I, resulting in bands of unspecific sizes.

c) After transfer to a nylon membrane, the DNA was hybridised to a radioactively labelled probe. In lane 1 the recloned, promoter converted plasmid shows two hybridised bands corresponding to the 7.7 and 3 kb fragments from the *Kpn*I digest. In lane 2, pMMTV-BAGgal shows only the 7.7 kb hybridised band as expected.

3.1.8.2 PCR Analysis of the Recloned Vector

PCR was used to further characterise the recloned plasmid. The primers used can be see in figure 3.14, one binding at the beginning of the MMTV U3 region in the 5' LTR and the other binding in the packaging signal. If the MMTV U3 region has been duplicated and translocated from the 3' LTR to the 5' LTR a 1.2 kb band would be expected in the PCR reaction. As expected the 1.2 kb PCR product can be seen in lane 1 from the recloned plasmid and a 1.9 kb product in lane 3 arising from the plasmid control pMMTV-BAGgal as the U3 of MMTV is only present in the 3'LTR. A product of 4.2 kb could also be obtained in pMMTVProCon as the MMTV U3 specific primer could also bind in the 3' LTR. However, in any PCR reaction, the shorter fragments are preferentially amplified. Hence, the 4.2 kb fragment is not amplified. Genomic DNA from Rat-2 cells that had been previously infected with the hybrid MMTV-BAGgal was used as a positive control and can be seen in lane 2. The resulting gel was then blotted to a nylon membrane (see 2.2.7.1.) and hybridised (see 2.2.7) to a 0.9 kb radioactively labelled (see 2.2.6.1) probe isolated from the U3 region of MMTV in order to check the specificity of the bands present. After hybridisation a strong band could be seen at the expected size. This shows the presence of the MMTV U3 region in the 5' LTR of the recloned plasmid.

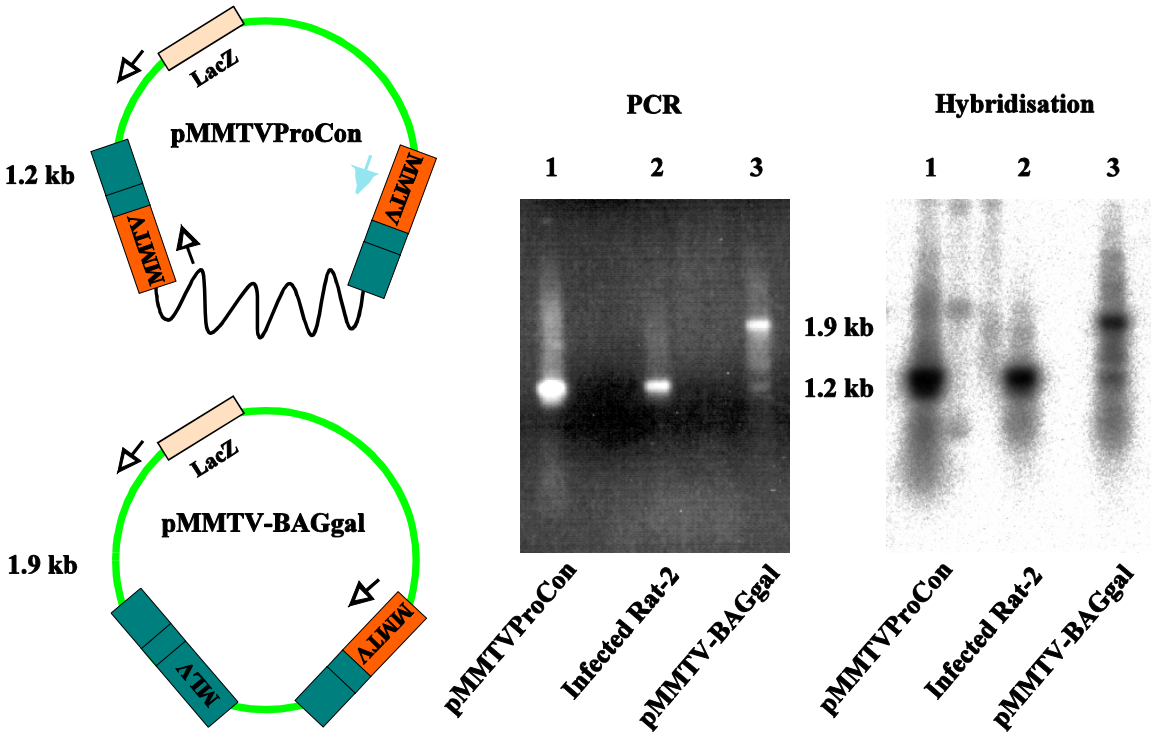

Figure 3.14: PCR Analysis of the Recloned Vector

1 ng of plasmid DNA was amplified by PCR using 40 pmol of each primer, one being specific for the MMTV U3 region and the other primer specific for the MLV packaging region. The PCR was perfomed under the following reaction conditions: denaturating for 1 min at 94°C, annealing for 2 mins at 55°C (P1-P2), and elongation for 3 mins at 68°C. 35 cycles were made. The primer pair P1-P2 gave a 1.2 kb PCR product (Lane 1) from the recloned plasmid (pMMTVProCon) and from infected Rat-2 cells (Lane 2) whereas a 1.9 kb PCR product (Lane 3) arose from pMMTV-BAGgal. 10 µl of the PCR product was separated on a 0.8% agarose gel and transferred to a nylon membrane. The membrane was then hybridised against a α^{32}P-labelled 0.9 kb MMTV U3 specific *Pst*I fragment from the plasmid p0.9pstI.

3.1.8.3 Sequencing of the Recloned Plasmid

Sequencing was used to show that the promoter conversion had taken place correctly at the level of DNA sequence and also to determine the sequence at the integration site. After promoter conversion it would be expected that the U3 of MMTV should be present in both LTRs. The recloned plasmid was digested with *Kpn*I, which recognises sites present in both LTRs, to separate the 5' and 3' LTRs and thus facilitate analysis of both LTRs separately. Two fragments were observed, a 7.7 kb fragment and a 3 kb fragment (Fig. 3.13). The 7.7 kb fragment containing the U3 region of MMTV present in the 3' LTR and a part of the R region and the 3 kb fragment containing the MMTV U3 region present in the 5' LTR and part of the R region. The 3kb fragment was used for sequencing as it contains the promoter converted LTR.

Two primers were chosen for the sequencing reactions (see 2.2.5.1.3), one of which was complementary to the U3 region of MMTV (S1) and the other of which was complementary to the R region of MLV (S2). Sequencing of the junction between the rat flanking sequence and at the 5' end of the provirus revealed that the *Sac*II restriction site and MMTV U3 sequences inserted into this site were intact, as expected. The MLV IR was also present, but it had been shortened 2 bp, as expected due to processing during the integration event (Goff *et. al*, 1992). Similarly, the sequence at the border between the MMTV U3 and MLV R regions in the 5' LTR revealed that the MMTV U3, *Mlu*I restriction site, and MLV R sequence were also intact (Fig. 3.15). Sequencing into the flanking regions, where the provirus had integrated, was also performed. Alignment of the obtained data with online databases (BLAST) showed no significant homology with known sequences.

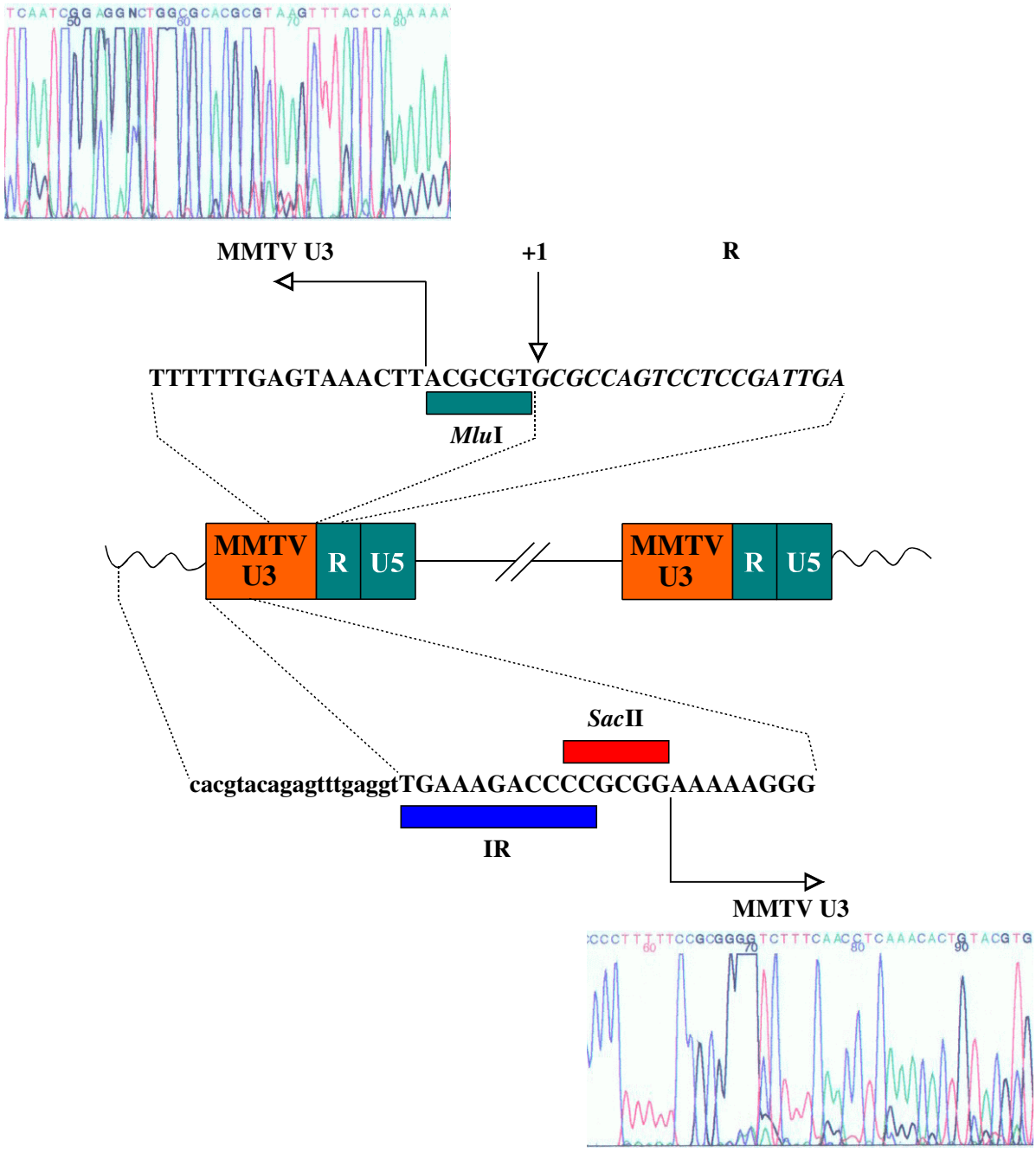

Figure 3.15: Sequencing of the Junction Regions of a Recloned Provirus.
5 µg of plasmid DNA was digested with the restriction enzyme *Kpn*I. A 3 kb fragment, which contains the MMTV U3 region present in the 5' LTR, was taken and extracted for sequencing.
Sequencing was carried out with an automatic sequencer (ABI 373a; Applied Biosystems). The reactions were performed with a dye terminator cycle sequencing kit (Applied Biosystems, see 2.2.5.1.3) according to the manufacturer's instructions. PCR mixtures contained 1 µg of plasmid DNA and 4 pmol of primer, one of which was complementary to the U3 region of MMTV (S1, 5'-CCTTGGCTGCTTCTC-3') and the other of which was complementary to the R region of MLV (S2, 5'-CCACAAGTCGGATGCAACTG-3'). Shown are the sequences at the U3-R junction of the 5' LTR and the integration site-U3 junction of the 5' LTR. The sequence analysis reveals that (i) the borders between the MMTV U3 and MLV R regions are intact after promoter conversion and (ii) the IR has been used for integration. The *Sac*II restriction enzyme cleavage site and the *Mlu*I site used to introduce the MMTV U3 region are marked. +1 indicates the position of the classic CAP site.

3.1.9 *In vitro* Infection and Expression Analysis

3.1.9.1 *In vitro* Hormone Inducible Expression of the Hybrid MMTV-BAGgal Vector in Non-Human Cells

Although MMTV promoter activity is restricted to a few cell types *in vivo* (Ross *et al*., 1990), this promoter is active in many cell types *in vitro*. The ability of glucocorticoid hormones to stimulate expression from this promoter has been extensively utilised in many cell types, including fibroblasts (Jaggi *et al*., 1986).

To examine the effect of glucocorticoid hormones on ß-galactosidase expression in MMTV-BAGgal infected cells, enzyme activity was determined and either quantitatively by using a light-emitting substrate in a photometric assay or in a qualitative assay using histochemical staining.

ß-galactosidase activity was analysed in cells (Rat-2, CK and NIH 3T3) stably infected with either the hybrid MMTV-BAGgal or the parental BAGgal vector. $3x10^5$ cells were plated out and either stimulated for 3 days with the glucocorticoid hormone dexamethasone at a concentration of 10^{-6} M or not stimulated at all. The protein lysate was prepared from hormone stimulated/non-stimulated infected or non-infected cell populations.

All of the tested infected cell populations showed expression of ß-galactosidase in the absence of glucocorticoids. However, addition of the synthetic glucocorticoid dexamethasone resulted in enhanced expression (Fig. 3.16), as expected, from the MMTV promoter. Levels of induction were between 3- and 40-fold, depending on the cell type; the best induction was observed in CrFK cells, one of the few cell lines known to be permissive for MMTV replication (Salmons *et al*., 1985). In contrast, levels of induction from the MLV promoter were maximally 1.5-fold. This low level glucocorticoid induction of murine C-type promoters has been previously reported (Miksicek *et al*., 1986).

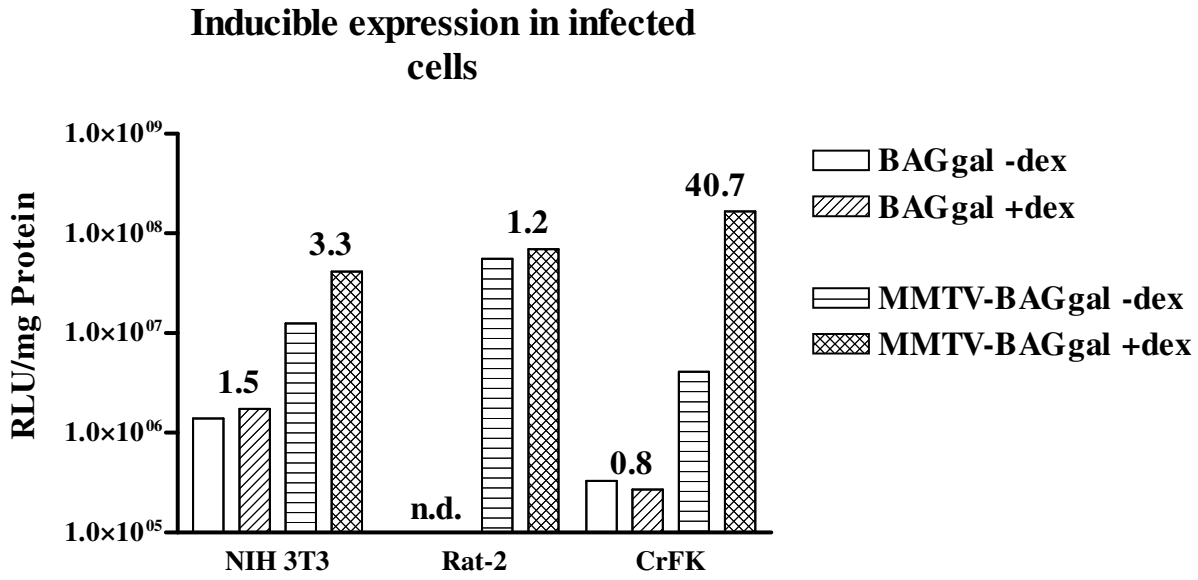

Figure 3.16: Dexamethasone Inducible Expression of ß-galactosidase in Transduced Cells
$3x10^5$ stably infected cells (NIH 3T3, Rat-2 and CrFK) were lysed and total cell protein extracted after 3 days +/- dexamethasone stimulation. 5 µg protein was analysed for ß-galactosidase enzymatic activity using the galactolight kit (Perkin Elmer). The expression of ß-galactosidase is measured in relative light units after a chemoluminescent substrate is added. The background ß-galactosidase activity was set to a value of 1 and the expression of the vectors then calculated in relationship to the background. The best induction was observed in MMTV-BAGgal infected CrFK cells, showing 40 times the expression in hormone stimulated cells compared to non-stimulated cells. Induction could also be seen to a lesser extent in NIH 3T3 and Rat-2 cells, being 3.3- and 1.2-fold respectively. The induction from the MLV promoter was 1.5-fold in NIH 3T3 and 0.8-fold in CrFK cells. No induction could be observed in Rat-2 cells.

Hormone induced ß-galactosidase expression was also analysed in a qualitative assay using histochemical x-gal staining (4-chrome-5-bromo-3indoyl-ß-D-galactosidse). The cells were treated in the same manner as described above for the chemiluminescent assay. After 3 days the cells were fixed and subsequently stained with an x-gal solution (see 2.5.3.2.1). 24 hours later the cells were examined using light microscopy. In the absence of dexamethasone, the background expression could be observed, but in the presence of dexamethasone more cells express the ß-galactosidase enzyme. This inducible expression of ß-galactosidase could be seen best in CrFK (Fig. 3.17). This correlates with the results seen in the quantitative assay.

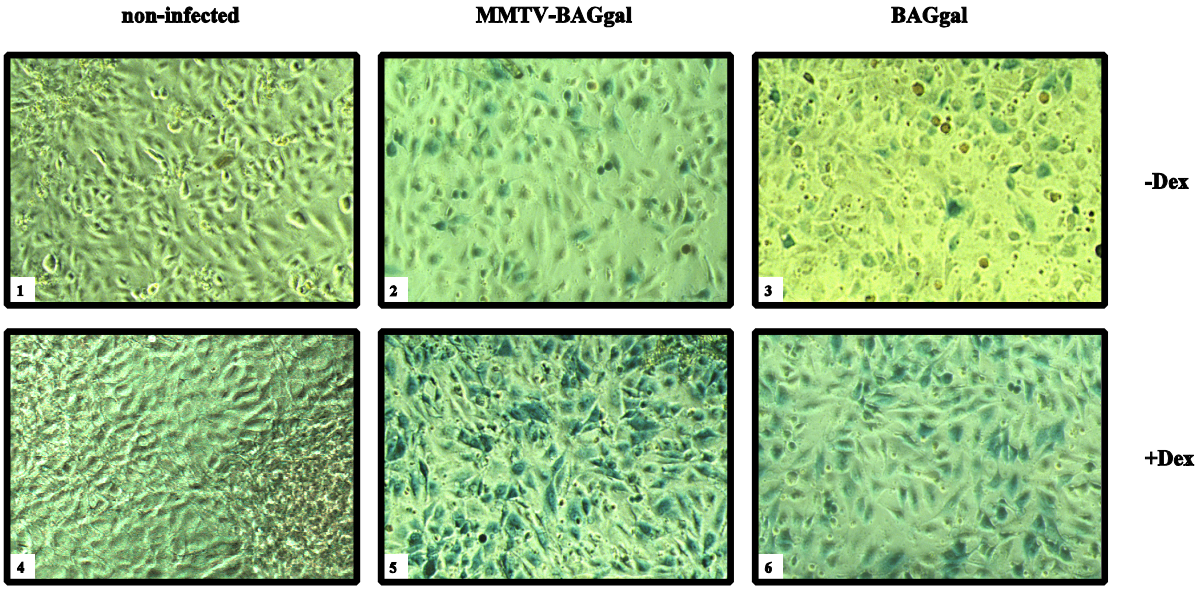

Figure 3.17: ß-galactosidase Expression in Infected CrFK Cells
Hormone inducible expression of ß-galactosidase was determined histologically by x-gal staining stably infected NIH 3T3, Rat-2 and CrFK cells. Cells were seeded out in duplicate and either stimulated for 3 days with dexamethasone (10^{-6} M) or remained unstimulated for the same time period. The cells were then fixed in 2% paraformaldehyde (see 2.5.3.2.1) and the expression of ß-galactosidase visualised by staining with x-gal (4-chrome-5-bromo-3-indoyl-ß-D-galactosidase). Non-infected CrFK cells without stimulation (panel 1) and with stimulation (panel 4) show no blue ß-galactosidase expressing cells. MMTV-BAGgal infected cells without stimulation (panel 2) show the basal level of expression of the vector with a small number of cells exhibiting ß-galactosidase expression. After hormone stimulation of these cells (panel 5) many dark blue, strongly expressing ß-galactosidase cells can be seen CrFK cells infected with the parental BAGgal vector (panel 3) shows some ß-galactosidase expressing cells, with slightly higher levels of expression being seen after stimulation with dexamethasone (panel 6). 200x magnification.

3.1.9.2 S1-Analysis

To investigate whether the initiation site of RNA transcribed from the MMTV promoter carried in the U3 region is maintained after insertion of the *Mlu*I restriction enzyme site, S1 analysis was performed. It is known that the spacing between the TATA box in the promoter and transcription initiation site is critical in determining the site of initiation and this method can be used to accurately pinpoint the start of transcription.

Total RNA was prepared from either stably infected Rat-2 cells grown 3 days in the presence or absence of dexamethasone or from non-infected cells and hybridised to a γ^{32}P-end-labelled *Bsa*I DNA fragment of the recloned hybrid MMTV-BAGgal vector (pMMTVProCon). If the classic MLV initiation site is used, a 89 bp fragment should be protected from S1 digestion (Fig. 3.18).

The probe actually protects a fragment of 90 nucleotides (Fig. 3.18, lanes 1 and 2), placing the site of transcription initiation 1bp upstream of the classic CAP site. This may be a result of the introducing of the *Mlu*I restriction site, which increases the spacing between the TATA box

within the MMTV promoter and the classical R-U5 border by 6 bp.

A clear induction of the signal could be observed in RNA derived from cells grown in the presence of dexamethasone (lane 1) compared to that from cells grown in the absence of the dexamethasone (lane 2). This confirms the data obtained in both qualitative and quantitative assays for ß-galactosidase expression.

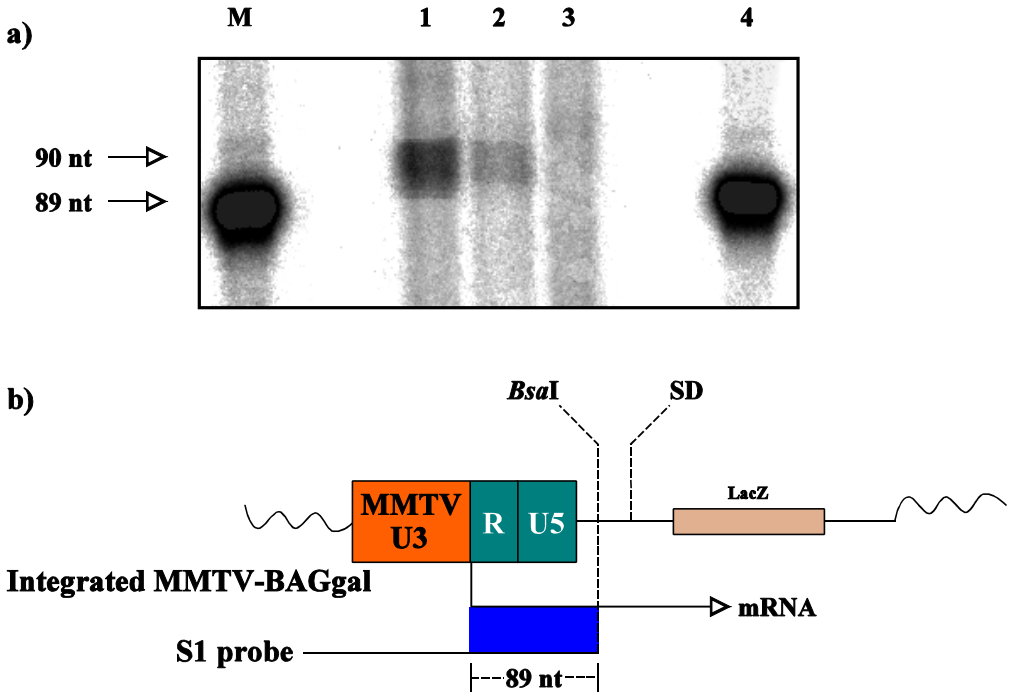

Figure 3.18: S1 nuclease Protection Analysis

a) Diagram of the S1 probe used. The probe was isolated after a *Bsa*I digest of the recloned hybrid MMTV-BAGgal plasmid pMMTVProCon. The protected fragment after S1 digestion was 90 nucleotides.

b) Total RNA was isolated from a stable infected population of MMTV-BAGgal infected cells grown in the presence or absence of dexamethasone (10^{-6} M). 40 µg total RNA was used per reaction. RNA from MMTV-BAGgal infected Rat-2 cells grown in the presence (lane 1) or absence (lane 2) of dexamethasone, as well as non infected Rat-2 cells (lane 3), was hybridised against a γ^{32}P-end-labelled *Bsa*I LTR DNA fragment as previously described (Günzburg *et al*., 1986). After S1 digestion, a protected fragment of a 90 bp was obtained, as determined with Phosphor imager software (Molecular Dynamics). The RNA of dexamethasone treated cells (lane 1) gives a stronger signal than that of non-stimulated cells (lane 2). No signal was obtained from the RNA derived from non-infected Rat-2 cells (lane 3). As a marker, *Hae*III-digested pBR322 was used, and the 89 nucleotide fragment is clearly visible (lane M).

3.1.10 *In vitro* Infection of Primary Human Tumour Cells and Cell Lines

After characterisation of the hybrid MMTV-BAGgal vector, its' activity in human breast tumour cells was investigated. Both different breast tumour cell lines and primary human breast cells that had been isolated from tumour biopsies were used for infection experiments and subsequent expression analysis. The human tumour cell lines and primary breast tumour tissue were cultivated either in matrigel or on collagen (see 2.4.3.2.3 and 2.4.3.2.2) to try to ensure the correct three dimensional cellular environment required for the differentiation of many cell types (Roskelley *et al*., 1994). In mammary epithelium the matrix of the matrigel as well as lactogenic hormones and glucocorticoids cooperate to direct accurate tissue-specific gene expression.The lactogenic hormone prolactin (3 µg/ml) as well as insulin (3 µg/ml) and dexamethasone (10^{-6}M)

were used to stimulate the cells before expression analysis.

3.1.10.1 Titre on Human Breast Tumour Cells

To investigate the viral titre of the recombinant retroviral vectors, infection experiments were made using T-47D cells. Virus supernatant harvested from a population of either MMTV-BAGgal or the parental BAGgal producing cells were used to determine the titre. This assay was made in a similar manner to the infection of NIH 3T3 cells described previously. NIH 3T3 cells were also infected in parallel with the same supernatant to check whether or not the infection experiment had worked technically. After approximately 14 days the mock-infected cells were seen to be dead, the number of resistant colonies was counted and the virus titre calculated as colony forming units per millilitre supernatant used.

Here it could be seen that less T-47D cells can be infected than NIH 3T3 cells using both the parental and hybrid vectors. Thus the T-47D cells are more difficult to infect than the NIH 3T3 cells (Table 3.2). Regardless of the virus used, the titre observed on T-47D cells was approximately 5-fold reduced when compared to the titre with the same virus on NIH 3T3 cells. The titres of the MMTV hybrid vectors (MMTV-BAGgal, LXPCMTVEGFP) were seen to be 2-4 fold lower than the parental vectors (BAGgal, LXSNEGFP).

Construct	Cell line	
	T-47D	NIH 3T3
	cfu/ml	
BAGgal	9×10^2	5.1×10^3
MMTV-BAGgal	4.2×10^2	2.2×10^3
LXSNEGFP	7.9×10^3	2.3×10^4
LXPCMTVEGFP	2.6×10^3	1.4×10^4

Table 3.2: Titre on T-47D Cells

Both T-47D cells and NIH 3T3 cells were used as a target cells for the infection with MMTV-BAGgal and BAGgal virus from producing populations. T-47D and NIH 3T3 cells were infected with supernatant from the same virus-producing cells. 24 hours after infection the target cells were diluted into 10 cm plates and a further 24 hours later G418 was added to the medium (NIH 3T3, 400 µg/ml; T-47D, 200 µg/ml). Approximately 14 days later, as soon as the mock-infected cells had died, the surviving colonies were counted and the titre calculated in colony forming units per millilitre infectious medium used (cfu/ml). The virus titre was calculated from three independent experiments.

3.1.10.2 Infection of Estrogen Receptor Positive Human Breast Tumour Cell Lines

Different types of human breast tumour cell lines such as MCF7, T-47D, ZR-75-1 were used for the infection experiments and expression analysis.

The MCF7, T-47D and ZR-75-1 cells were cultured in a monolayer and then infected with either MMTV-BAGgal or BAGgal. Due to the fact that these cells are more difficult to infect than the NIH 3T3 cell line, the virus particles were pseudotyped with the G protein from the vesicular stomatitis virus in order to obtain sufficient infected clones to allow the creation of a cell population (normally > 50 clones). The virus-producing cells were transfected with 10 µg of the plasmid pHCMV.G (Fig. 3.7) 48 hours pre-infection. The cells were washed with PBS 24 hours post transfection and fresh medium added. The human breast cells were infected 24 hours later and the selection began 48 hours after that. G418 at different concentrations was used for selection (MCF7, 600 µg/ml; T-47D, 200 µg/ml; ZR-75-1, 400 µg/ml). After approximately 2 weeks the mock-infected cells were found to be dead and the surviving infected clones were pooled to form populations.

To examine the expression of ß-galactosidase with or without hormone stimulation in MMTV-BAGgal infected cells, enzyme activity was determined by either using a photometric quantitative ß-galactosidase assay or qualitatively using histochemical staining.

For the quantitative assay, ß-galactosidase activity was analysed in cells (MCF7, T-47D and ZR-75-1) stably infected with either the hybrid MMTV-BAGgal or the parental BAGgal vector. $3x10^5$ cells were plated out in matrigel and either stimulated for 3 days [with prolactin (3 µg/ml), insulin (3 µg/ml) and dexamethasone (10^{-6} M)] or not stimulated at all. The protein lysate was prepared from hormone stimulated/non-stimulated infected or non-infected cell populations.

All of the tested infected cell populations showed expression of ß-galactosidase in the absence of hormonal stimulation. However, addition of hormones, especially the synthetic glucocorticoid dexamethasone resulted in enhanced expression (Fig. 3.19), as expected, from the MMTV promoter. Treatment with lactogenic hormones is not essential for induction of MMTV. Prolactin and insulin were shown to be sufficient to induce differentiation of established mammary epithelial cells *in vitro* (Danielson *et al*., 1984; Doppler *et al*., 1989; Reichmann *et al*., 1989). They also allow the breast or breast tumour cells to retain their original morphology to a certain extent. Levels of expression in the presence of hormones were between 9.1 and 1270 fold the expression seen in uninfected cells, depending on the cell type; the best induction was observed in T-47D cells (Fig. 3.19). The presence of hormones showed a dramatic increase in expression in T-47D cells (122 fold) whereas lower levels of induction could be seen in ZR-75-1 (13 fold) and MCF7 cells (2.6 fold). Low levels of hormone induction were also observed in cells infected with the parental BAGgal vector, levels approximately 3-fold the non hormone stimulated values being seen in T-47D cells.

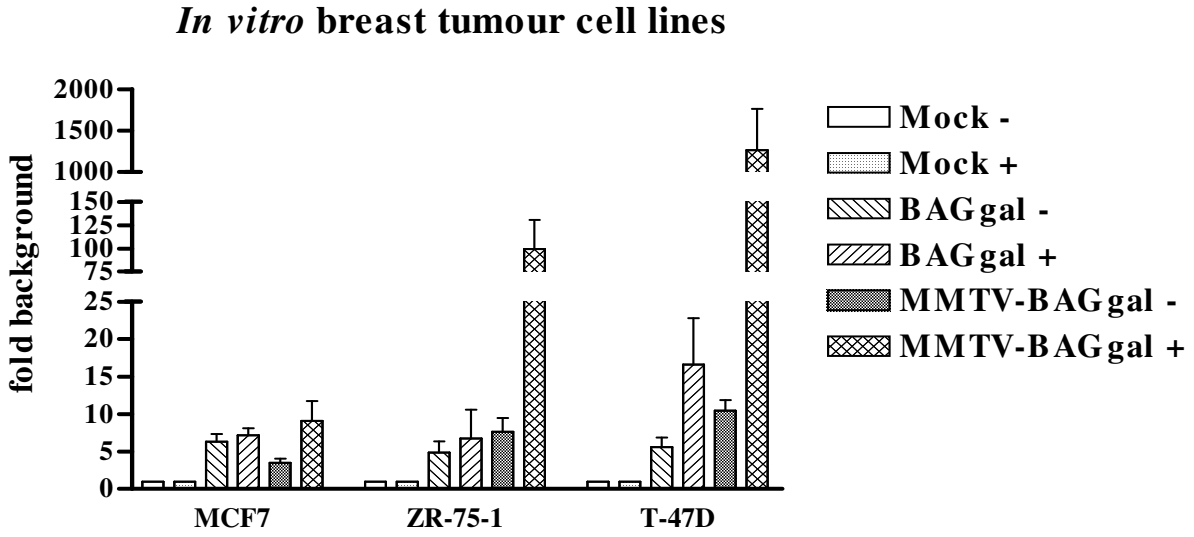

Figure 3.19: Dexamethasone Inducible Expression of ß-galactosidase in Transduced Human Breast Tumour Cells Lines.

The breast tumour cell lines MCF7, T-47D and ZR-75-1 were stably infected with either the hybrid MMTV-BAGgal vector or the parental BAGgal vector and cell populations established. $3x10^5$ infected cells that had been cultured in matrigel were lysed and total cell protein extracted after 3 days +/- hormone treatment [prolactin (3 µg/ml), insulin (3 µg/ml) dexamethasone (10^{-6} M)]. 10 µg protein was analysed for ß-galactosidase enzymatic activity using the galactolight kit (Perkin Elmer). The expression of ß-galactosidase is measured in relative light units after a chemoluminescent substrate is added. The background ß-galactosidase activity was set to a value of 1 and the expression of the vectors then calculated in relationship to the background. The best expression/induction was observed in MMTVBAGgal infected T-47D cells, levels of 1270-fold background being observed. Expression and induction could also be seen in MMTV-BAGgal infected MCF7 and ZR-75-1 cells, being 9.1 and 99.3 fold background respectively. The fold background expression observed from the MLV promoter was maximally 16.6 fold in T-47D cells. The experiment was repeated 3 times.

Hormone induced ß-galactosidase expression was also analysed in a qualitative assay using histochemical x-gal staining (4-chrome-5-bromo-3indoyl-ß-D-galactosidse). The cells were treated in the same manner as described above for the chemiluminescent assay. After 3 days the cells were fixed and subsequently stained with an x-gal solution. 24 hours later the cells were examined using light microscopy. In the absence of dexamethasone, the background expression could be observed, but in the presence of dexamethasone more cells express the ß-galactosidase enzyme. The inducible expression of ß-galactosidase could clearly be seen in T-47D (Fig. 3.20) as well as ZR-75-1 and MCF7 cells. The induction observed when using this assay correlates with the results seen in the quantitative ß-galactosidase assay (Fig. 3.19).

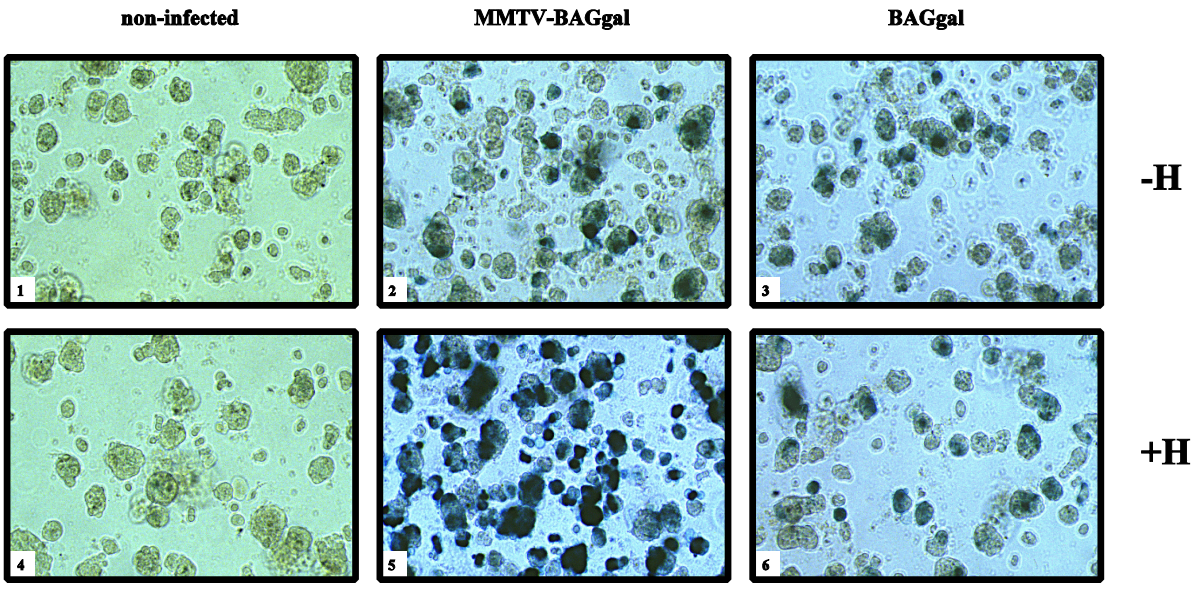

Figure 3.20: ß-galactosidase Expression in Infected Human Breast Tumour Cell Lines
Hormone inducible expression of ß-galactosidase was determined histologically by x-gal staining stably infected T-47D, ZR-75-1 or MCF7 cells. Cells were seeded out in duplicate and either stimulated for 3 days with hormones [prolactin (3 µg/ml), insulin (3 µg/ml) dexamethasone (10^{-6} M)] or remained unstimulated for the same time period. The cells were then fixed in 2% paraformaldehyde (see 2.5.3.2.1) and the expression of ß-galactosidase visualised by staining with x-gal (4-chrome-5-bromo-3-indoyl-ß-D-galactosidase). Non-infected T-47D cells without stimulation (panel 1) and with stimulation (panel 4) show no blue ß-galactosidase expressing cells. MMTV-BAGgal infected cells without stimulation (panel 2) show the basal level of expression of the vector with a small number of cells exhibiting ß-galactosidase expression. After hormone stimulation of these cells (panel 5 many dark blue, strongly expressing ß-galactosidase cells can be seen. T-47D cells infected with the parental BAGgal vector (panel 3) shows some ß-galactosidase expressing cells, with slightly higher levels of expression being seen after stimulation with dexamethasone (panel 6). Similar results were observed in both ZR-75-1 and MCF7 cells. 200x magnification.

3.1.10.3 Infection of Estrogen Receptor Negative Human Breast Tumour Cell Lines

An estrogen receptor negative human breast tumour cell line, MDA-MB-435S, was used for further *in vitro* analysis experiments. Here, the pLXSN based pLXPCMMTVEGFP retroviral vector was used. The reporter gene in this vector is EGFP instead of ß-galactosidase and carries a similar modification of the 3' LTR as in pMMTV-BAGgal. It should also undergo reverse transcription in a similar manner leading to expression being controlled by the U3 region of MMTV after infection of target cells. As it is well known that the MDA-MB-435S cells are highly metastatic (Lesoon-Wood *et al.*, 1995), the use of EGFP as a marker gene should allow easier detection of expression in an *in vivo* mouse model where these cells have been transplanted and metastases allowed to develop.

MDA-MB-435S as well as T-47D breast tumour cells were infected with either pLXPCMMTVEGFP or pLXSNEGFP. As it had already been shown that the U3 of MMTV was active and inducible in T-47D cells they were used as a positive control in this series of experiments.

MDA-MB-435S and T-47D cells were cultured in a monolayer system and then infected with either the hybrid LXPCMMTVEGFP or the parental LXSNEGFP vector. As in the infection experiments that have been previously described, the recombinant particles were here also pseudotyped with the VSV-G protein (see 3.1.1.5.3.2) in order to obtain enough clones to establish a population (>50 clones) after selection (400 µg/ml G418 for MDA-MB-435S cells and 200 µg/ml for T-47D cells). The stably infected cells were then seeded out in a 3D culture system and either stimulated for 3 days with prolactin (3 µg/ml), insulin (3 µg/ml) dexamethasone (10^{-6} M) or not stimulated at all. The expression of the enhanced form of the green fluorescent protein (EGFP) was examined using fluorescence microscopy. In the absence of hormones, LXPCMMTVEGFP infected cells exhibited basal levels of expression, whereas addition of hormones leads to higher expression levels (Fig. 3.21). LXSNEGFP infected cells showed expression of EGFP regardless of the state of hormone stimulation.

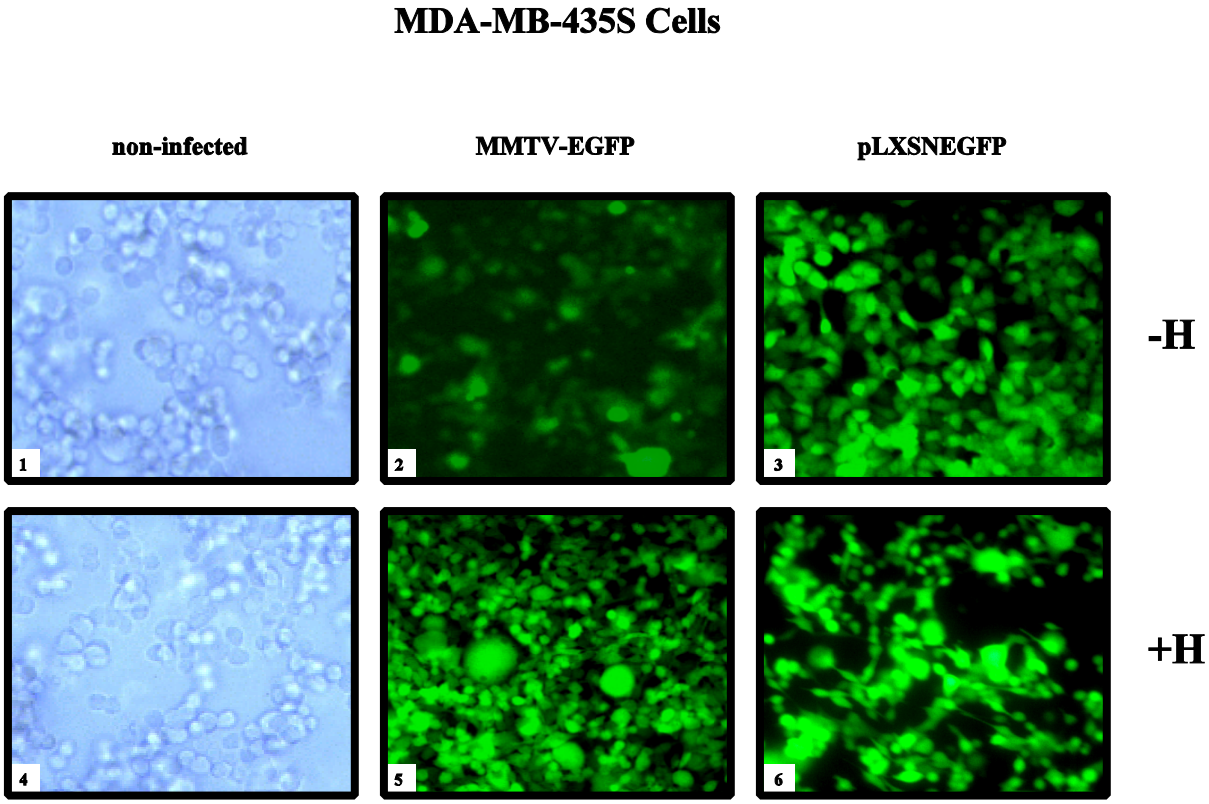

Figure 3.21: Dexamethasone Inducible EFGP Expression in Transduced MDA-MB-435S Human Breast Tumour Cell Lines

The breast tumour cell lines MDA-MB-435S and T-47D were stably infected with either the hybrid LXPCMMTVEGFP or the parental LXSNEGFP vector and cell populations established. Infected cells that had been cultured in matrigel were examined using fluorescent microscopy after 3 days +/- hormone treatment [prolactin (3 µg/ml), insulin (3 µg/ml) dexamethasone (10^{-6} M)].

Non-infected MDA-MB-435S cells without stimulation (panel 1) and with stimulation (panel 4) show no green EGFP expressing cells. LXPCMMTVEGFP infected cells without stimulation (panel 2) show the basal level of expression of the vector with a small number of cells exhibiting EGFP expression. After hormone stimulation of these cells (panel 5) many green, strongly expressing EGFP cells can be seen. MDA-MB-435S cells infected with the parental LXSNEGFP vector (panel 3) show strong EGFP expressing cells, with similarly high levels of expression being seen after stimulation with dexamethasone (panel 6). Similar results were observed in the control T-47D cells. 200x magnification.

3.1.10.4 Infection of Human Non-Breast Tumour Cells

After it had been shown that the hybrid MMTV-BAGgal vector was active in human breast tumour cell lines it was important to analyse the activity of this vector in human non-breast tumour cells. Panc-1 cells, derived from a ductal pancreatic tumour as well as EJ cells arising from a bladder carcinoma were used (see 2.4.1.4)

Panc-1 and EJ cells were infected with either the hybrid MMTV-BAGgal or the parental BAGgal vector in an identical manner to the infections of human breast tumour cell lines (see, 3.1.10) and selected for approximately 2 weeks with G418 (Panc-1, 400 µg/ml; EJ, 800 µg/ml).

The stably infected cells were then seeded out in matrigel or on collagen and either stimulated for 3 days with hormones or not stimulated at all. The cells were treated in exactly the same manner as the breast tumour cells lines to ensure that any differences observed in the expression was not due to the cultivation conditions.

To examine the expression of ß-galactosidase with or without hormone stimulation in MMTV-BAGgal infected cells, enzyme activity was determined either by using a photometric quantitative ß-galactosidase assay or qualitatively using histochemical staining. Expression of the ß-galactosidase was observed in EJ cells but not in Panc-1 cells (Fig. 3.22). Although expression could be observed in MMTV-BAGgal infected EJ cells, the levels are low when compared to most infected human breast tumour cell lines.

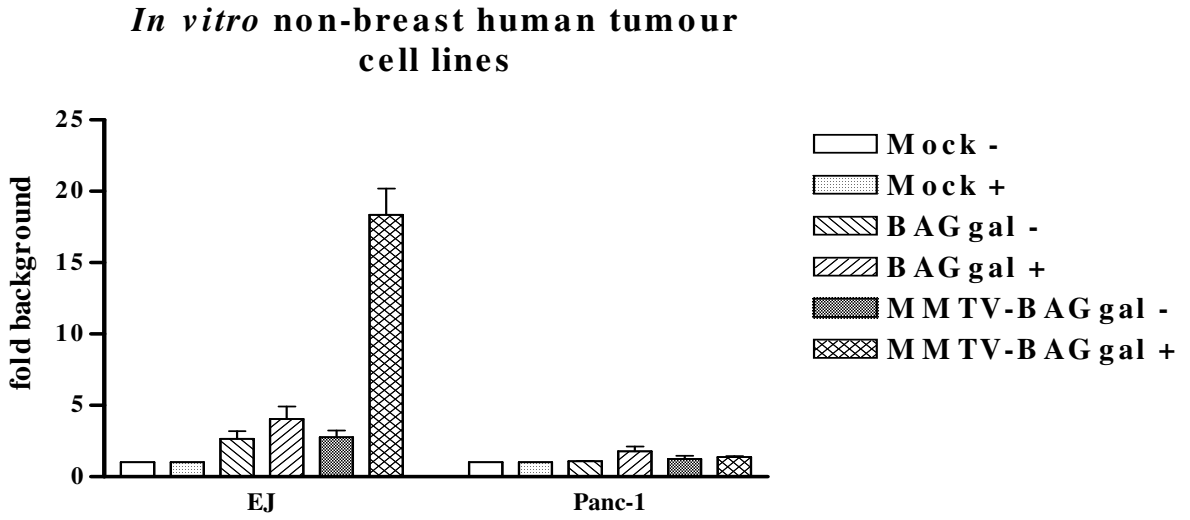

Figure 3.22: Dexamethasone Inducible Expression of ß-galactosidase in Transduced Human Non-Breast Tumour Cells Lines

The non-breast tumour cell lines Panc-1 and EJ were stably infected with either the hybrid MMTV-BAGgal vector or the parental BAGgal vector and cell populations established. $3x10^5$ infected cells that had been cultured in matrigel were lysed and total cell protein extracted after 3 days +/- hormone treatment [prolactin (3 µg/ml), insulin (3 µg/ml) dexamethasone (10^{-6} M)]. 10 µg protein was analysed for ß-galactosidase enzymatic activity using the galactolight kit (Perkin Elmer). The expression of ß-galactosidase is measured in relative light units after a chemoluminescent substrate is added. The background ß-galactosidase activity was set to a value of 1 and the expression of the vectors then calculated in relationship to the background. Expression was observed in MMTV-BAGgal infected EJ cells. No expression could be seen in Panc-1 cells. The experiment was repeated 3 times and the standard error of the mean is shown.

Hormone induced ß-galactosidase expression was also analysed in a qualitative assay using histochemical x-gal staining (4-chrome-5-bromo-3-indoyl-ß-D-galactosidase). The cells were treated in the same manner as described above for the chemiluminescent assay. After 3 days the cells were fixed and subsequently stained with an x-gal solution. 24 hours later the cells were examined. A low level of inducible expression of ß-galactosidase could clearly be seen in hybrid MMTV-BAGgal infected EJ cells (Fig. 3.23) but not in Panc-1 cells.

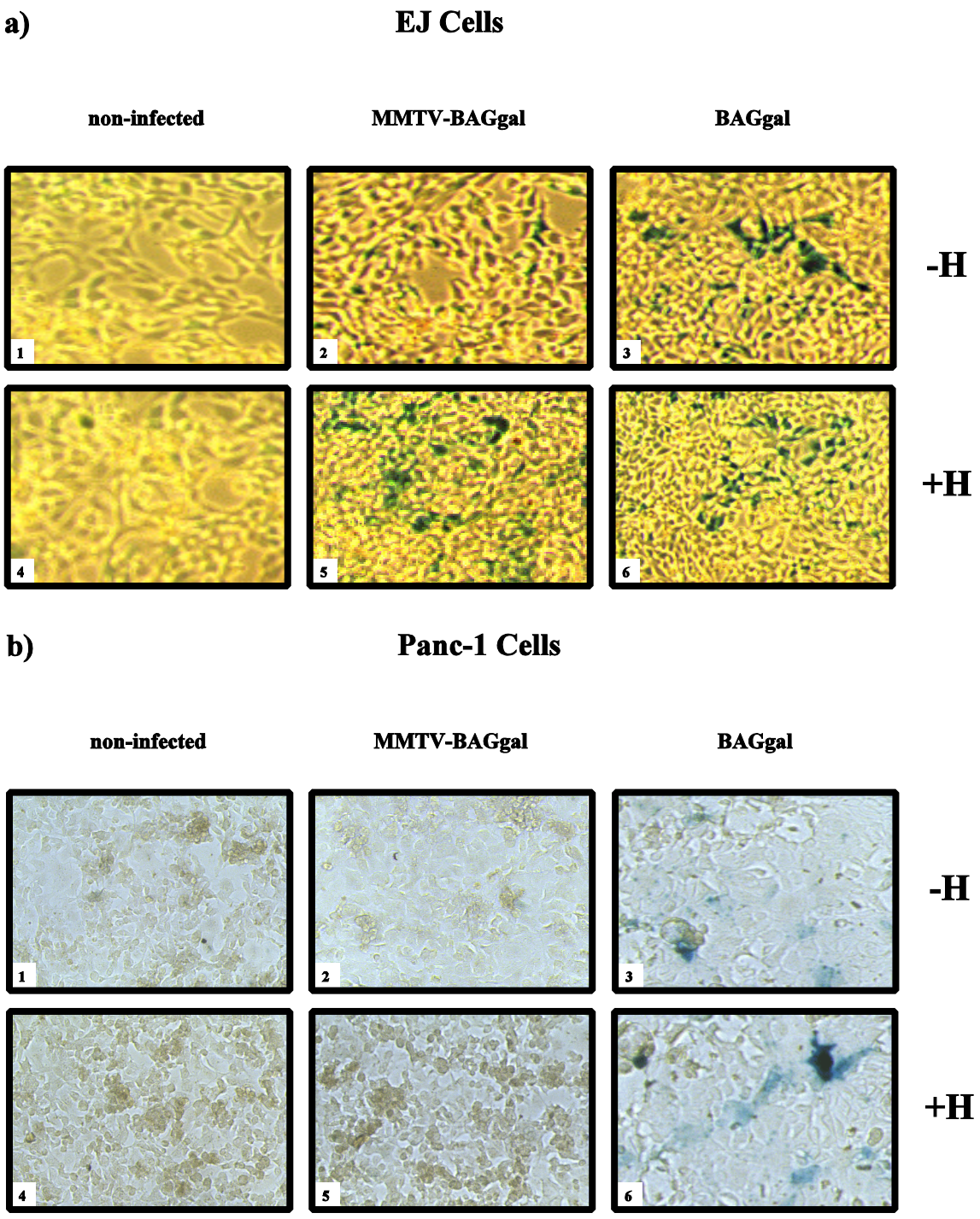

Figure 3.23: ß-galactosidase Expression in Infected Human non-Breast Tumour Cell Lines
Hormone inducible expression of ß-galactosidase was determined histologically by x-gal staining stably infected EJ (a) and Panc-1 cells (b). Cells were seeded out in duplicate and either stimulated for 3 days with hormones or remained unstimulated for the same time period. The cells were then fixed in 2% paraformaldehyde and the expression of ß-galactosidase visualised by staining with x-gal (4-chrome-5-bromo-3-indoyl-ß-D-galactosidase). Non-infected cells without stimulation (panel a) 1 and b) 1) and with stimulation (panel a) 4 and b) 4) show no blue ß-galactosidase expressing cells. MMTV-BAGgal infected cells both with and without stimulation (panels a) 2 & b) 5 respectively) show few blue ß-galactosidase expressing cells. Cells infected with the parental BAGgal vector both with and without stimulation (panels a) 3 & b) 6 respectively) show ß-galactosidase expressing in cells. 100x magnification.

3.1.10.5 Infection of Primary Human Breast Tumour Cells

Human breast tumour tissue that had been obtained after a biopsy was used for these experiments. The tissue was first cultivated in a monolayer system (see 2.4.3.2.1), infected and then further cultivated on collagen to further three-dimensional growth. 3 days after the tissue had been taken into cell culture, primary cells were infected with either one of the hybrid MMTV vectors (MMTV-BAGgal or LXPCMMTVEGFP) or the corresponding parental vector as a control (BAGgal or LXSNEGFP).

For the histological x-gal assay, ß-galactosidase activity was analysed in cells transiently infected with either the hybrid MMTV-BAGgal or the parental BAGgal vector. Both infected and non- infected cells were plated out on collagen and either stimulated for 3 days [with prolactin (3 µg/ml), insulin (3 µg/ml) and dexamethasone (10^{-6} M)] or not stimulated at all. If the primary cells had been successfully infected and the promoters controlling the expression of the transgene are active then a ß-galactosidase expression should be seen. In this case ß-galactosidase expression could be seen in a few cells infected with MMTV-BAGgal after hormone stimulation and also in a few BAGgal infected cells with and without hormone (Fig. 3.24). This may be due to a low transduction efficiency of the primary cells grown in monolayer.

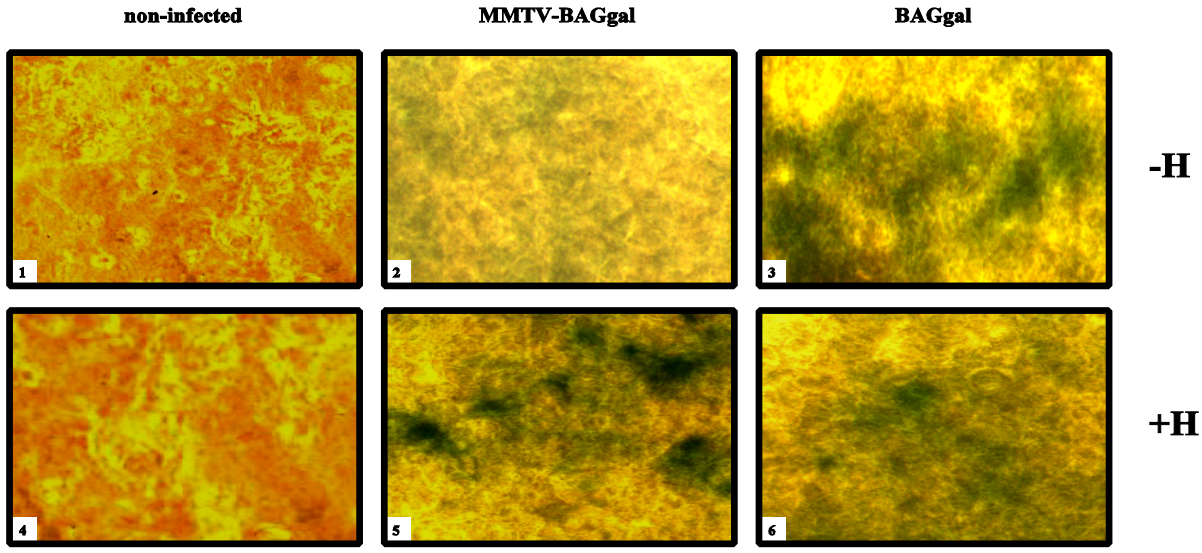

Figure 3.24: ß-Galactosidase Expression in Infected Primary Human Breast Tumour Cells
Primary human breast tumour cells isolated from a biopsy were first cultivated in a monolayer system, infected and then further cultivated on collagen to further three-dimensional growth. 3 days after the tissue had been taken into cell culture, primary cells were infected with either one of the hybrid MMTV vectors (MMTV-BAGgal or LXPCMMTVEGFP) or the corresponding parental vector as a control (BAGgal or LXSNEGFP). Cells were than stimulated for 3 days with hormones or remained unstimulated for the same time period. The cells were fixed in 2% paraformaldehyde and the expression of ß-galactosidase visualised by staining with x-gal (4-chrome-5-bromo-3-indoyl-ß-D-galactosidase). Non-infected primary human tumour cells with stimulation (panel 1) as well as those that remained unstimulated (panel 4) show no blue ß-galactosidase expressing cells. MMTV-BAGgal infected cells without stimulation show no expressing cells (panel 2) whereas with stimulation expression can be seen (panel 5). Primary human tumour cells infected with the parental BAGgal vector show some ß-galactosidase expression in both cells without (panel 3) with stimulation (panel 6).

In parallel, primary human breast tumour cells infected with either the hybrid LXPCMMTVEGFP or the parental LXSNEGFP were selected in G418 (400 µg/ml). After approximately 2 weeks mock-infected cells were found to be dead and the surviving infected clones were pooled to form populations (30 clones/population in this case). It has already been demonstrated that it is possible to culture primary human mammary tumour cells for long periods (one to six months; Yang *et al*., 1980; Ethier *et al*., 1993; Croce *et al*., 1998). Infected and non- infected cells were plated out on collagen and either stimulated for 3 days [with prolactin (3 µg/ml), insulin (3 µg/ml) and dexamethasone (10^{-6} M)] or not stimulated at all.

The expression of the enhanced form of the green fluorescent protein (EGFP) was examined using fluorescence microscopy. In the absence of hormones, LXPCMMTVEGFP infected cells exhibited basal levels of expression, whereas addition of hormones leads to higher expression levels (Fig. 3.25). LXSNEGFP infected cells showed expression of EGFP regardless of the state of hormone stimulation.

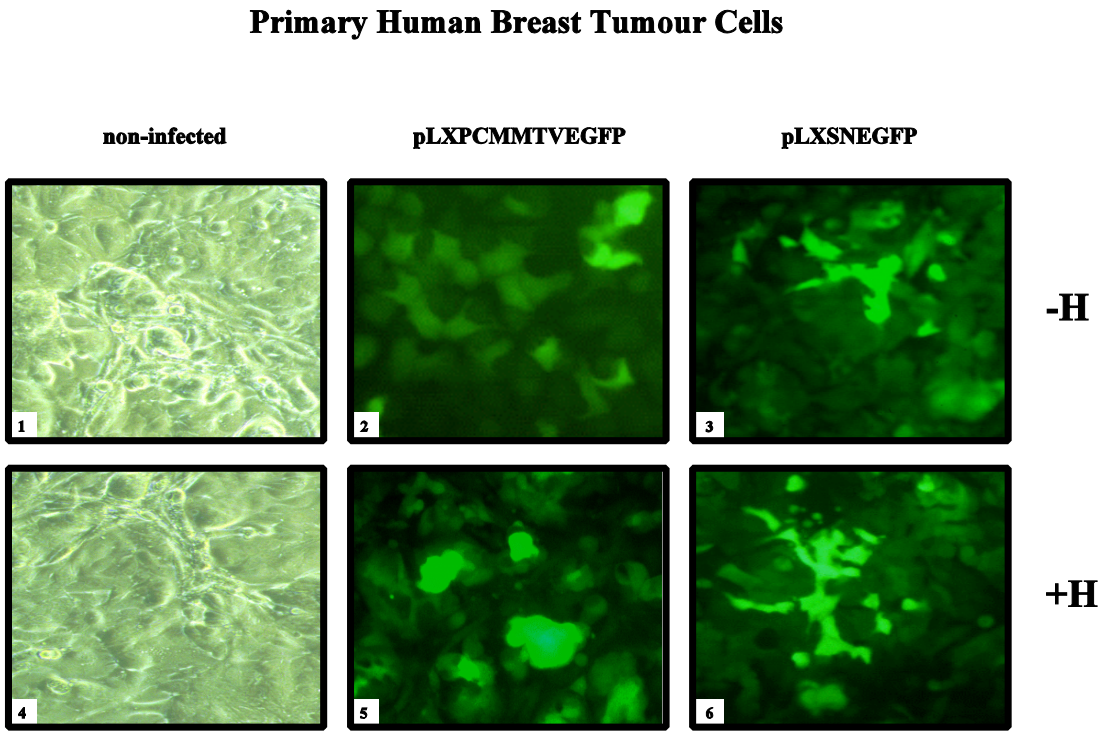

Figure 3.25: EGFP Expression in Infected Primary Human Breast Tumour Cells
Primary human breast tumour cells were stably infected with either the hybrid LXPCMMTVEGFP or the parental LXSNEGFP vector and cell populations established. Infected cells that had been cultured on collagen were examined using fluorescent microscopy after 3 days +/- hormone treatment [prolactin (3 µg/ml), insulin (3 µg/ml) dexamethasone (10^{-6} M)].
Non-infected primary human tumour cells both without stimulation (panel 1) and with stimulation (panel 4) show no green EGFP expressing cells. LXPCMMTVEGFP infected cells without stimulation (panel 2) show the basal level of expression of the vector with a small number of cells exhibiting EGFP expression. After hormone stimulation of these cells (panel 5) many green, strongly expressing EGFP cells can be seen. Primary human tumour cells infected with the parental LXSNEGFP vector (panel 3) show EGFP expressing cells, with similarly high levels of expression being seen after stimulation with hormones (panel 6). 400x magnification.

3.1.11 *In vivo* Expression Analysis of Xenografts in Severe Combined Immunodeficiency Mice (SCID)

Xenotransplantation was performed with infected cells derived from both breast and non-breast cells. To determine whether or not the *in vitro* ß-galactosidase/EGFP expression of the MMTV-BAGgal hybrid vector is mirrored in an *in vivo* setting, human tumour cells that had been stably infected with the hybrid vector were taken up in matrigel before being injected into SCID mice. The use of matrigel embedded cells has been described as being particularly useful when transplanting human breast tumours cells. It has been observed that especially breast tumour cells injected in the presence of matrigel exhibited close histopathalogical resemblances to surgical breast specimens after the tumours that arose had been recovered. Co-injection with matrigel can increase the incidence of tumours and enhanced the tumour growth. Estrogen supplementation was found also to increase the growth rate of such tumours (Rae-Venter *et al.*, 1980; Osborne *et al.*, 1985). An estrogen pellet (1.7 mg) was therefore transplanted into the

SCID/bg mice 24 hours before the transplantation took place (see 2.6.1). Such pellets release estrogen for a period of approximately 90 days.

3.1.11.1 Transplantation of Infected Breast and Non-Breast Cells into SCID/bg Mice

Human breast or non-breast tumours cells that had previously been infected with either the MMTV-BAGgal hybrid vector or BAGgal were cultivated in a monolayer cell culture system. $1x10^7$ cells per SCID mouse were prepared for the implantation (see 2.6.1.1). These cells were mixed together with matrigel on ice and immediately injected into the mammary fad pad (mfp) of SCID/bg mice. 4 mice were injected per construct and cell line. The mice were then examined 2 times per week and any tumours measured. 5 to 8 weeks later tumours could be observed in all treated mice, the time depending on the cell line used. The mice were sacrificed when the tumour had reached a size of 10 to 15 mm. The tumour was removed from the surrounding tissue and both RNA and protein prepared for further analysis (Fig. 3.26).

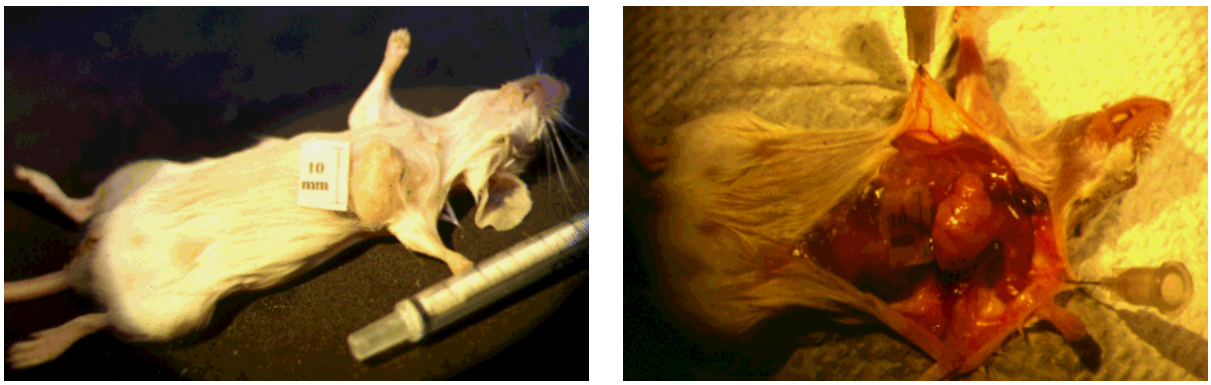

Figure 3.26: Transplantation of Infected Breast and non Breast Cells into SCID/bg mice
Mice were housed in microisolator cages, fed only autoclaved food and water, and handled only with gloves. Female mice that weighed between 17 and 20 g were anaesthetised with ketamin (Ketalar 1%, 0.2 ml/20 g mouse i.p). Antibiotic (Borgal^R 24%) was added to the drinking water on the day of implantation. For the implantation, a small area of skin was shaved (~2x2 cm) and disinfected. A small incision was made in the skin and the oestrogen pellet implanted. Depending on the size of the wound, the cut was either sewn together (by a veterinarian) or simply closed. 24 hours later $1x10^7$ cells that had been previously mixed with Matrigel were injected into the mammary fat pad. After a 5-8 week incubation period, the mice were killed and the tumour immediately removed from the surrounding tissue. A SCID/bg mouse with tumour derived from primary mammary tumour cells can be seen.

3.1.11.2 Protein Expression Analysis

Protein was extracted from the tumours of 3 mice per tumour type. Tumours were homogenised on ice in lysis buffer (see 2.5.3.1). The tumour extracts were then treated with a cocktail of protease inhibitors [PMSF (0.2 mM) and leupeptin (5 µg/ml)] in conjunction with a heat inactivation step, just before analysis, in order to reduce high levels of endogenous ß-galactosidase activity that had been previously observed in tissue samples (Shaper *et al.*,1994). Every human breast tumour showed high levels of ß-galactosidase expression from the MMTV-BAGgal hybrid vector as compared to the parental BAGgal (Fig. 3.27a).

The T-47D cells showed expression levels from the MMTV-BAGgal hybrid vector approximately 250 times that of the background, whereas the BAGgal levels were, on average only 1.4 times that of the empty cells. This high level of expression could also be shown histologically in both T-47D and ZR-75-1 tumours (Fig. 3.28a) after fixation (see 2.5.3.2.2) and staining.

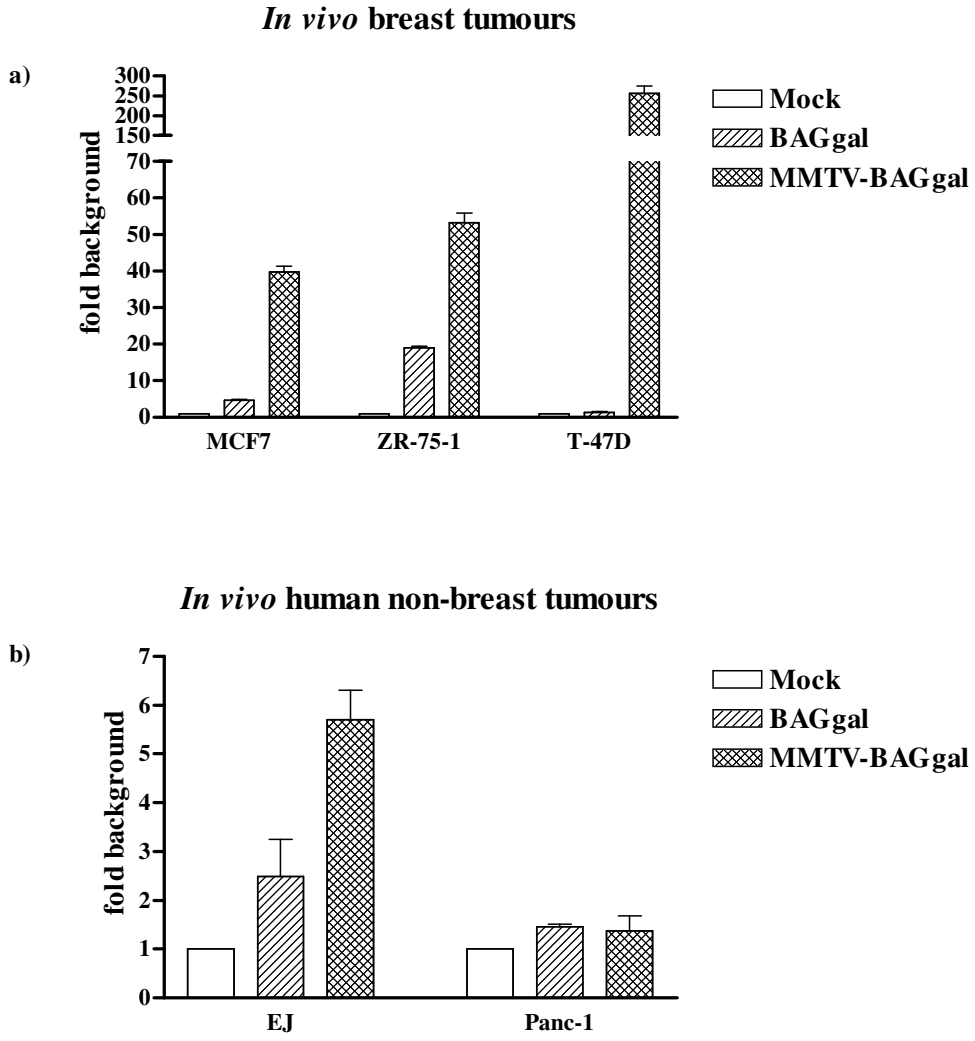

Figure 3.27: Analysis of ß-galactosidase Expression in Breast and non-Breast Tumour Cell Xenografts in SCID/bg mice

a) Expression of ß-galactosidase in human breast cell derived tumours in SCID/bg mice.
10 µg of protein that had been previously been extracted from tumours that had arisen after implantation of tumour cells was used for a quantitative ß-galactosidase assay (Galactolight, Perkin Elmer). The expression of ß-galactosidase is measured in relative light units after a chemoluminescent substrate is added. The background ß-galactosidase activity was set to a value of 1 and the expression of the vectors then calculated in relationship to the background. High levels of expression could be seen from the hybrid MMTV-BAGgal construct in MCF7, ZR-75-1 and T-47D cells. Expression from the BAGgal vector was much lower by comparison.
b) Expression of ß-galactosidase in human non-breast cell derived tumours in SCID/bg mice
The experiment was performed in the same manner as a). Expression of both the hybrid MMTV-BAGgal vector as well as the parental BAGgal could be seen although the levels of expression are much lower than observed in the breast cell derived tumours. No significant expression could be seen in Panc-1 cells. The absolute levels of expression from both BAGgal and MMTV-BAGgal vectors were much lower in both EJ and Panc-1 cells derived tumours than any of the human breast cell derived tumours, with the exception of BAGgal vector in T-47D cells.

In the non-breast tumours cell lines examined, EJ and Panc-1, the expression levels from the MMTV-BAGgal hybrid vector were much lower than those observed in the breast cell derived tumours (Fig. 3.27b), the highest levels being obtained in EJ cells (~6x background for MMTV-BAGgal). No significant expression could be seen in Panc-1 cells (Fig. 3.28b) Histological analysis showed similar results, with only very low expression detectable in EJ cells (not shown).

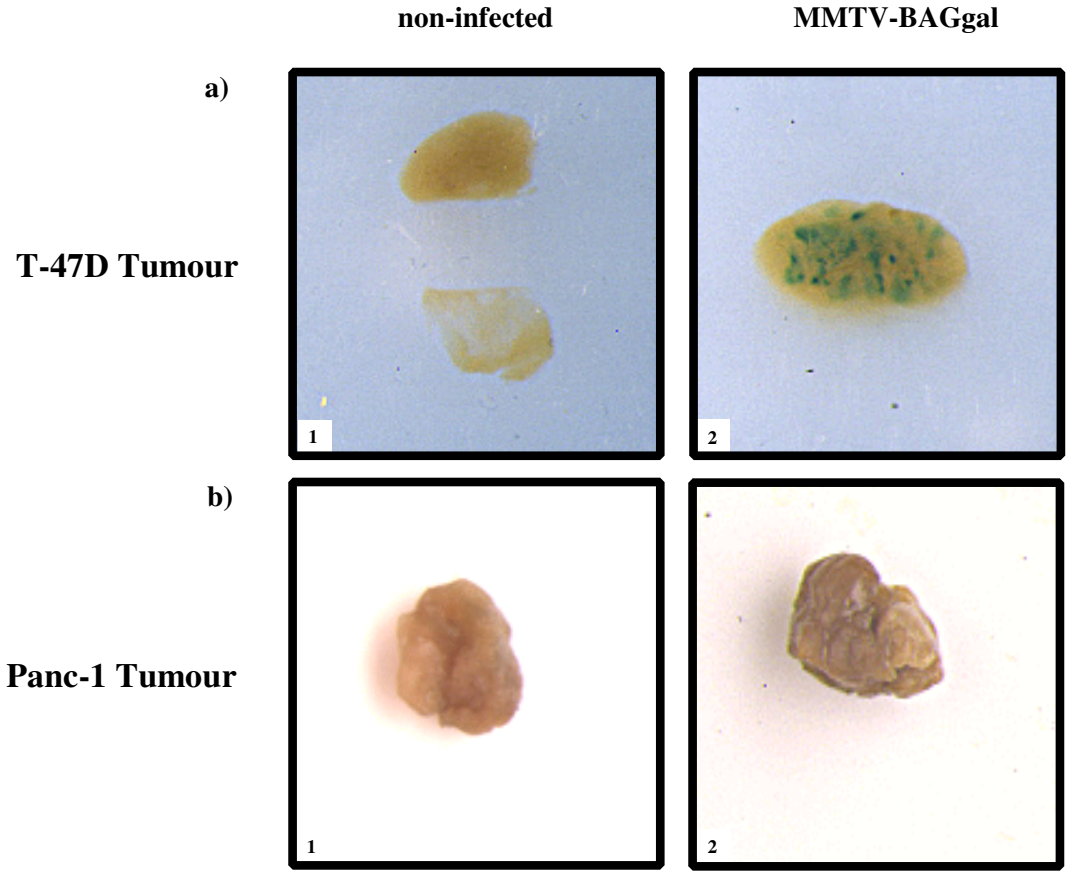

Figure 3.28: Histological Analysis of ß-galactosidase Expression in Breast and non-Breast Tumour Cell Xenografts in SCID/bg mice

a) X-gal staining of breast cell derived tumours.
A T-47D derived tumour was dissected and cut into small pieces before being fixed. In 4% paraformaldehyde for 24 hours at 4°C. The tissue was then stained with x-gal (2.5.3.2.2) before being embedded in paraffin and photographed. Blue staining indicates areas of ß-galactosidase expression. Similar results were also obtained for ZR-75-1 tumours.
b) X-gal staining of non-breast cell derived tumours.
The experiment was performed in the same manner as a) No expression, normally indicated by blue areas, can be seen in the Panc-1 derived tumour.

3.1.11.3 RNA Expression Analysis

RNA analysis was performed to show the expression levels of ß-galactosidase in the tumours arising from breast and non-breast derived cells. Northern blot analysis was made as it not only indicates the levels of expression but also allows the length of the transcript to be shown. Total RNA was isolated from a tumour from 1 of the 4 mice per construct per cell and run over a denaturing formaldehyde gel (see 2.2.2.3.2) and, after being transferred to a nylon membrane via capillary blotting (see 2.2.7.2), was hybridised to a radioactively labelled 0.4 kb ß-galactosidase specific fragment. The expected 7 kb fragment that could be seen in all human breast cell derived tumours infected with both the hybrid MMTV-BAGgal as well as the parental BAGgal vector (lanes 1, 2, 3, 6, 7 and 8) corresponding to the full length vector transcript. The BAGgal vector does, in fact, give a slightly shorter transcript than the MMTV-BAGgal vector. This is due to the presence of the larger MMTV U3 region in the 3' LTR, which makes the transcript approximately 700 bp longer (Fig. 3.29). The strongest ß-galactosidase expression could be observed in those human breast cell derived tumours that had been infected with the hybrid MMTV-BAGgal vector before implantation. The ß-galactosidase expression could also be detected in bladder carcinoma cell derived tumours (EJ, lanes 4 and 8) where the MLV promoter showed higher levels of expression than the promoter of MMTV. However, in pancreatic cell derived tumours (Panc-1, lanes 5 and 9), no expression could be detected from either the MMTV-BAGgal or BAGgal vectors. The same membrane was then stripped and hybridised to a radioactively labelled 1.2 kb ß-actin specific fragment. It shows the presence of similar quantities of a 2.2 kb ß-actin specific transcript in each lane.

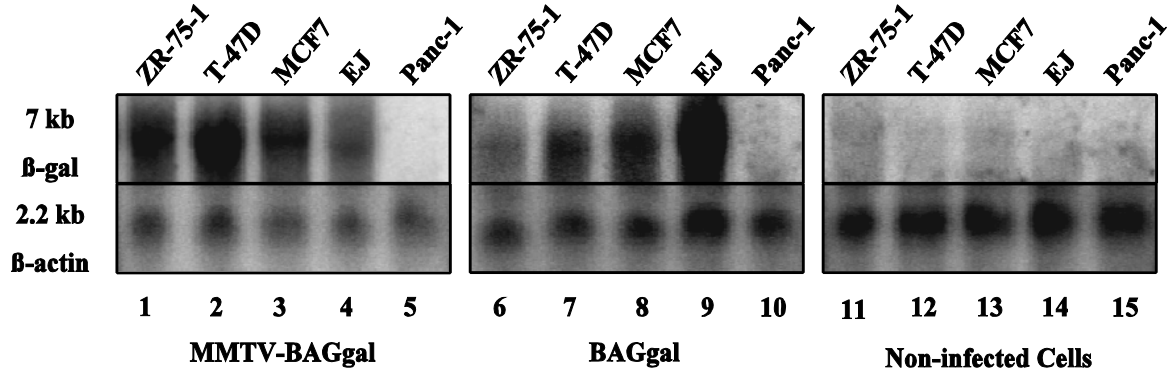

Figure 3.29: Northern Blot Analysis of Human Tumours from SCID/bg Mice

20µg of total RNA was separated on a denaturing formaldehyde gel transferred to a nylon membrane using capillary blotting. The membranes was then hybridised to an α^{32}P labelled 0.4 kb ß-galactosidase specific fragment (from pBAGgal after *Mlu*I digestion) and, following documentation and stripping, also to an α^{32}P labelled 1.2 kb ß-actin fragment [from pAL41 (Alonso *et al*., 1986) after *Pst* I digestion]. Lane 1, RNA from an MMTV-BAGgal infected ZR-75-1 tumour; lane 2, RNA from an MMTV-BAGgal infected T-47D tumour; lane 3, RNA from an MMTV-BAGgal infected MCF7 tumour; lane 4, RNA from an MMTV-BAGgal infected EJ tumour; lane 5, RNA from an MMTV-BAGgal infected Panc-1 tumour; Lane 6, RNA from a BAGgal infected ZR-75-1 tumour; lane 7, RNA from a BAGgal infected T-47D tumour; lane 8, RNA from a BAGgal infected MCF7 tumour; lane 9, RNA from a BAGgal infected EJ tumour; lane 10, RNA from an MMTV-BAGgal infected Panc-1 tumour. Lanes 11-15 represent non-infected cells.

3.1.12 Establishment of Transgenic Mice

The *in vivo* tissue specifity of MMTV in the context of retroviral vector was tested using the recloned MMTV-BAGgal vector (pMMTVProCon) in order to establish transgenic mice (in collaboration with the Institute of Animal Breeding and Genetics at the University of Veterinary Sciences in Vienna). The recloned plasmid pMMTVProCon was first linearised with the restriction enzyme *Asp*700 I and purified over an agarose gel. The fragment was then resuspended in TE buffer before being given to the Institute of Animal Breeding and Genetics. There it was introduced via microinjection into freshly fertilised eggs from the mouse strain HIM OF-1 (Fig. 3.30). After a short incubation period of 12 hours, the eggs were implanted into foster mice.

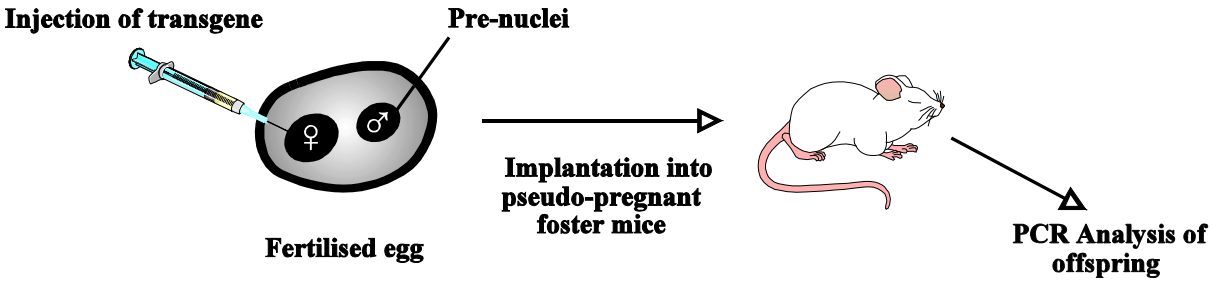

Figure 3.30: Establishment of Transgenic Mice using the Recloned Plasmid pMMTVProCon
The restriction enzyme *Asp*700 I linearised plasmid MMTVProCon was taken and, after purification following agarose gel electrophoresis, was microinjected into previously fertilised mouse oocytes. After 12 hours the microinjected eggs were implanted into foster mice.

3.1.12.1 DNA Analysis of Transgenic Mice

6 female founder mice (F_0) were produced after implantation of microinjected oocytes. When the mice had reached the age of approximately 7 weeks tail clips were provided from these founders. Genomic DNA was isolated from the provided material (see 2.2.2.1.3) and examined for the presence of the recloned vector using PCR. The ß-galactosidase specific primer pair Baggal1 and Baggal2 was used for this purpose (Fig. 3.31). Genomic DNA that had been isolated from a non-transgenic mouse was used to show that the primer being used did not amplify a non-specific product.

This DNA analysis showed that 3 of the 6 female founder mice were positive for the transgene (Table 3.3). These positive mice were then used for further breeding experiments. Two separate F_1 lines were then provided by the Institute of Animal Breeding and Genetics and were from then onwards were haused in the animal facility in the Institute of Virology. The F_1 animals were analysed in a similar manner to the F_0 founders. This analysis showed that only 1 positive F_0 mouse had been able to transfer the transgene to the F_1 generation. The following analyses were then made with this one transgenic line.

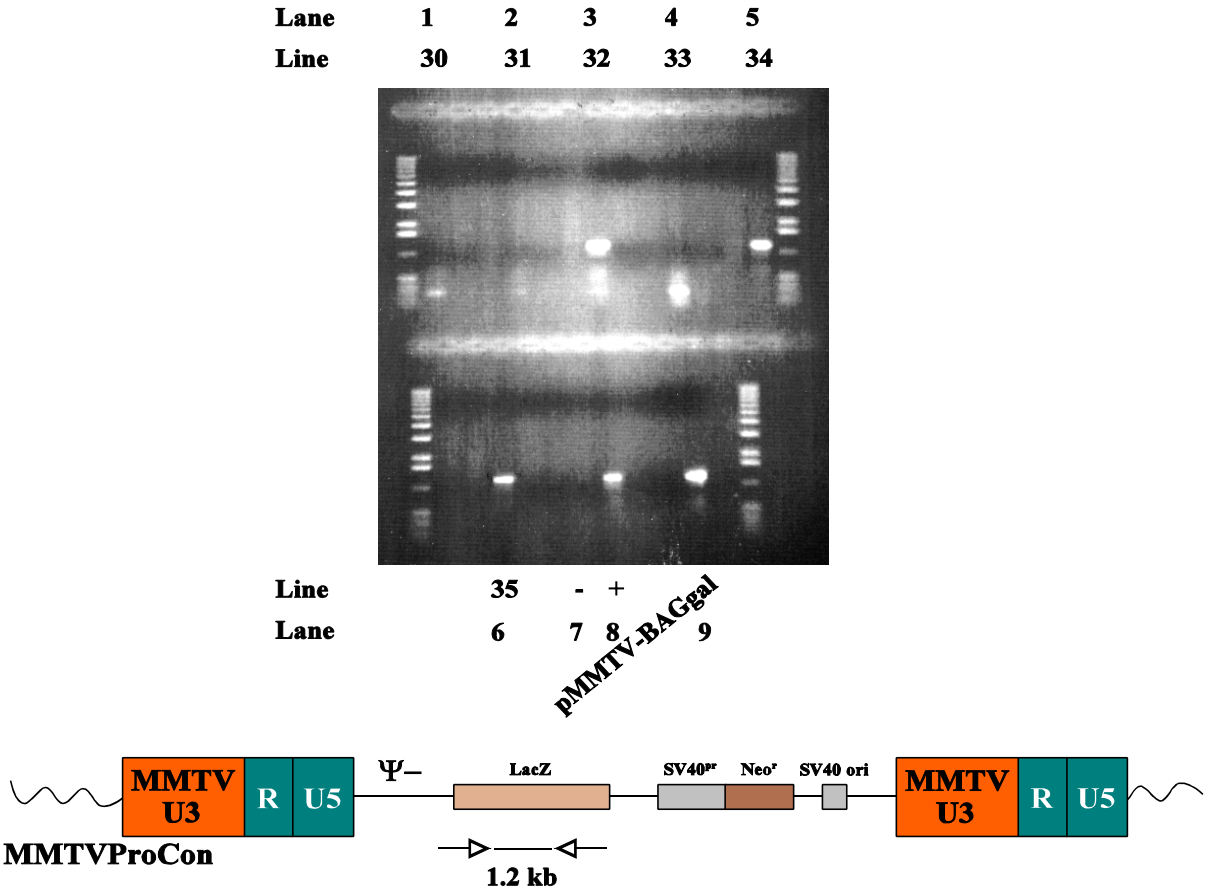

Figure 3. 31: DNA Analysis of Transgenic Mice

Genomic DNA was isolated from the tail clips of transgenic mice. 1 µg of DNA was used per PCR reaction. The following ß-galactosidase specific primer pair was used to show the presence of the transgene: Baggal1 (5'-TACAACGTCGTGACTGGGAA-3') and Baggal2 (5'-TTCATCCACCACATACAGGC-3'). These primers should give a 1.2 kb product if the transgene is present. Both 1 µg of genomic DNA isolated from Rat-2 cells infected with the MMTV BAG hybrid vector and 1ng of the original plasmid pMMTVProCon were used as positive controls.

6 female founder F_0 mice were analysed. 3 mice were found to be positive: lane 3, transgenic mouse line 32: lane 5, trangenic mouse line 34: lane 6, transgenic mouse line 35. Lanes 1, 2 and 4 with transgenic mouse lines 30, 31 and 33 respectively were found to be negative. Genomic DNA from a non-transgenic mouse can be seen in lane 7 and the positive controls in lanes 8 (genomic DNA from infected Rat-2 cells) and 9 (from pMMTVProCon).

F_0	34		35	
	+	-	+	-
F_1	20♀	5♀	0♀	14♀
	17♂	8♂	0♂	17♂

Table 3.3: Analysis of the F_1 Generation of the MMTVProCon Transgenic Mice
The analysis of the genomic DNA was made in a similar manner to the analysis of the F_0 generation. The lines 34 and 35 were provided for analysis. Line 34 showed a total of 20 positive and 5 negative female mice and 17 positive and 8 negative male mice. Line 35 showed a total of 14 negative female mice and 17 negative male mice. No positive mice could be detected in this line.

3.1.12.2 Southern Blot Analysis of Transgenic Mice

Genomic DNA was isolated (see 2.2.2.1.2) from 4 transgenic mice from line 34. Southern blot was the used to investigate the copy number and integration pattern of the transgene present. The genomic DNA was first digested with the restriction enzyme *Bgl*II (Fig. 3.32b). This enzyme cleaves only once at the beginning of the neomycin resistance gene. After separation of the digested DNA using agarose gel electrophoresis, the DNA was transferred to a nylon membrane and then hybridised using a radioactively labelled probe specific to the neomycin gene. The use of a neomycin specific probe allows the copy number and pattern of integration to easily be shown.

Fragments of approximately 14.5, 11.4 and 7.6 kb can be seen in all transgenic mice analysed. This indicates that 3 different sites of integration are present (Fig. 3.32d). After examining the intensity of the hybridised bands it was hypothesised that multiple head to tail or head to head integrations had taken place. The transgenic mice were established with an *Asp*700 I linearised pMMTVProCon as described above. In order to examine the hypothesis that concatamers of the linear form of pMMTVProCon are present in the genome of the transgenic mice, it was necessary to determine the position of the *Asp*700 I site in relationship to the LTRs. This allows the possible concatameric forms to be calculated. It also allows the mapping of the integration sites to a certain extent. In figure 3.32a, two double restriction digests were made to pinpoint the position of the *Asp*700 I site. The *Asp*700 I/*Cla*I and *Asp*700 I/*Bgl*II digests showed that the *Asp*700 I site was approximately 300 bp downstream of the 3' LTR. This allowed the map shown in figure 3.32b to be calculated and subsequently the integration possibilities to be estimated (Fig. 3.33) The hybridised blot was then analysed using a Phosphor Imager (Molecular Dynamics Storm 860). Using the Fragment™ software it was possible to compare the strength of the bands with known DNA amounts in order to estimate the copy number. This showed that, on average, 8 integrated copies were present.

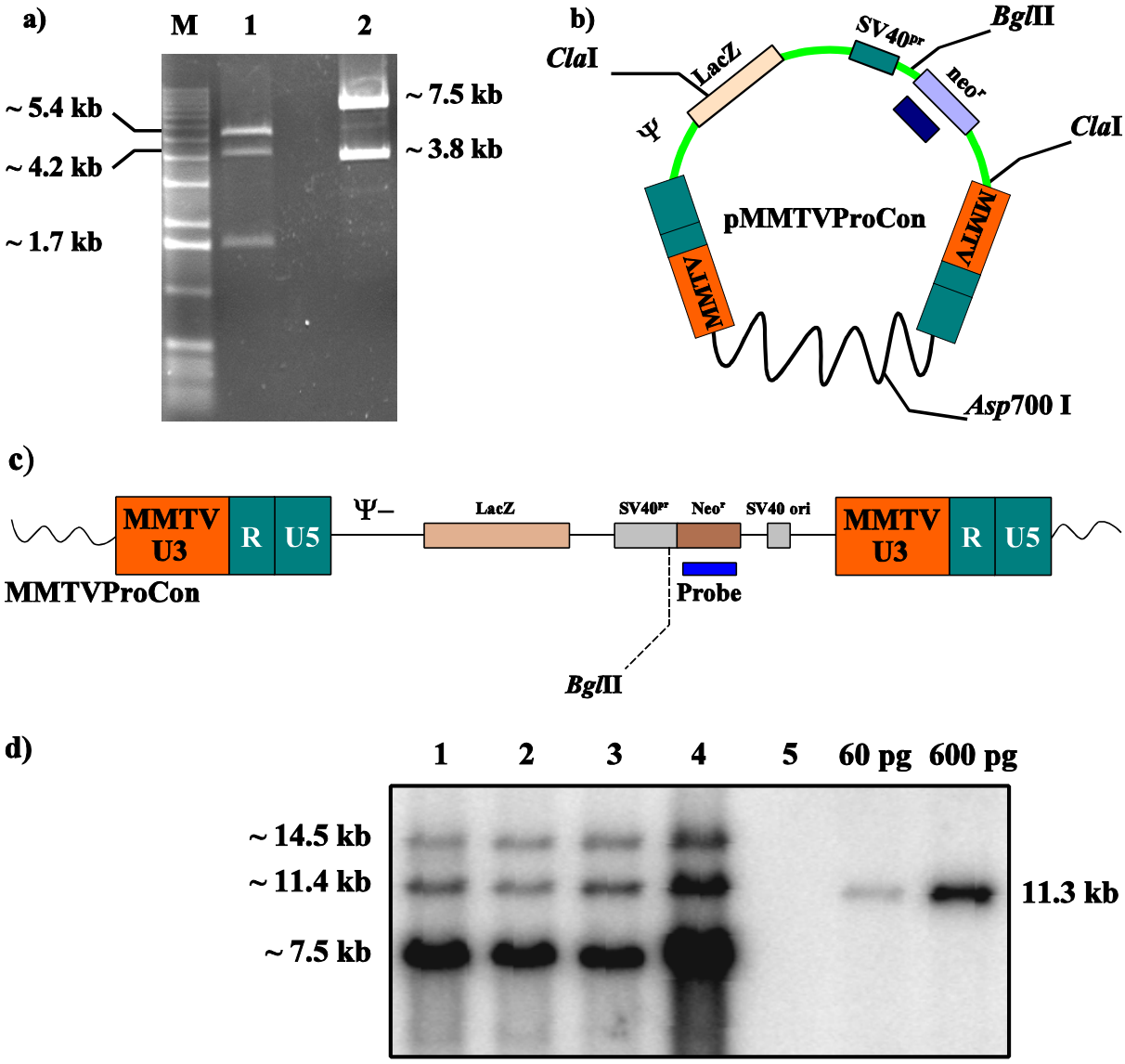

Figure 3.32: Southern Blot Hybridisation of MMTVProCon Transgenic Mice

a) Test restriction digests. Two double digests were used to determine the position of the *Asp*700 I site. In lane 1, an Asp700 I/ClaI digest shows 3 bands of approximately 5.4, 4.2 and 1.7 kb whereas the Asp700 I/BglII digest in lane 2 shows fragments of ~7.5 and ~3.8 kb. This allowed the position to be determined as being ~300 bp downstream of the 3' LTR as shown in b).

c) Diagram of the linearised plasmid pMMTVProCon. 20 µg of genomic DNA was digested with the restriction enzyme *Bgl*II which cuts once only in the neomycin resistance gene of the vector.

d) After agarose gel electrophoresis the DNA was transferred to a nylon membrane before being hybridised to a radioactively labelled neomycin specific probe. The 1kb neomycin probe was prepared by digesting the plasmid pMMTVProCon with the restriction enzymes *Bcl*I and *Eco*RI. Lane 1: DNA from transgenic mouse 7, lane 2: DNA from transgenic mouse 23, lane 3: DNA from transgenic mouse 11, lane 4: DNA from transgenic mouse 13, lane 5: DNA from a non-transgenic mouse. In lanes 6 and 7 DNA from the non-transgenic mouse had been mixed with 60 or 600 pg pMMTVProCon plasmid DNA before digestion. These lanes were used both as a positive control and also as standards for the quantification of the copy number. The intensity of the signal in lane 6 (60 pg) is taken as amounting to the signal that would arise from one integrated copy. The blot was then analysed using a Phosphor Imager (Molecular Dynamics Storm 860) using the program Fragment™. This program measures the intensity of the hybridised bands and then compares them to the control band in lane 6 (60 pg). The average values calculated were 8 copies.

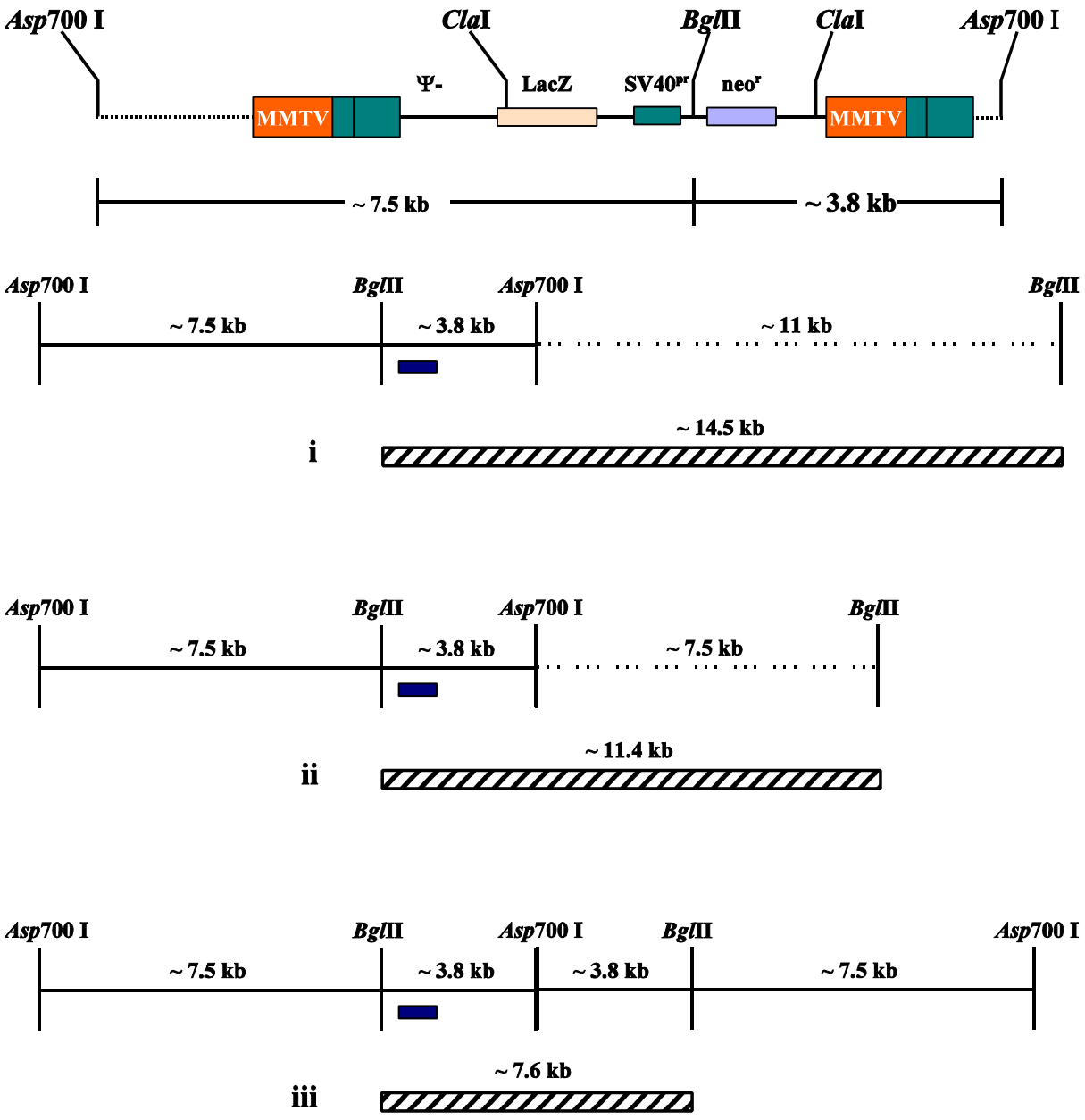

Figure 3.33: Possible Integration Sites in Transgenic Mice.
The 3 bands seen in figure 3.32d can be explained in the following manner. i) If the next *Bgl*II site 3' of the integration site was approximately 11 kb from the end of the 3' LTR this would result in a hybridised band of ~ 14.5 kb. ii) If, however, the next site were only 7.5 kb downstream this would give a band of ~ 11.4 kb. This band is probably not a concatameric form, as it is of a similar strength to the 14.5 kb band that is not a product of a concatamer, although its' size could correspond to a head to tail form. iii) If the linearised plasmid recombined in a tail-to-tail manner before integrating into the genome a 7.6 kb fragment would be the result.

3.1.13 Expression Analysis of MMTVProCon Transgenic Mice

The next step was to see if the proviral transripts could be found in the organs of the MMTVProCon transgenic mice. Total RNA was isolated from both transgenic and non-transgenic mice and used for both northern blot analysis as well as RT-PCR.

3.1.13.1 RT-PCR Analysis

The RNA that had been previously extracted from the organs and tissues of both transgenic and non-transgenic mice was treated with RNase free DNase in order to eliminate any DNA contamination that may have occured during the isolation process. The DNase was then removed via phenol extraction and 1µg of the cleaned RNA then used in the RT-PCR (see 2.2.5.2.2). The primer pair Bag1540 and Bag2478c was used to amplify a specific 0.9 kb product (Fig. 3.34a). Two control reactions per organ were made. First, to see if the RNA samples had any remaining DNA contamination present, in other words to see if the DNase treatment had worked properly, one reaction was made without the reverse transcriptase activity (inactivation of RT at 95°C for 10 minutes) before the PCR was made. The second control reaction was made using the ß-actin specific primers mouse ß-actin and mouse ß-actinc (Wei *et al.*, 1997) which amplify a specific 569 bp product. This reaction shows that the RNA that used in the RT-PCR was intact and, as the ß-actin expression should be the same in all the samples, acts as a standard allowing the comparison of expression levels.

A total of 8 MMTVProCon transgenic mice were analysed. Figure 3.34b shows one of the RT-PCR analyses (mouse 21). The expected band from the amplified fragment can clearly be seen in the spleen (lane 7) and in the mammary gland (lane 8). The control RT-PCR reaction also shows that the signal originates from RNA and cannot have arisen from a DNA contamination. In other mice that were analysed it could also be shown that the MMTV promoter preferentially directs the expression of linked genes to the mammary gland and spleen, mirroring the expression pattern of MMTV proviruses. However, ectopic expression could also be observed in the ovary or brain (Table 3.4), a finding that has also been shown by others.

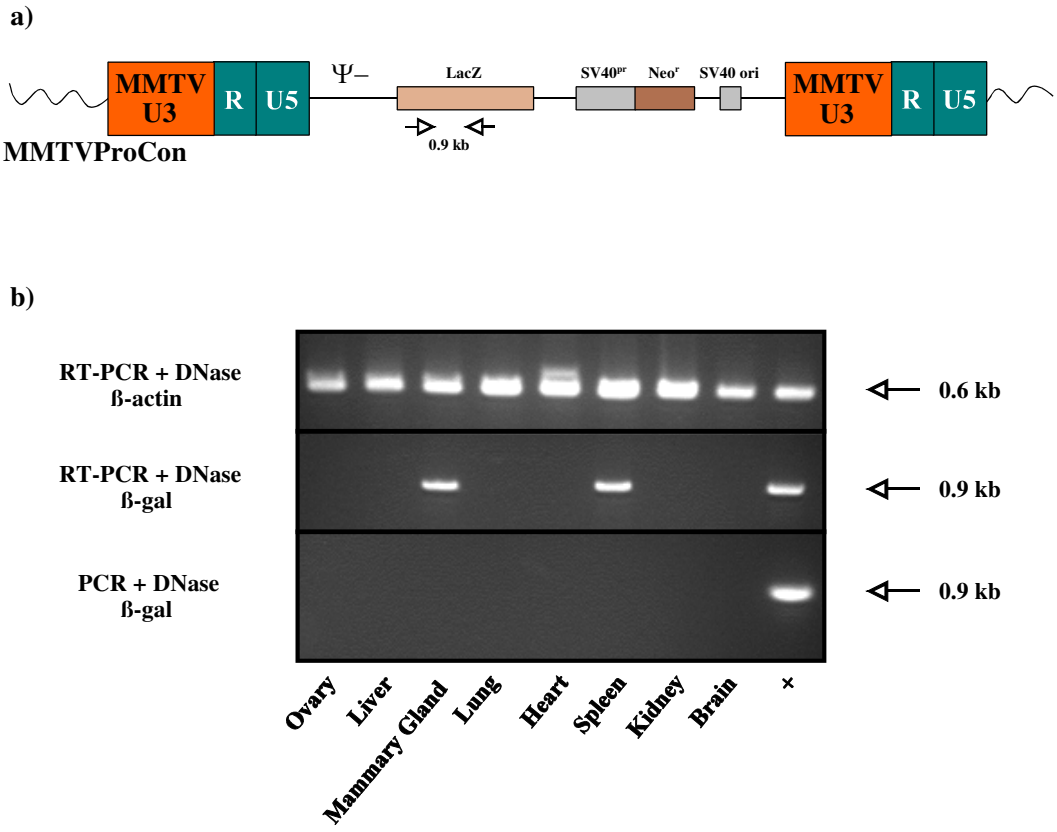

Figure 3. 34: RT-PCR Analysis of MMTVProCon Transgenic Mice.

a) Diagram of the linearised plasmid pMMTVProCon. The following ß-galactosidase specific primer pair was used to show transcription of the transgene: Bag1540 (5'-TTACGGCCAGGACAGTCGTT-3') and Bag2478c (5'-CGATACAGCGCGTCGTGATT-3'). These primers should amplify a specific 0.9 kb product if ß-galactosidase is expressed. The ß-actin control RT-PCR was made using the primer pair mouse ß-actin (5'-ATGGATGACGATATCGCTG-3') and mouse ß-actinc (5'-ATGAGGTAGTCTGTCAGGT-3') (Wei *et al.*, 1997) which amplify a specific 569 bp product (indicated in figure b as 0.6 kb).

b) Total RNA was isolated from various MMTVProCon transgenic mice organs. After treatment with RNase free DNase and subsequent phenol extraction 1µg RNA was used per RT-PCR reaction (Titan RT-PCR Kit, Roche). PCR conditions were as recommended in the manufacturers instructions (see 2.2.5.2.2). The RT-PCR products were then separated over a 1.5% agarose gel. The expected 0.9 kb band can be seen in the mammary gland as well as the spleen. In the control RT-PCR (without RT activity) the bands cannot be seen, indicating that they originate from RNA rather that a DNA contamination.

Line	32									
Animal	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Ovary	-	+	+	+	+	-	-	+	-	-
Liver	-	-	-	-	-	-	-	-	-	-
Mammary Gland	+	+	+	+	-	+	+	+	+	+
Lung	-	-	-	-	-	-	-	-	-	-
Heart	-	-	-	-	-	-	-	-	-	-
Spleen	+	+	+	+	-	+	-	+	-	+
Kidney	-	+	+/-	-	-	-	-	-	-	-
Brain	-	+	-	+	+	-	-	-	-	-

Table 3.4: Expression Pattern of the MMTVProCon in Transgenic Animals
Expression was mainly seen in the mammary gland and spleen, although some ectopic expression could be detected in other organs.

3.1.13.2 Northern Blot Analysis of MMTVProCon Transgenic Mice

To see the length of the transcript, northern blot analyses were performed used. RNA was isolated from various organs and 20 µg from each organ was run over a denaturing formaldehyde gel (see 2.2.2.2.2). After being transferred via capillary blot to a nylon membrane the fixed RNA was hybridised to a radioactively labelled 1.2 kb ß-galactosidase fragment. A 7 kb band, corresponding to the full length RNA transcript from the MMTVProCon hybridising to the ß-galactosidase probe could clearly be seen in both spleen and mammary gland. This is in concurrence with the results seen in the RT-PCR analysis. After the blot had been documented using a Phosphor Imager (Molecular Dynamics Storm 860), the blot was stripped and reprobed with a radioactively labelled 1.2 kb ß-actin specific fragment. As the levels of ß-actin expression should be the same in all the samples, it was then possible to compare the intensity of the ß-galactosidase expression between samples using the ß-actin levels as a reference.

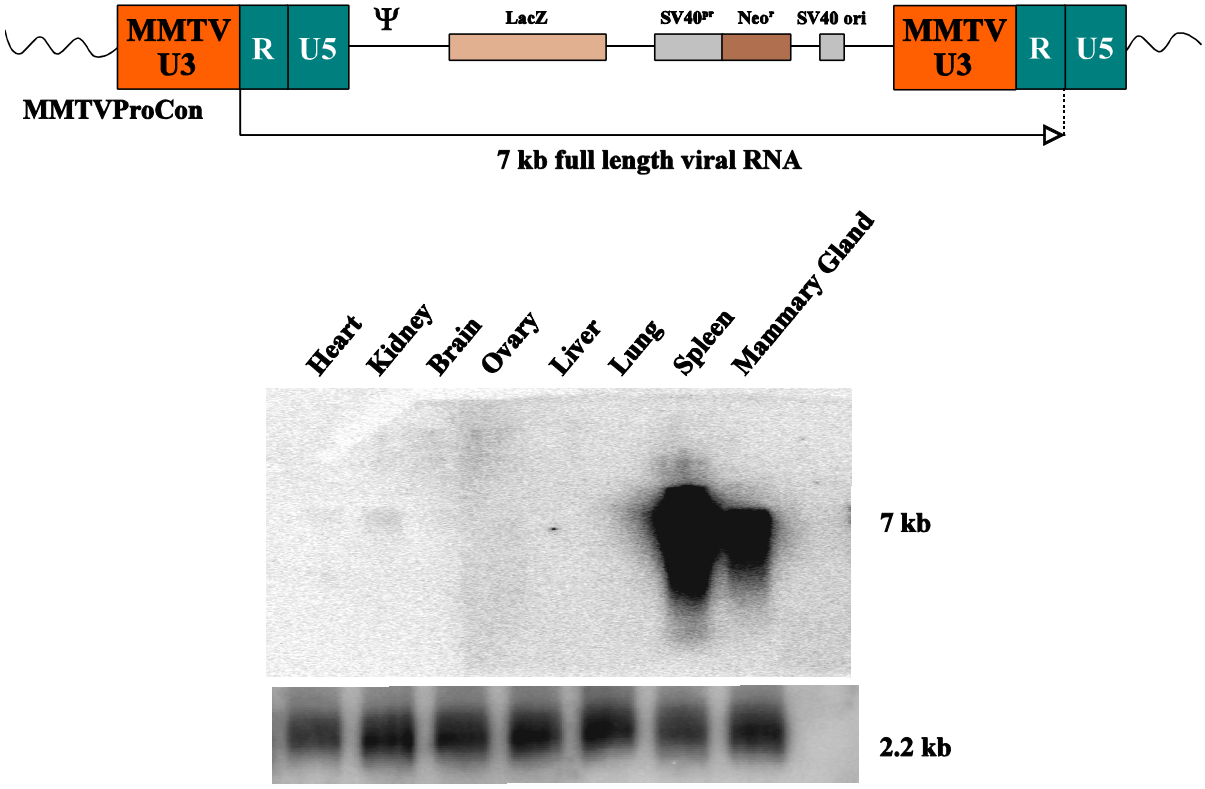

Figure 3.35: Northern Blot Analysis of MMTVProCon Transgenic Mice

20 µg of total RNA from a pregnant mouse was separated on a denaturing formaldehyde gel and subsequently transferred to a nylon membrane via capillary blotting. The membrane was then hybridised to an α^{32}P labelled 1.2 kb ß-galactosidase specific fragment (amplified using the PCR) and, after stripping, re-probed with an α^{32}P labelled 1.2 kb ß-actin fragment (from the plasmid pAL41 after *Pst*I digestion). A 7 kb band, corresponding to expression of the full length MMTVProCon RNA, can be clearly seen in the spleen and mammary gland. Weak expression could also be shown to be present in the kidney and heart.

3.2 Heterologous Cellular Promoter (WAP NRE Region)

The Whey Acidic Protein Negative Regulatory Element (WAP NRE) was chosen as a mammary specific heterologous cellular promoter. The WAP promoter was originally cloned from the genome of rodents and has been used in the context of various transgenes to direct expression of linked sequences preferentially to the mammary gland of pregnant and lactating mice (Hennighausen *et al*., 1990). Until recently, it was thought that the WAP gene is specific for rodents. However, a WAP homologue has recently been found in pigs (Simpson *et al*., 1998). A human homologue has not yet been identified. Nevertheless, it may be expected that the underlying regulatory mechanisms controlling the expression of many, if not all, milk genes are similar among mammals.

It has previously been shown in transgenic mice that both a 2.5 kb as well as a 100 bp fragment of whey acidic protein promoter (Fig. 3.36) can direct expression of human growth hormone (hGH) to the mammary gland (Günzburg *et al*., 1991). In further cell culture studies, a negative regulatory element (NRE) was found in the WAP promoter that limits its' expression (Kolb *et al*., 1994) and a factor binding to the NRE has been characterised (Kolb *et al*., 1995). The ability of this NRE to give expression in the mammary glands of transgenic mice was not tested. However, in this study a 405 bp fragment of the WAP promoter, containing the NRE, was analysed for mammary specific expression in transgenic mice before the establishment of a modified retroviral vector with this cellular promoter.

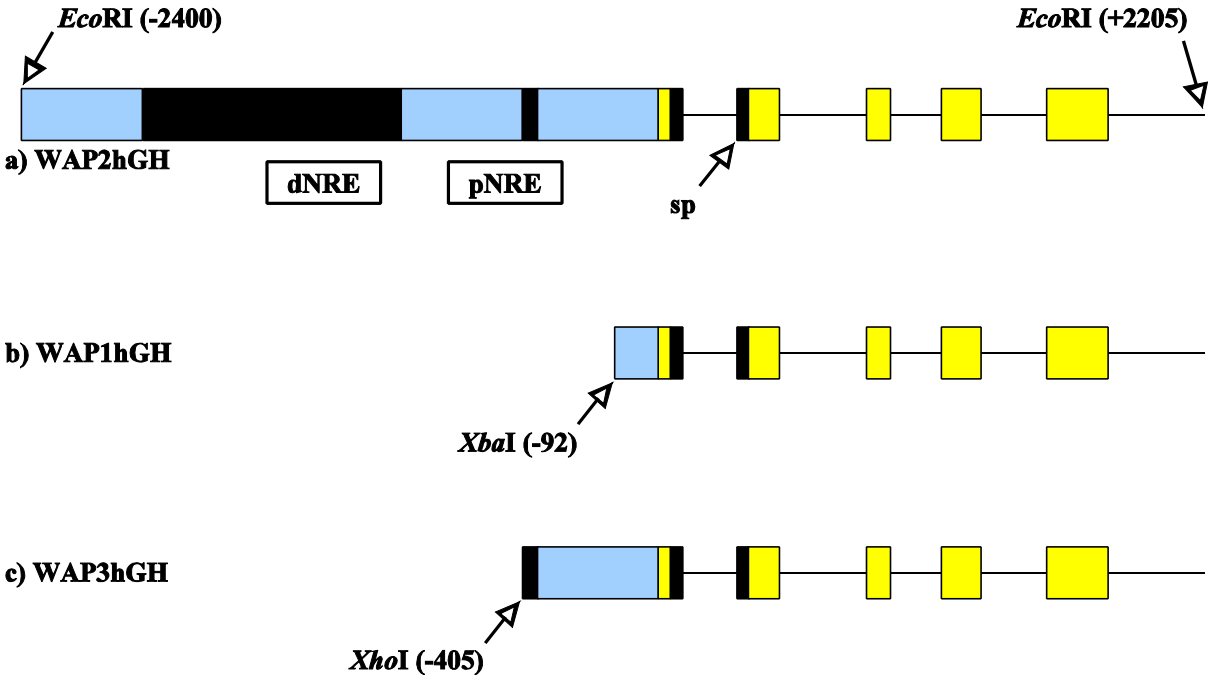

Figure 3.36: WAP Constructs
Schematic diagram showing the linkage of the WAP promoter sequences (blue) to the five exons of the human growth hormone (hGH) gene (yellow). Different lengths of the 5' sequences of the WAP gene were used to direct the expression of hGH in transgenic mice. a) Wap2hGH containing 2.4 kb, b) Wap1hGH containing 110 bp and c) Wap3hGH containing 450 bp of the WAP promoter, including the NRE (Günzburg *et al.,* 1991).

3.2.1 Establishment of Transgenic Mice

The plasmid pWAP3hGH, carrying a 405 bp fragment of the WAP promoter coupled to the human growth hormone gene (hGH), was used in order to establish transgenic mice (in collaboration with the Institute of Animal Breeding and Genetics at the University of Veterinary Sciences in Vienna). The plasmid was first linearised with the restriction enzyme *Eco*RI and purified over an agarose gel. The fragment was then resuspended in TE buffer before being given to the Institute of Animal Breeding and Genetics. There it was introduced via microinjection into freshly fertilised eggs from the mouse strain HIM OF-1 (Fig. 3.37). After a short incubation period of 12 hours the eggs were implanted into foster mice.

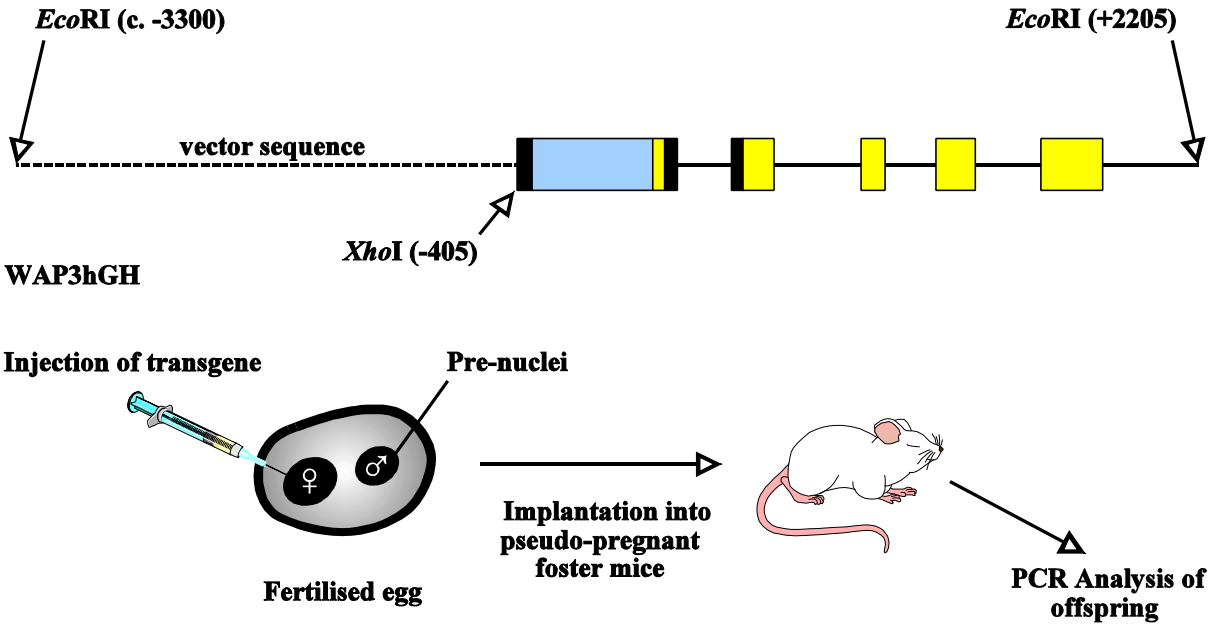

Fig. 3.37: Establishment of Transgenic Mice Using the Plasmid WAP3hGH
The plasmid WAP3hGH that had been linearised with the restriction enzyme *Eco*RI (giving a total size of 6.77 kb) was taken and, after purification following agarose gel electrophoresis, was microinjected into previously fertilised mouse oocytes. After 12 hours the microinjected eggs were implanted into foster mice.

3.2.1.1 DNA Analysis of Transgenic Mice

16 founder mice (F_0) were produced by the Institute of Animal Breeding and Genetics after implantation of microinjected oocytes. When the mice had reached the age of 7 weeks tail clips were provided from these founders. Genomic DNA was isolated from the provided material (see 2.2.2.1.3) and examined for the presence of WAP3hGH sequences using PCR (see 2.2.5.1.1). One primer binding in the WAP promoter (WAPprom3) and another which binds to exon 3 of the hGH gene (hGHex3) were used for this purpose (Fig. 3.38a). Genomic DNA that had been isolated from a non-transgenic mouse was used to show that the primers being used did not amplify a non-specific product.

This DNA analysis showed that 2 female mice (Fig. 3.38b) and 1 male founder mouse (not shown) were positive for the transgene (Table 3.5). These positive mice were then used for further breeding experiments. Two separate F_1 lines were then provided by the Institute of Animal Breeding and Genetics and were kept from then onwards in the animal facility in the

Institute of Virology. The F_1 generation was analysed in a similar manner to the F_0 founders. This analysis showed that the F_0 mice had been able to transfer the transgene to the F_1 generation. 36 positive F_1 animals were identified (Table 3.5) after breeding further with 2 F_0 transgenic lines (53 and 54). Offspring arising after breeding of these two lines were then used in further experiments.

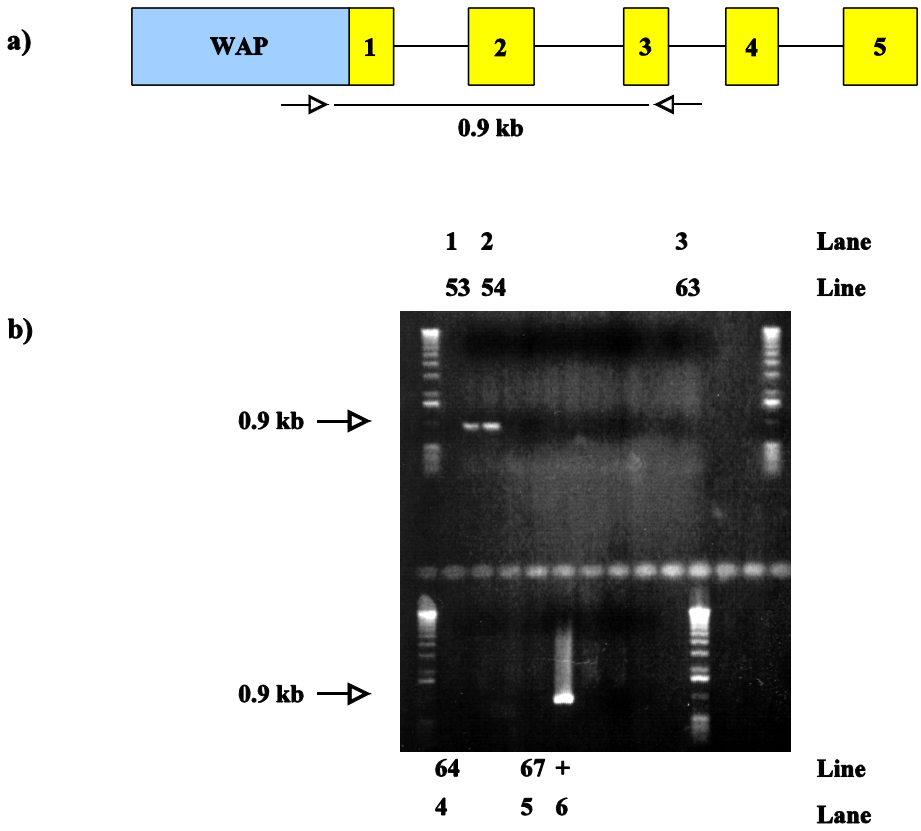

Figure 3. 38: DNA Analysis of Transgenic Mice

a) Genomic DNA was isolated from the tail clips of transgenic mice. 1 µg of DNA was used per PCR reaction (see 2.2.5.1.1). WAP promoter specific and hGH exon 3 specific, primers were used to show the presence of the transgene: WAPprom3 (5'-GTGTGGCCAAGAAGGAAGTG-3') and hGHex3 (5'-GGTGTCGGAATAGACTCTGA-3'). These primers should give a 921 bp product if the transgene is present. 1ng of the plasmid WAP3hGH was used as a positive control.

b) 16 founder F_0 mice were analysed. 3 mice were found to be positive: lane 1, transgenic mouse line 53: lane 2, trangenic mouse line 54. The other lanes with transgenic mouse lines 55 to 67 were found to be negative. The positive male founder line 22 is not shown in this PCR. Genomic DNA from a non-transgenic mouse can be seen in the lane next to the positive control (1 ng of the plasmid WAP3hGH).

F_0	53		54		22
F_1	+	-	+	-	not further bred
	8♀	8♀	14♀	6♀	
	7♂	12♂	7♂	10♂	

Table 3.5: Analysis of the F_1 Generation of WAP3hGH Transgenic Mice
The analysis of the genomic DNA was made in a similar manner to the analysis of the F_0 generation. The lines 53 and 54 were provided for analysis. Line 53 showed a total of 15 positive (8 female, 7 male) and 20 negative mice (8 female, 12 male). Line 54 showed a total of 21 positive (14 female, 7 male) and 16 negative mice (6 female, 10 male).

3.2.1.2 Southern Blot Analysis of Transgenic Mice

Southern blot was then used to investigate the copy number and integration pattern of the transgene present. Genomic DNA was isolated from 2 transgenic mice (see 2.2.2.1.2) from both lines 53 and 54. The genomic DNA was first digested (see 2.2.4.1) with the restriction enzyme *Bgl*II (Fig. 3.39a). This enzyme cleaves only once in the WAP3hGH plasmid. After separation of the digested DNA using agarose gel electrophoresis, the DNA was transferred to a nylon membrane (see 2.2.7.1) and then hybridised using a radioactively labelled probe specific to the hGH gene (see Fig. 3.39a). The use of an hGH specific probe allows both the copy number and pattern of integration to easily be shown.

Fragments of approximately 6.5 and 5.5 kb can be seen in mouse 53/33 and fragments of approximately 11 and 5.5 kb can be seen in mouse 54/9. This indicates that 2 different sites of integration may be present (Fig. 3.39b). After examining the intensity of the hybridised bands it was hypothesised that multiple head to tail or head to head integrations had taken place, which results in the strong 5.5 kb band (which also had the same size as the plasmid control). This could happen when, after the linearisation of the plasmid with a restriction enzyme, the fragments form concatamers before being incorporated into the genetic information of the fertilised ooyctes (Fig. 3.40). The other, weaker, bands present are the result of different integration sites of non-concatameric origin. These bands, comprising chimeras of transgene and flanking mouse DNA, also hybridise to the specific probe used. The hybridised blot was then analysed using a Phosphor Imager (Molecular Dynamics Storm 860). Using the Fragment™ software it was possible to compare the strength of the bands with know DNA controls in order to estimate the copy number. Line 53 was calculated to have 71 copies whereas line 54 had 155 copies.

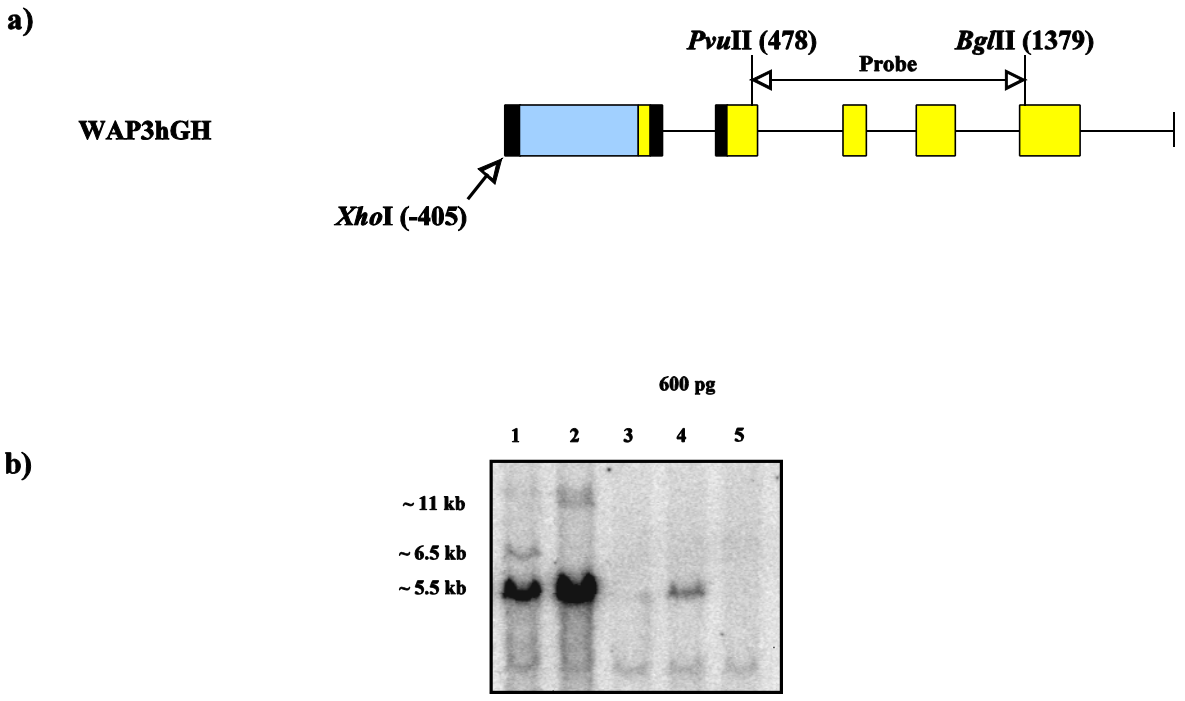

Figure 3.39: Southern Blot Hybridisation of WAP3hGH Transgenic Mice

a) Diagram of the linearised plasmid pWAP3hGH. 20 µg of genomic DNA was digested with the restriction enzyme *Bgl*II (see 2.2.4.1) which cuts once only in the vector.

b) After agarose gel electrophoresis the DNA was transferred to a nylon membrane before being hybridised to a radioactively labelled 0.9 kb hGH specific probe. The hGH probe was prepared by digesting pWAP3hGH with the restriction enzymes *Bgl*II and *Pvu*II. Lane 1: DNA from transgenic mouse 53/33, lane 2: DNA from transgenic mouse 54/9, lane 3: DNA from the non-transgenic mouse. In lanes 4 and 5 DNA from the non-transgenic mouse had been mixed with 600 pg or 60 pg of pWAP3hGH plasmid DNA that had also been digested with the restriction enzyme *Bgl*II before loading. The lane 4 was used both as a positive control and also as a standard for the quantification of the copy number. The intensity of the signal in lane 4 (600 pg) was taken as amounting to the signal that would arise from 10 integrated copies. The blot was analysed using a Phosphor Imager (Molecular Dynamics Storm 860) using the program Fragment™. This program measures the intensity of the hybridised bands and then compares them to the control band in lane 6 (600 pg). Line 53 was calculated to have 71 copies whereas line 54 had 155 copies.

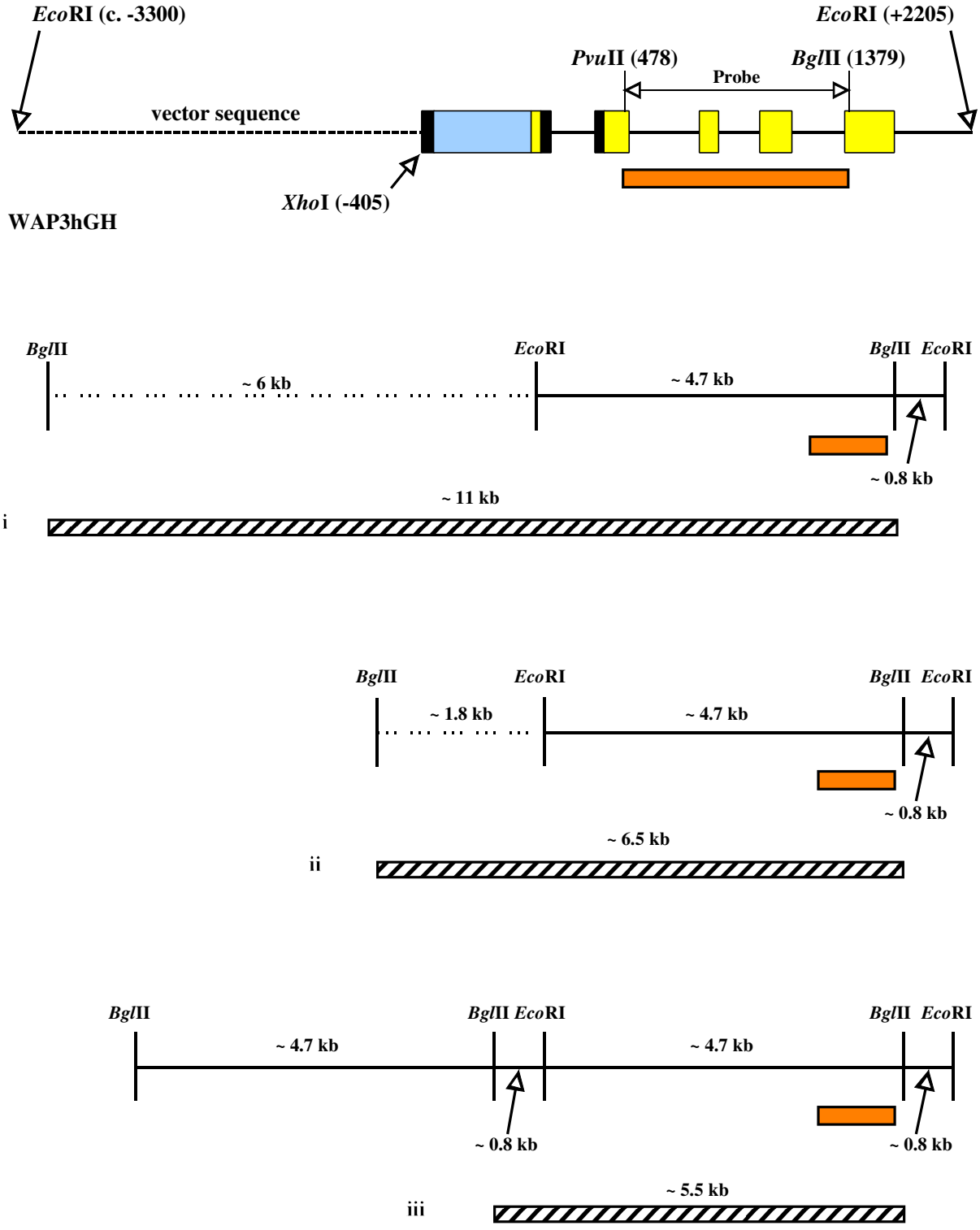

Figure 3.40:Integration Pattern

Integration sites of WAP3hGH transgenic mice. i) If the next *Bgl*II site is situated ~ 6 kb upstream of the integrated construct then a hybridised band of ~ 11 kb would arise. ii) If, however, the distance was only 1.8 kb upstream, the resulting band would have a size of ~ 6.5 kb. iii) If the construct were to form a head to tail concatamer before incorporation into the genome a 5.5 kb band would arise.

3.2.1.3 Expression Analysis of WAP3hGH Transgenic Mice

The next step was to examine the expression pattern in several organs of the WAP3hGH transgenic mice. Total RNA was isolated (see 2.2.2.2.2) from both transgenic and non-transgenic mice and used for both RT-PCR (see 2.2.5.2.2) as well as quantitative real time RT-PCR analysis (see 2.2.5.2.3).

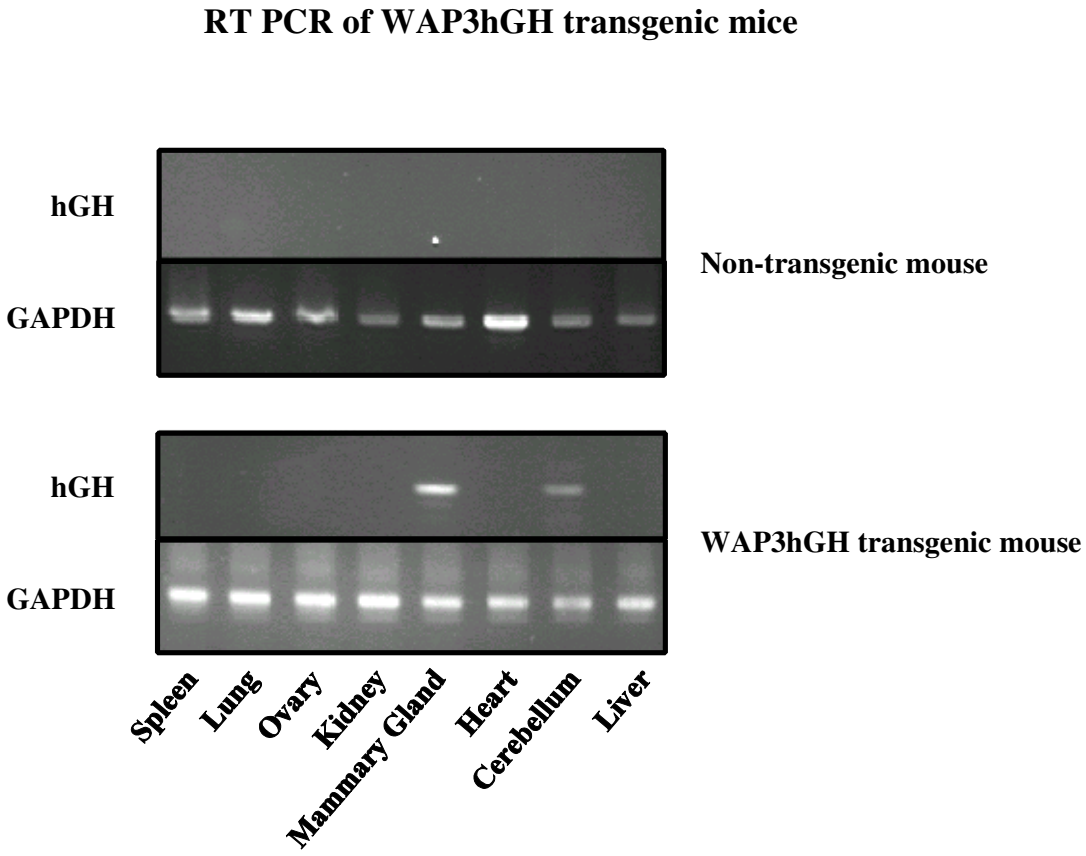

Figure 3.41: RT-PCR Analysis of WAP3hGH Transgenic Mice
Total RNA was isolated from different organs from both a pregnant transgenic (Line 54/26) as well as a non-transgenic mouse. Two RT-PCR (2.2.5.2.2) reactions were performed: the first using primers specific for hGH RNA binding in exon 2 (5'-TCCCTGCTCCTGGCTTTT-3') and in exon 5 (5'-ACAGAGCGGCACTGCACGAT-3') and the second using GAPDH specific primers (mouse-gapdh, 5'-TTGTGCAGTGCCAGCCTCGT-3' (Cheng *et al.*, 1997) and mouse-gapdhc 5'-GGATGACCTTGCCCACAGCCT-3 (intron spanned, made in our lab) as an internal control. hGH expression could only be observed in the mammary gland and cerebellum of the female transgenic mouse. No expression was detected in any other organs or anywhere in the non-transgenic animal.

Mammary gland and cerebellum both showed expression of hGH whereas other organs of the transgenic mice did not show detectable hGH expression.

Organs that tested positive in the RT-PCR (see Fig. 3.41) were then examined in the ABI Prism 7700 sequencing detector system (Perkin Elmer) using a quantitative real time RT-PCR assay. Here two male transgenic mice were also examined even though its' organs were seen to be negative in the RT-PCR. As the real time RT-PCR is a very sensitive method this would allow even very low levels of expression to be detected. This assay is based upon the 5' nuclease activity of the Taq polymerase acting on a Taq Man^{TM} fluorogenic probe. The RNA was diluted

1:10 and 1:1000 before use. Two different reactions were made, the first with hGH specific primers and probes and the second with internal primers and a probe specific to rRNA.

Due to the method of quantification in this assay a high threshold level was set. This means that only those samples with very high expression levels appear to be positive in figure 3.42. In examined mammary tissue, expression could be detected in all samples from pregnant mice and some of the non-pregnant mice whereas neither the male (lanes 11 and 12) nor non-transgenic (lane 13) samples showed any expression. When the cerebellum was tested, expression could be seen once again in the pregnant mice, with lower levels seen in the non-pregnant mice compared to the breast samples. Once again, no expression could be seen in either the male (lanes 24 and 25) or the non-transgenic mice (lane 26).

This shows that the shortened WAP promoter, which retains the NRE, has not lost its' mammary specificity. The expression seen in the cerebellum has previously been described (Günzburg *et al*., 1991) and was thus to be expected.

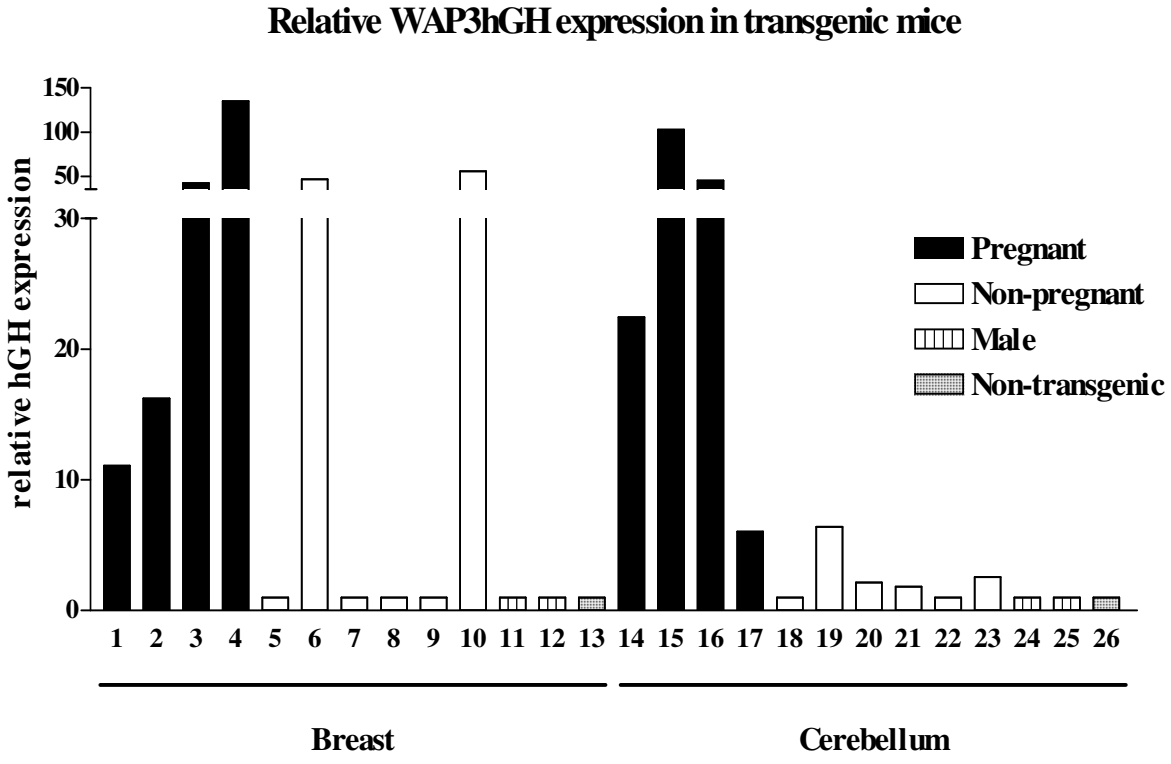

Figure 3. 42: Relative WAP3hGH Expression in Transgenic Mice

RNA samples from the mammary gland and cerebellum of female pregnant and non-pregnant WAP3hGH transgenic mice as well as male WAP3hGH transgenic mice and female non-transgenic mice were tested for their hGH expression using the ABI Prism 7700 real time RT-PCR system. Expression in mammary tissue samples could be seen only in pregnant (lanes 1, 2, 3 and 4) and non-pregnant (lanes 6 to 10) mice. No expression was seen in either male transgenic or female non-transgenic animals (lanes 11,12 and 13). In the cerebellum, hGH expression could again be seen in all pregnant mice (lanes 14, 15, 16 and 17) with lower levels seen in the non-lactating samples (lanes 19, 20, 21 and 23). Again no expression could be seen in either male transgenic or female non-transgenic animals (lanes 24, 25 and 26).

3.2.2 S1-Analysis of Endogenous WAP Expression in Murine Mammary Tumours

Although the WAP promoter has been described as being mammary specific, it was not known whether the promoter is active in mammary tumours. To investigate whether the endogenous WAP promoter is activ in mouse mammary tumours from the GR inbred mouse strain, S1 analysis (see 2.2.5.2.1) were performed.

Total RNA was prepared from mammary mouse tumours either lactating or non-lactating GR mice. RNA from mammary tissue of a pregnant mouse was also used in the S1 analysis, since it is known to express WAP (Hennighausen *et al*., 1990). RNA from the liver of the same mouse was also subjected to S1 analysis as no expression would be expected here. 40 µg of total RNA was hybridised to a γ^{32}P-end-labelled *Bbs*I DNA fragment of the WAP gene. The probe should protect a 98 bp fragment after S1 digestion (Fig. 3.42).

A clear expression of the WAP gene could be observed in RNA derived from all breast tumours in both lactating and non-lactating mice, as well as in breast tissue from pregnant mice. No expression was seen in the liver as expected (Fig. 3.42). RNA from GR cells was taken and, in parallel, was hybridised to a γ^{32}P-end-labelled *BstE*I DNA fragment of the MMTV LTR in order to verify that the S1 analysis had worked and showed the expected protected fragment of 110 nucleotides (Fig. 3.42).

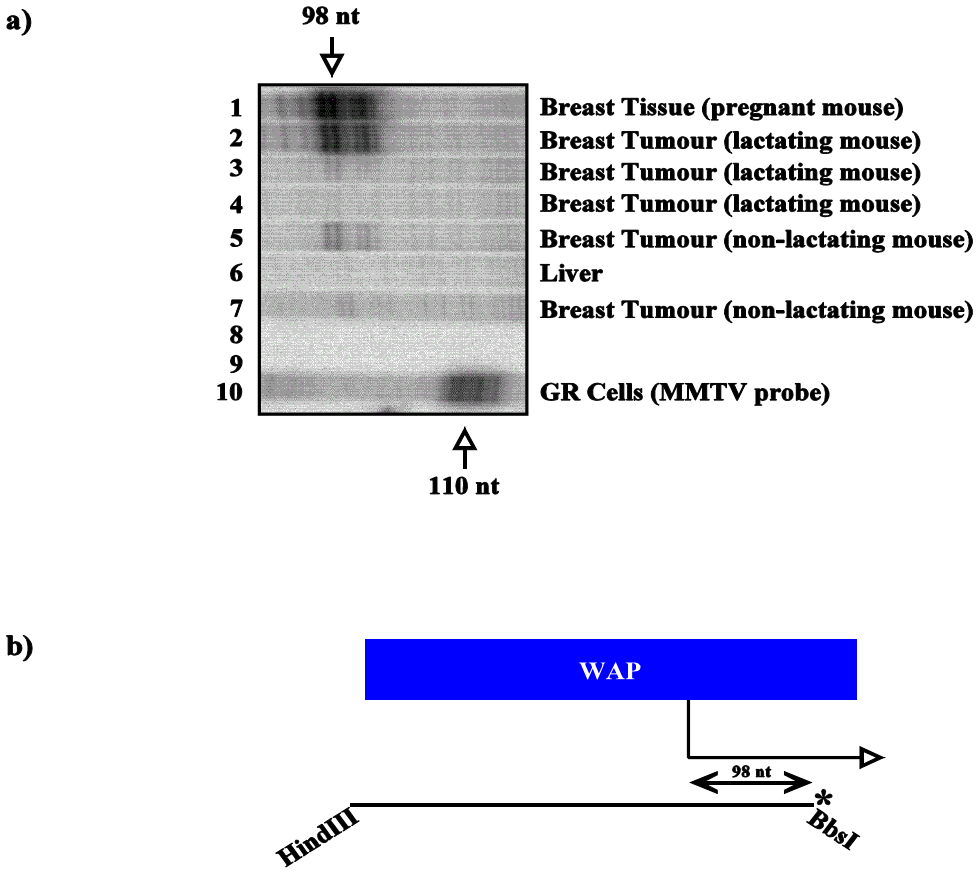

Figure 3.43: S1 Nuclease Protection Analysis

a) Total RNA was isolated from tumours from both lactating and non-lactating GR mice. RNA isolated from the mammary tissue of a lactating mouse was used as a positive control and RNA isolated from the liver of the same mouse used as the negative control. 40 µg total RNA was used per reaction and was hybridised against a γ^{32}P-end-labelled specific fragment (see. 2.2.5.2.1). After S1 digestion, a WAP specific protected fragment of 98 nucleotides in tumours or an MMTV specific fragment of 110 nucleotides in GR cells is expected. A signal could be seen in the RNA of tumours from both lactating (lanes 2 and 3) and non-lactating mice (lanes 5 and 7). However, comparison of the levels of expression could not be made as internal controls, which allow equal loading of the lanes to be verified, were not performed in this instance. No signal was observed from the RNA derived from liver of a lactating mouse (lane 6). In lane 9 the 110 nucleotide fragment from GR cells can clearly be seen, indicating that the S1 analysis had worked without technical problems. *Hae*III-digested pBR322 was used as a marker.

b) Diagram of the S1 probe used. A WAP specific probe was isolated after a *Bbs*I digest of the plasmid puc19-WAP, which contains a 649 bp fragment of the WAP promoter and gene. The protected fragment after S1 digestion was 98 nucleotides. The MMTV specific probe not shown (kindly provided by S. Wintersperger) protects a fragment of 110 nucleotides after S1 digestion.

3.2.3 Construction of Modified Retroviral Vectors

The retroviral vectors BAG (Price *et al*., 1987) and LXSN (Miller and Rosman, 1989) were used as the basis for construction of the hybrid retroviral vectors as described in 3.1.2.

3.1.17.1 Construction of the Hybrid Retroviral Vector WAP-BAGgal

pCon6 was also used for the construction of the WAP-BAGgal vector. It is a derivate of pBAG, where the 3' U3 region of the 3' LTR has been replaced by a polylinker (Saller, 1994). The inverted repeat (IR) remains intact, as it is necessary for integration. The polylinker facilitates the incorporation of heterologous promoters into the U3 region of the 3'LTR. The strategy used ensured that the inverted repeat at the 5' border of the U3 region was left intact and also that unique *Sac*II and *Mlu*I sites were inserted to facilitate the introduction of the WAP NRE. The complete 405 bp WAP NRE region was isolated by PCR using specific primers carrying *Sac*II or *Mlu*I restriction enzyme cleavage sites and inserted into the pCON6 plasmid (Fig. 3.45). The resulting plasmid was named pWAP-BAGgal. In the majority of the following experiments pWAP-BAGgal was used as the presence of a prokaryotic origin of replication in the vector allows recloning of integrated proviruses for later molecular characterisation.

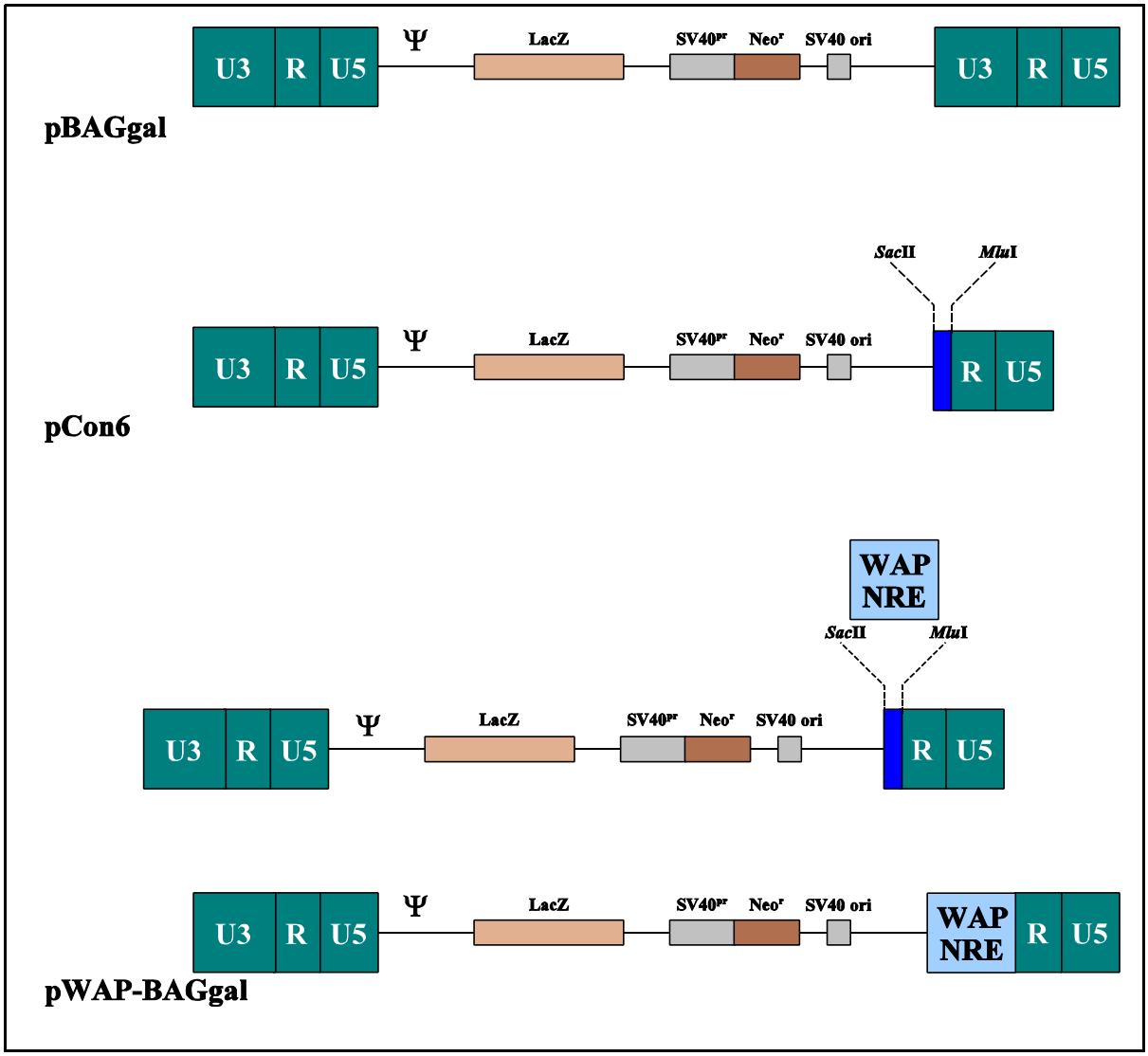

Figure 3.45: Construction of pWAP-BAGgal
The complete U3 region except the inverted repeat (IR) of the 3' LTR within the BAG vector was deleted by a PCR mediated approach. This gave the plasmid pCON6 (Saller, 1994). A 405 bp WAP NRE fragment was amplified by PCR using the plasmid pWAP2hGH as the template and primers carrying either an *Mlu*I or *Sac*II extension. The product was digested with *Sac*II and *Mlu*I and ligated to the *Sac*II/*Mlu*I digested pCON6 to give the plasmid pWAP- BAGgal (8377 bp), in which the ß-galactosidase (lacZ) gene is under the transcriptional control of the MLV promoter after transfection.

In order to construct the plasmid pLXPCWAPEGFP, pLXPCMMTVEGFP was used. The pLXPCMMTVEGFP was digested with the restriction enzymes *Sac*II and *Mlu*I and ligated with 405 bp WAP NRE fragment, which was isolated from pWAP- BAGgal after digestion with the restriction enzymes *Sac*II and *Mlu*I (Fig. 3.46). This plasmid was used to produce recombinant virus for infection experiments in which the quantification of expression was not required.

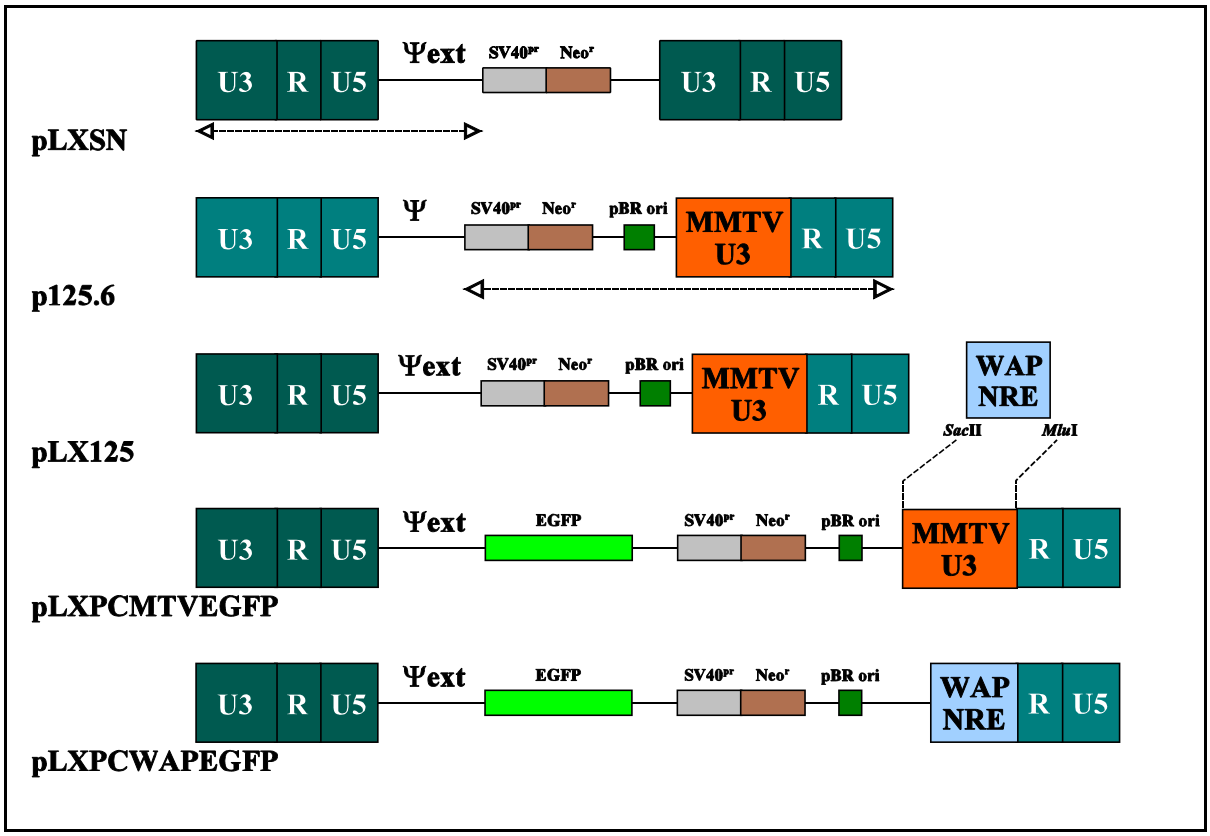

Figure 3.46: Construction of pLXPCWAPEGFP
pLXPCMMTVEGFP was digested with the enzymes *Mlu*II and *Sac*II and a 7460 bp fragment, containing the vector with exception U3 region of 3' LTR, isolated. The 405 bp WAP NRE fragment was isolated from pWAP-BAGgal with the restriction enzymes *Mlu*II and *Sac*II and ligated to the 7460 bp fragment of pLXPCMMTVEGFP. The resulting 8047 bp long plasmid was named pLXPCWAPEGFP.

3.2.4 Production of Recombinant Retroviral Particles

To produce recombinant virus particles either the hybrid pWAP-BAGgal, pBAGgal, pLXSNEGFP or the hybrid pLXPCWAPEGFP vector was introduced by lipofection (see 2.4.7.2) into the amphotropic packaging cell line PA317 (Miller and Buttimore, 1986). Recombinant viruses produced from these cells are capable of infecting human, mouse and other mammalian cell types. PA317 cells have been engineered to produce the *gag*, *pol* and *env* proteins of an amphotropic MLV and, after the introduction of a retroviral vector, only make replication defective vector particles capable of introducing their genes into target cells. The PA317 packaging cell line, in addition to a deletion in the Ψ region, lacks part of the 5'LTR and the 3' LTR has been replaced with the polyadenylation signal from SV40. Two recombination events between the retroviral vector and the so-called packaging construct are necessary to generate replication competent virus.

Transfected PA317 cells were selected in medium containing G418, an anolog of neomycin (Neo^r) (see 2.4.6.1). When the non-transfected cells had died, both clones and populations of stable, virus-producing cells could be obtained. After transfection, the viral RNA is under the control of the MLV promoter in the 5' LTR. The transfection efficiencies can be seen in table

3.6, with no significant difference between all vectors investigated. The supernatant from these stable virus-producing cells could then be used for the infection of several cell lines (Fig. 3.47).

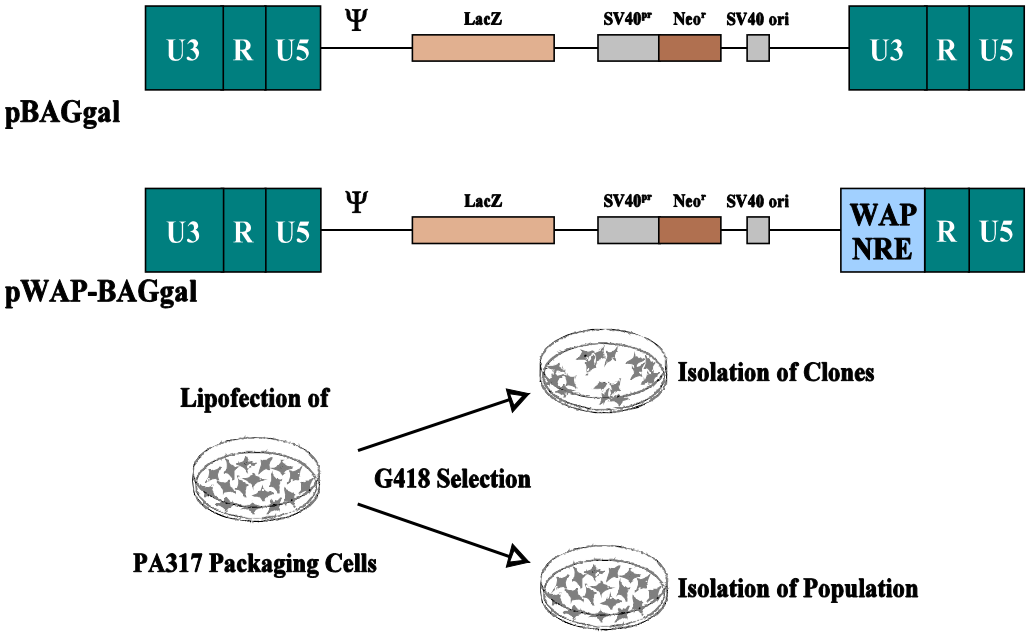

Figure 3.47: Production of Recombinant Retroviral Particles
pBAGgal, pWAP-BAGgal, pLXSNEGFP and pLXPCWAPEGFP were introduced separately by lipofection into the PA317 packaging cell line. After neomycin selection (Neo^r) stable virus-producing cells were established. The transcription of the viral RNA is under the control of MLV U3 region in the 5' LTR. The virus present in the supernatant of these cells is capable of infecting all types of mammalian cells.

Construct	Transfection Efficiency
pBAGgal	420
pWAP-BAGgal	350
pLXSNEGFP	460
pLXPCWAPEGFP	320

Table 3.6:The Efficiency of Lipofection
2 µg of plasmid DNA was used for the lipofection of $5x10^5$ PA317 cells (see 2.4.7.2). Transfected cells were selected (See 2.4.6.1) on neomycin (G418, 400 µg/ml). After two weeks the mock control (untransfected cells) was observed to be dead. Colonies could be counted in the transfected cells and the transfection efficiency calculated. The efficiency of lipofection is shown as colonies per µg transfected plasmid DNA.

3.2.5 Effect of U3 Region Replacement on Viral Titre

After establishment of the infection system, it was important to investigate if there was any virus production and, if so, to know if the replacement of the 3' U3 region with the cellular promoter WAP NRE had any effect on virus production and infection efficiency. Therefore, the infectivity of the virus and the titre were examined.

Initially, viruses harvested from either a population of WAP-BAGgal or BAGgal virus-producing cells were used to determine retroviral titre. The supernatant was assayed for the ability to confer G418 resistance to infected cells. The most commonly used target cell line is NIH 3T3 cells. This was done to show that the replacement of the U3 region, as expected, does not affect the infection spectrum of viral particles arising from the PA317 packaging cell line. No appreciable difference in titre is expected since the neomycin resistance gene conferring G418 resistance is expressed from the constitutive SV40 promoter in all vectors.

The virus titre was calculated from three independent experiments. 24 hours before infection the medium from the virus-producing cells was changed (see 2.4.8.3.1). The supernatant was filtered (45 µm) and after addition of polybrene (8 µg/ml final concentration) was used to infect several cell lines. 24 hours after infection the target cells were diluted into 10 cm plates and a further 24 hours later G418 was added to the medium. Approximately 14 days later, as soon as the negative control was seen to be dead, the number of resistant colonies was counted (see 2.4.8) and the virus titre calculated as colony forming units per millilitre supernatant used.

The titre of transducing particles obtained from a population of WAP-BAGgal hybrid vector virus-producing cells was slightly reduced in comparison to that obtained from a population of the parental BAGgal vector producing cells when assayed on NIH 3T3 cells. The WAP-BAGgal titre being on average 7.1×10^3 cfu/ml compared to 8.6×10^3 cfu/ml in the parental BAGgal vector (Fig. 3.48a).

The titre of several different virus-producing clones was also investigated. Similar results could be observed here as were seen in the populations, although a clonal variation could, of course, be observed. The BAGgal clones here showed titres of 8×10^3 and 6.5×10^3 cfu/ml compared to the WAP-BAGgal clones which gave titres of 5.2×10^3 and 4.2×10^3 cfu/ml (Fig. 3.48a).

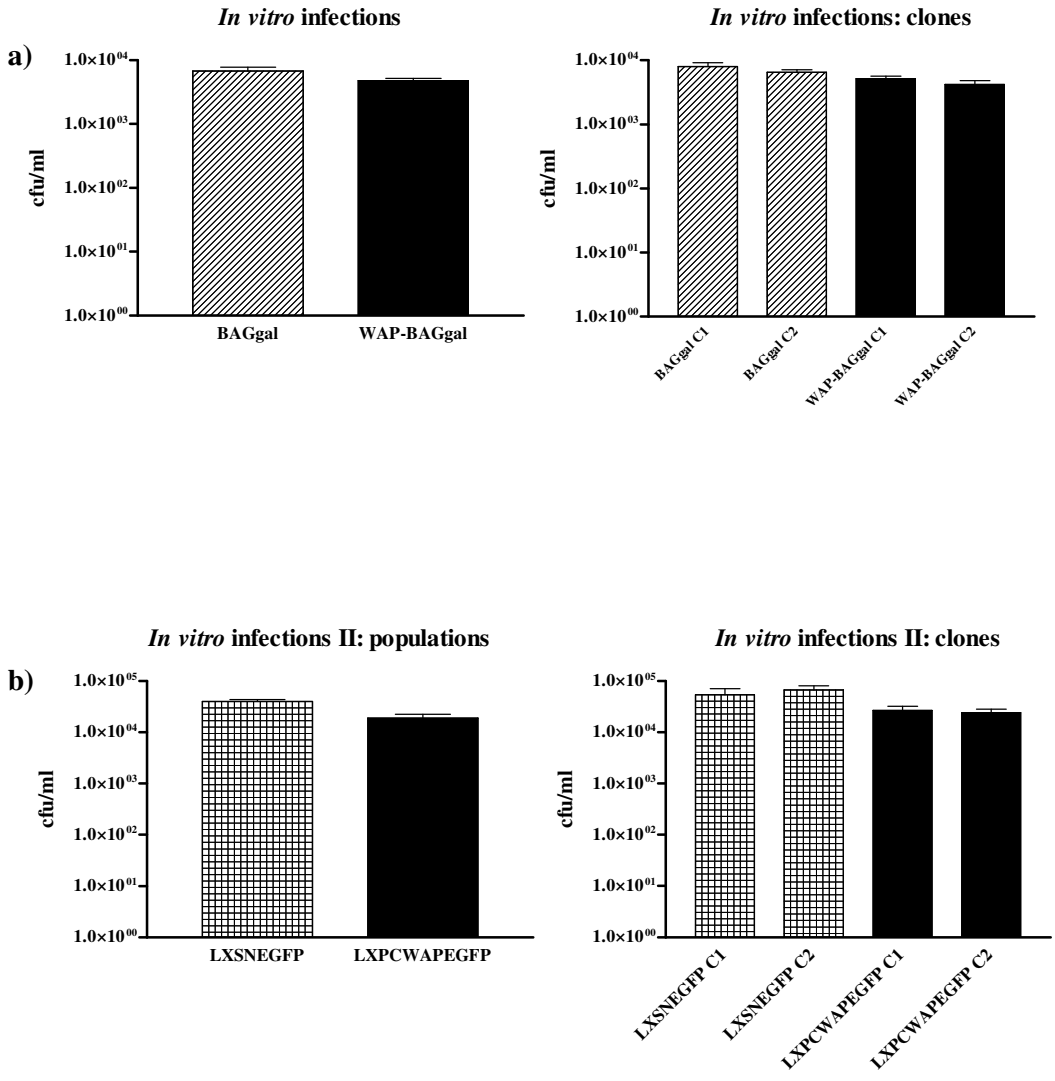

Figure 3.48: Infection of Cell Lines with Hybrid and Parental Retroviral Vectors

a) NIH 3T3 cells were infected with supernatant from either the parental BAGgal retroviral particle producing cell populations (see 2.4.8.3.1) or that from the hybrid WAP-BAGgal population. 24 hours after infection, the NIH 3 T3 cells were diluted into 10 cm plates and a further 24 hours later G418 was added to the medium (NIH 3T3, 400 µg/ml). Approximately 14 days later, as soon as the mock-infected control cells were dead, the surviving colonies were counted and the titre calculated (see 2.4.8) as colony forming units per millilitre infectious medium used (cfu/ml).

b) NIH 3T3 cells were infected with supernatant from either two virus-producing clones of the parental BAGgal retroviral vector or that from two virus-producing clones from the hybrid LXPCWAPEGFP vector. 24 hours after infection, NIH 3T3 cells were diluted into 10 cm plates and a further 24 hours later G418 was added to the medium (400 µg/ml). Approximately 14 days later, as soon as the mock-infected control cells were dead, the surviving colonies were counted and the titre calculated as colony forming units per millilitre infectious medium used (cfu/ml).

After it could be shown that the hybrid retroviral vectors were capable of producing infectious recombinant viral particles and that the replacement of the U3 region did not change the infection spectrum, a second set of infection experiments, using pLXSNEGFP and pLXPCWAPEGFP were also performed on NIH 3T3 cells. In comparison to the BAG based vectors, those arising from pLXSN carry a longer packaging signal and would therefore be expected to have a higher titre. This was actually the case, LXPCWAPEGFP giving a titre of 1.9 x 10^4 cfu/ml compared to the parental LXSNEGFP, which showed 3.5 x 10^4 cfu/ml (Fig. 3.48b). Two virus-producing clones were also investigated and, as seen in the BAGgal vs. WAP-BAGgal experiments, the results obtained mirrored those shown by the populations. Here the LXPCWAPEGFP clones gave titres of 2.7 x 10^4 and 2.4 x 10^4 cfu/ml compared to the LXSNEGFP clones which showed a titre of 5.4 x 10^4 and 6.7 x 10^4 cfu/ml (Fig. 3.48b).

Thus it could be demonstrated that the modification of the U3 region of the 3' LTR had no drastic effect upon viral tire and that, as expected, the pLXSN based vectors had a higher titre that those based upon pBAG, presumably because of the extended packaging signal.

3.2.6 Improvement of Virus Titre

3.2.6.1 Pseudotyping with VSV-G

As mentioned in 3.1.5.1, retroviruses have a restricted host range (Kavanaugh *et al*., 1994). They use specific cell surface proteins as receptors to get into cells and attach to the receptors via their envelope glycoproteins. This interaction is very often species specific, and in some cases even tissue specific. As a result, retroviruses usually have a quite limited host range. However, the retroviral core particles can be pseudotyped with VSV-G envelope protein (Yee *et al*., 1994), which has a notoriously broad range, infecting many cell types and permits the production of high titre retroviral vectors for efficient transgene delivery. The wide host range and high titre of pseudotyped retroviral vectors could be useful for *in vivo* gene therapy applications.

The VSV-G pseudotyped vectors can reach titres of 10^5-10^6 cfu/ml (Yee *et al*., 1994), and by ultracentrifugation can be concentrated to titres of greater than 10^9 cfu/ml. The receptor for VSV-G is a phospholipid (Schlegel *et al*., 1983) that is widespread, thus giving the virus a wide host range (Burns *et al*., 1993).

Unfortunately, the VSV-G protein is toxic to cells in which it is expressed. Pseudotyped vector particles were derived by transient expression of the VSV-G gene after transfection of an expression plasmid into virus-producing cells. The presence of VSV-G in the envelope of pseudotyped particles has no noticeable toxic effect on the cells that they infect.

10 µg of the plasmid pH-CMV.G was transfected into the virus-producing cells 48 hours prior to infection. 24 hours later the cells were washed and fresh medium was added. The production of a functional VSV-G protein could be clearly observed as typical syncytia (multiple nuclei containing cells) were seen under the microscope after transfection. After a further 24 hours, NIH-3T3 cells were infected (Fig. 3.49) and subsequently selected as in previous infection experiments. The results of these experiments are shown in figure 3.50 are take from three independent experiments.

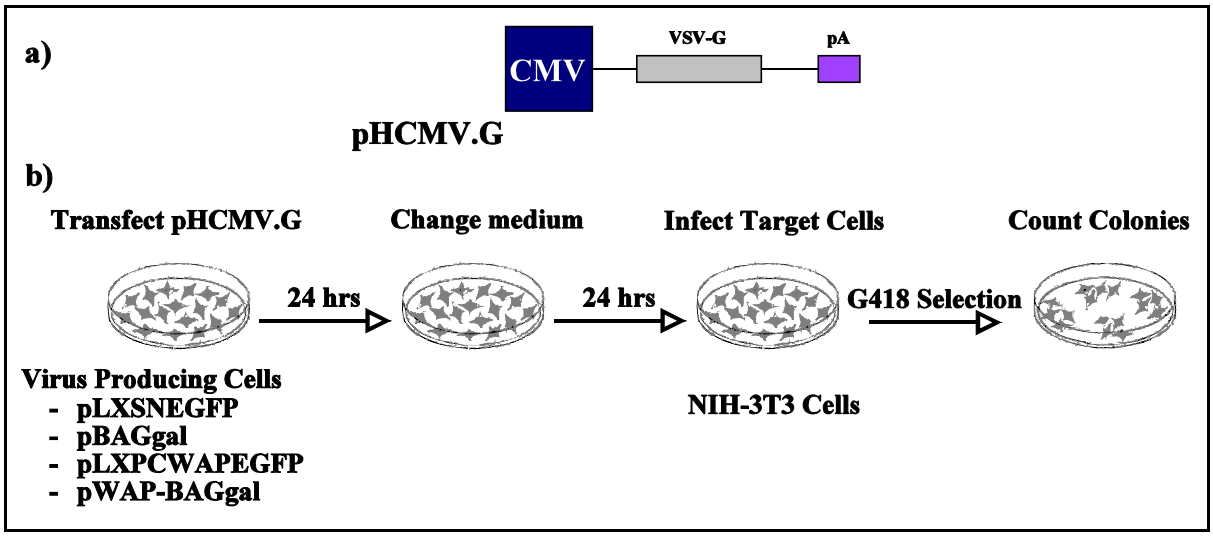

Figure 3.49: Pseudotyping with VSV-G

a) The plasmid pH-CMV.G (courtesy J.C. Burns) carries the gene that codes for the G protein of the vesicular stomatitis virus (VSV) under the transcriptional control of the immediate early promoter of the cytomegalavirus (CMV). In order to select this plasmid in bacteria an ampicillin resistance gene is also present**.** No eukaryotic selection marker is necessary, as a stable cell line expressing VSV-G cannot be made due to the toxicity of the G protein.

b) Supernatant from virus-producing cells that had been transfected with 10 µg pH-CMV.G were used 48 hours later to infect NIH 3T3 cells. A further 48 hours later, the infected cells were put into selective medium containing G418 (400 µg/ml). As soon as the mock-infected cells had died, the surviving colonies in the infected cells were counted and the titre calculated in colony forming units per millilitre (cfu/ml).

Here it can be seen that pseudotyping of recombinant retroviral particles with VSV-G as expected, leads to an increase in titre. The BAGgal titre from a population was seen to increase from 6.6 x 10^3 to 1.6 x 10^4 cfu/ml after addition of VSV-G. Similarly, an increase from 4.1 x 10^3 to 1.1 x 10^4 cfu/ml could be observed in the WAP-BAGgal population (Fig. 3.50a). Two virus-producing clones per construct were also analysed, the results being comparable to those seen in the virus-producing populations tested (Fig. 3.50b).

A second round of experiments was also made, to investigate the virus production arising from the LXSN based WAP vector pLXPCWAPEGFP and to compare it's titre with the parental pLXSNEGFP. The LXPCWAPEGFP titre from a population was seen to rise from 1.9 x 10^4 to 5.3 x 10^4 cfu/ml after addition of VSV-G whereas the titre of the parental pLXSNEGFP was seen to increase from 4.4 x 10^4 to 2 x 10^5 cfu/ml (Fig. 3.51a). Similar results could also be seen in virus-producing clones (Fig. 3.51b).

Here it can be seen that the hybrid WAP vector, irrespective of whether it was BAG or pLXSN based, could be successfully pseudotyped with VSV-G and that the modification of the U3 region of the 3' LTR does not have a drastic effect upon virus titre after the incorporation of a cellular promoter element. It has also been shown that it is possible to concentrate VSV-G pseudotyped vectors and this may be important for a future *in vivo* application. However, in this study it was of primary interest to characterise the modified vectors.

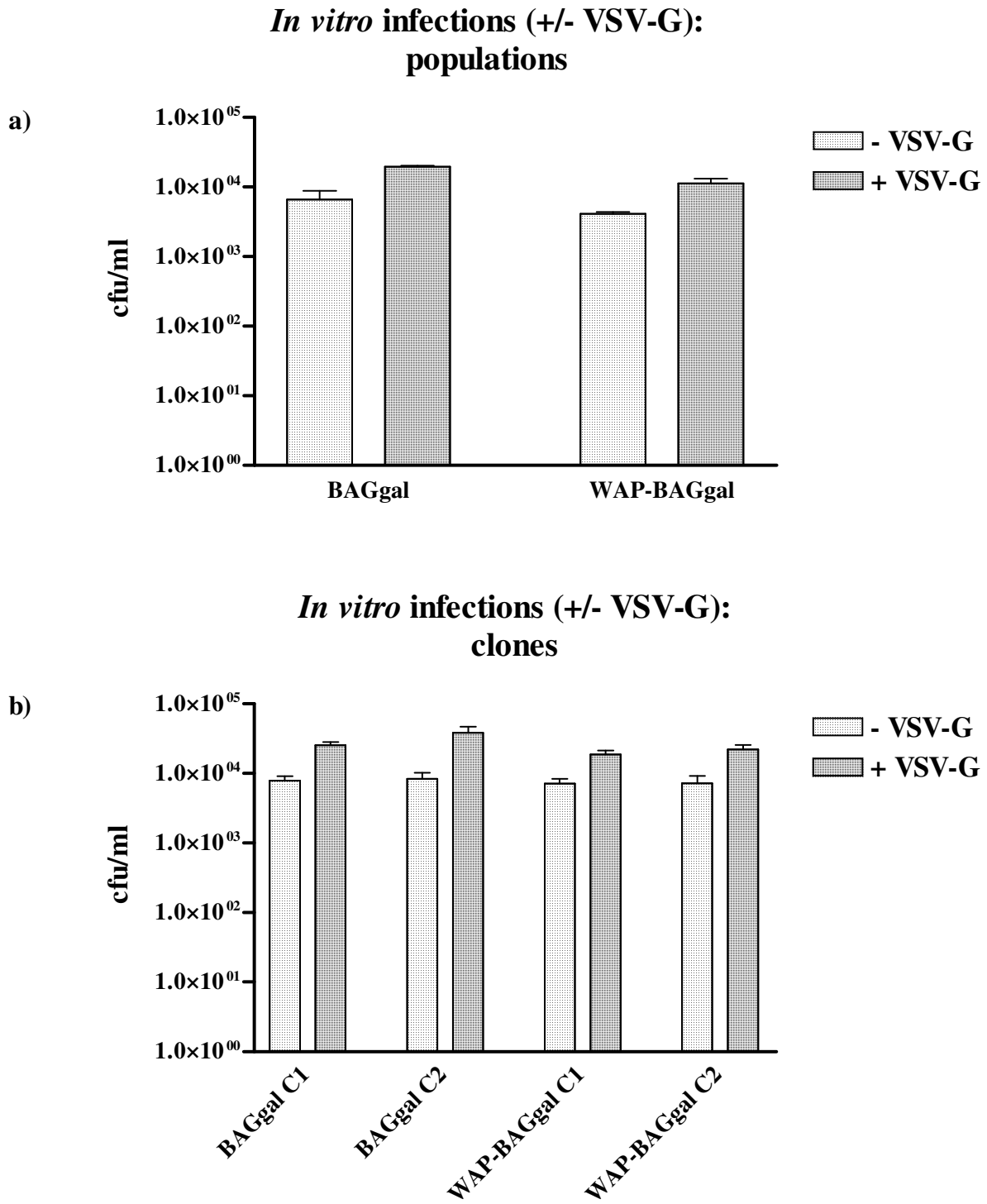

Figure 3.50: Infection of NIH 3T3 Cell with VSV-G Pseudotyped Retroviral Vectors
Infection experiments were performed as described in figure 3.49.
a) Infection of NIH 3T3 cells with the supernatant of populations of either the parental vector BAGgal or the modified vector WAP-BAGgal with or without the addition of the VSV-G protein
b) Infection of NIH 3T3 cells with the supernatant of two clones from either the parental vector BAGgal or the modified vector WAP-BAGgal with or without the addition of the VSV-G protein. The results are shown as the mean of three independent experiments (error bars show the standard error of the mean).

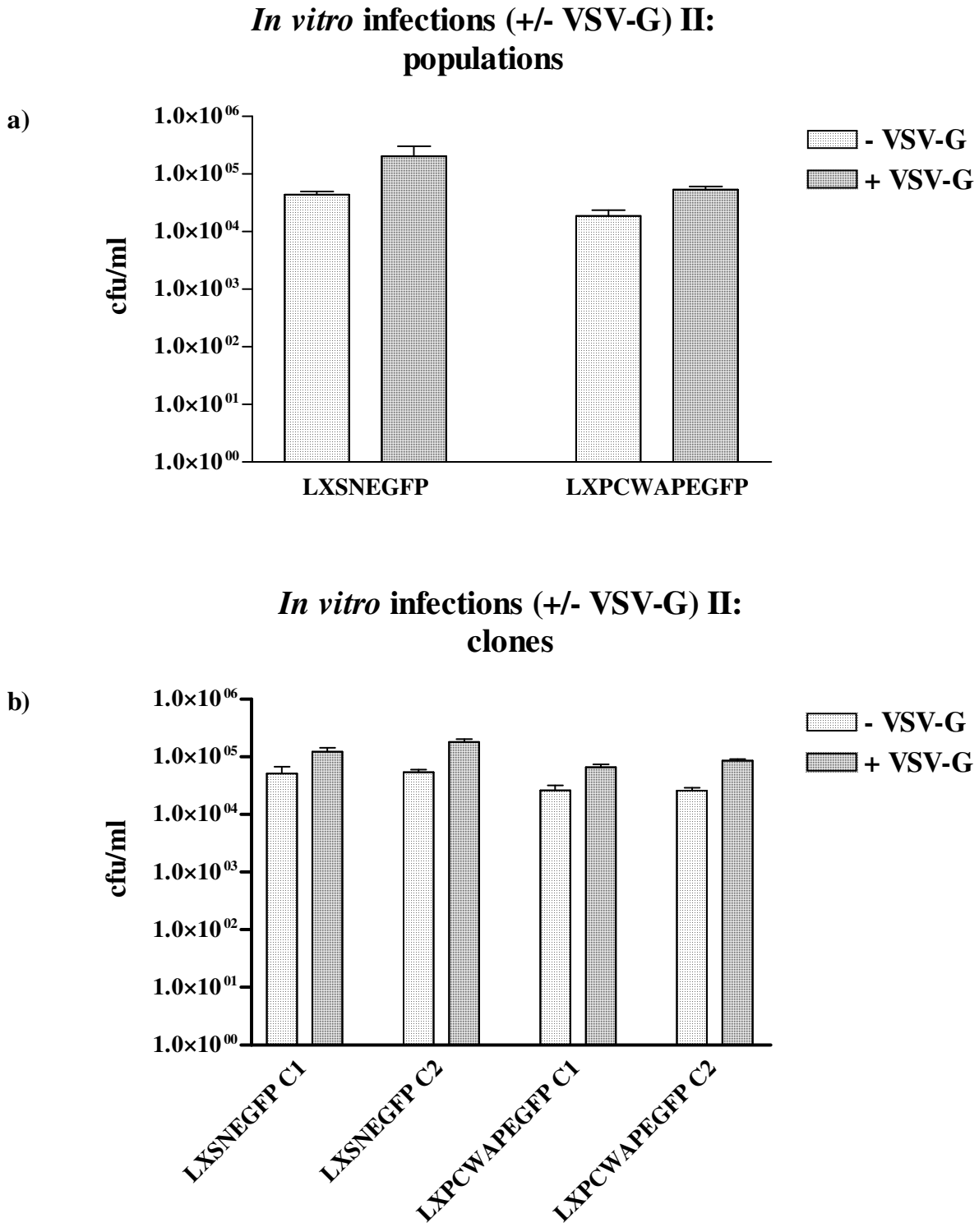

Figure 3.51: Infection of NIH 3T3 Cell with VSV-G Pseudotyped Retroviral Vectors
Infection experiments were performed as described in figure 3.49.
a) Infection of NIH 3T3 cells with the supernatant of populations of either the parental vector LXSNEGFP or the modified vector LXPCWAPEGFP with or without the addition of the VSV-G protein.
b) Infection of NIH 3T3 cells with the supernatant of two clones from either the parental vector LXSNEGFP or the modified vector LXPCWAPEGFP with or without the addition of the VSV-G protein. The results are shown as the mean of three independent experiments (error bars show the the standard error of the mean).

3.2.7 Analysis of Promoter Conversion

In the retroviral life cycle, after infection and during reverse transcription, the U3 region of the 3'LTR is duplicated and one copy is translocated to the 5'LTR where it then controls expression of the structural genes or in the case of retroviral vectors it then controls the reporter gene, in this case ß-galactosidase or EGFP. To confirm that the infected cells had acquired the vector constructs and to verify that the WAP NRE promoter was now present in the 5' LTR of the vector provirus, DNA was isolated from stably infected and non-infected populations of CrFK, T-47D and MCF7 cells and used for both PCR and Southern hybridisation.

Three different primer pairs were chosen for the PCR. Primers specific for either the WAP NRE (P1 and P3) or MLV R (P4) sequences in combination with a second primer complementary to the ß-galactosidase gene (P2) present in all constructs were used for this analysis (Fig. 3.52).

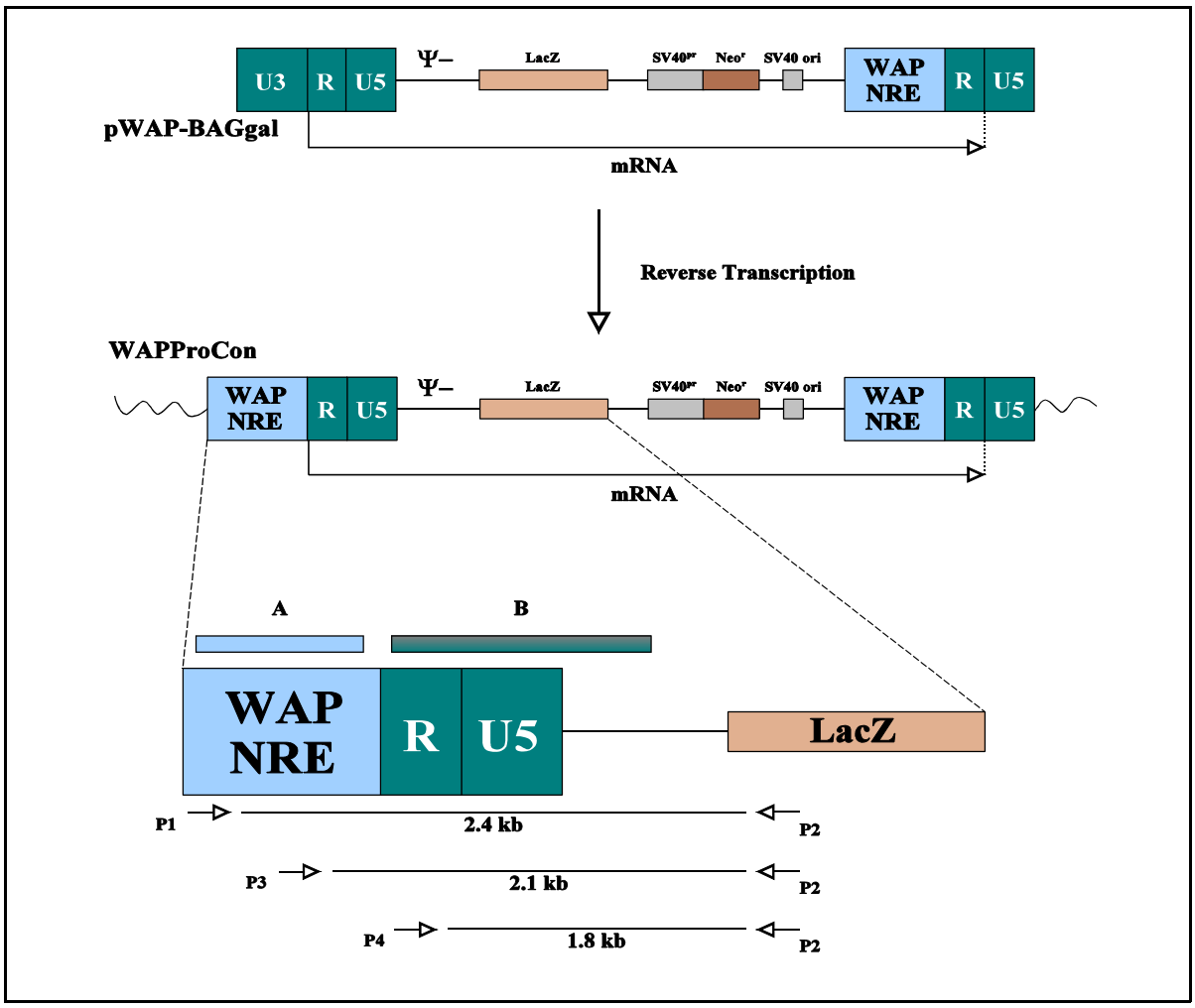

Figure 3.52: PCR Strategy
The position of primers specific for the WAP NRE region (P1, 5'-AGATGTAGCCACGAACTC-3'; P3, 5-GTGTGGCCAAGAAGGAAGTG-3') or MLV R sequences (P4, 5'-GCGCCAGTCCTCCGATTGA-3') together with a primer specific for the ß-galactosidase gene (P2 5'-TTCATCCACCACATACAGGC-3') are shown, as are the expected sizes of the PCR products that can be obtained by using these primers and WAP (labelled fragment A) and MLV (labelled fragment B) probes that can be used to detect them.

After electrophoresis of the PCR products they were transferred to a nylon membrane (see 2.2.5.1.2) and were then hybridised to either a WAP or an MLV specific probe (Fig. 3.52, labelled fragments A and B respectively).

DNA prepared from cells infected with the WAP BAGgal hybrid virus gave PCR products of 2.4 kb and 2.1 kb after amplification with the P1-P2 and P3-P2 primer pairs, respectively. These products hybridised, as expected, to both WAP specific (Fig. 3.53, lanes 1, 3, 5, 7, 9, and 11) and MLV specific (Fig. 3.53 lanes 1, 3, 5, 7, 9 and 11) hybridisation probes. In the non-infected cells (Fig. 3.53) lanes, 2, 4, 6, 8, 10 and 12) neither PCR amplification nor hybridisation could be observed.

In contrast, the primer pair P4-P2 gave a PCR product of 1.8 kb that hybridised to the MLV probe (Fig. 3.53, lanes 13, 15, and 17) but not to the WAP probe (Fig. 3.53, lanes 13, 15, and 17). Here too, in the non-infected cells (Fig. 3.53, lanes 14, 16, and 18) neither PCR amplification nor hybridisation could be observed. This data is consistent with the occurrence of promoter conversion.

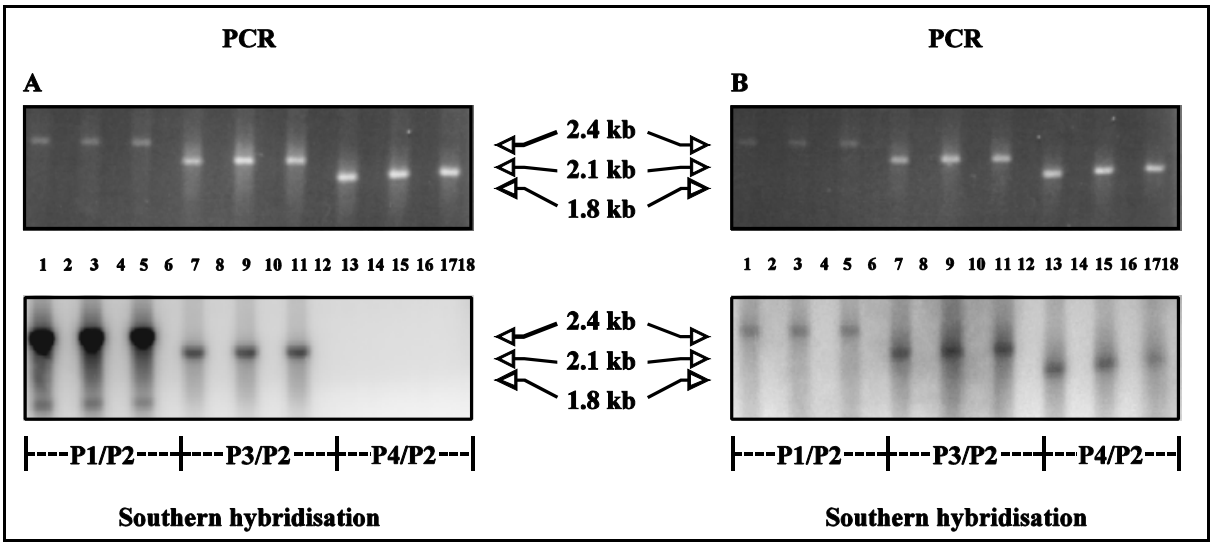

Figure 3.53 :PCR Products and Hybridisation

PCR analysis of infected cell DNA for the presence of WAP-BAGgal sequences. 1 µg of genomic DNA was amplified by PCR using 40 pmol of each primer specific for the WAP promoter region or MLV R sequences and of a primer specific for the ß-galactosidase gene. The PCRs were performed under the following reaction conditions: denaturating for 1 min at 94°C, 2 mins annealing at 55°C (P1-P2), 53°C (P3-P2) or 56°C (P4-P2), and elongation for 3 mins at 68°C for 35 cycles. The primer pairs P1-P2 gave a 2.4 kb PCR product (lanes 1,3 and 5); P3-P2 gave a 2.1 kb PCR product (lanes 7, 9, and 11); P4-P2 gave a 1.8 kb PCR product (lanes 13, 15, and 17). 10 µl of each PCR product was separated on a 0.8% agarose gel, transferred to a nylon membrane and hybridised to α ^{32}P-labelled probes. Promoter conversion was detected by using two primers specific for the WAP promoter region (P1 and P3) in combination with a primer specific for the ß-galactosidase gene (P2). An MLV R region specific primer (P4) was also used in combination with P2. Nylon membranes were hybridised against a 0.2 kb WAP specific fragment (A) from the plasmid pWAP- BAGgal or an MLV specific 0.3 kb PCR fragment (B) (which was amplified from pWAP- BAGgal with the primer pair, 125pos, 5'-AGATGTAGCCACGAACTC-3' and 125posc2, 5'-GGTCGGCCAGATACAGAGCTAGTTA-3'), as shown. Lanes 1,7 and 13, infected CrFK cells; lanes 2, 8 and 14, non-infected CrFK cells; lanes 3, 9 and 15, infected T-47D cells; lanes 4, 10 and 16, non-infected T-47D cells; lanes 5, 11 and 17, infected MCF7 cells; lanes 6, 12 and 18 non-infected MCF7 cells.

3.2.8 Recloning of the Integrated Provirus

To analyse the structure of the hybrid provirus (named pWAPProCon), integrated proviruses were recloned from infected CrFK cells and characterised by Southern blot, PCR or sequencing. The hybrid WAP-BAGgal retroviral vector carries a prokaryotic origin of replication (Fig. 3.45), allowing the integrated provirus to be recloned from genomic DNA of infected cells. Genomic DNA was isolated from a population of WAP-BAGgal infected CrFK cells (Fig. 3.54). The genomic DNA was then digested with the restriction enzyme *BspH*I, which does not cut within the vector provirus. The digested DNA was ligated and transformed in to *Escherichia coli* (2.3.1). After selection on kanamycin containing medium a number of plasmids, each carrying an integrated provirus, were isolated. Restriction enzyme mapping was then used to show that the promoter conversion had taken place correctly in the proviruses isolated and also to allow one to be chosen for further investigation.

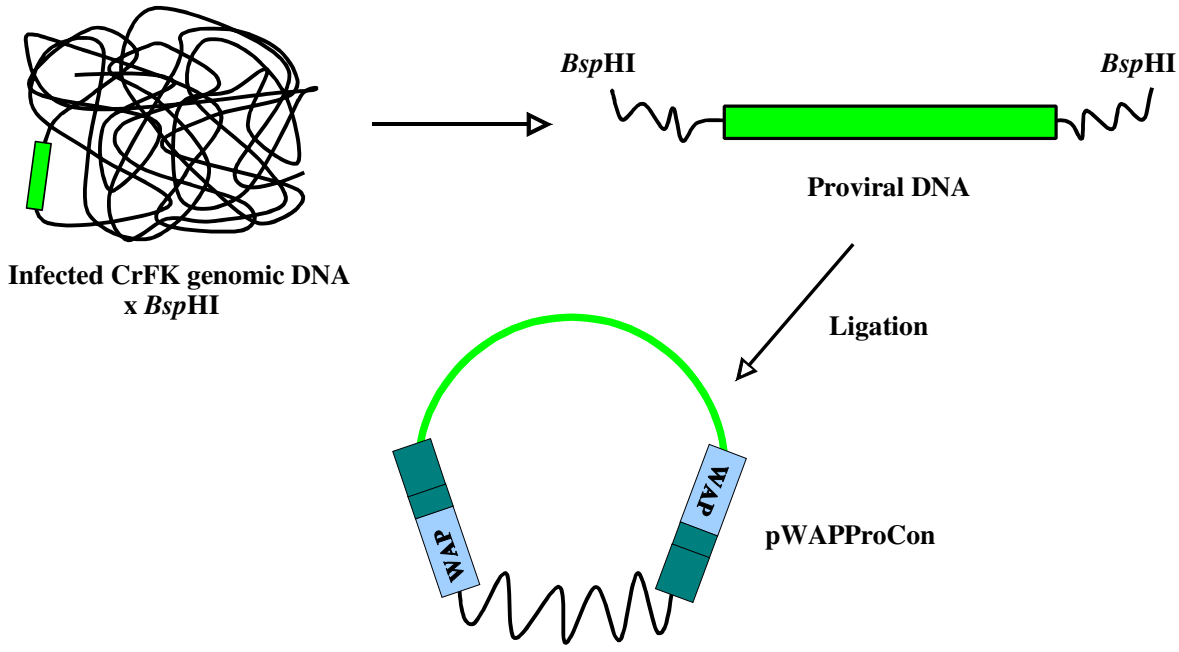

Figure 3.54: WAPProCon Recloning Strategy
Genomic DNA from CrFK cells that had previously been infected with the WAP-BAGgal vector was digested with the restriction endonuclease *Bsp*HI, which has no recognition site within the vector. It was then ligated and transformed into *E. coli*. After selection on kanamycin, colonies could be isolated which carried a promoter converted recloned provirus.

3.2.9 Investigation of the Recloned Vector

The recloned plasmid was analysed using many different molecular biological methods to show that a promoter converted provirus had been isolated and that the conversion had taken place correctly.

3.2.9.1 Southern Blot Analysis

The recloned plasmid was digested with the restriction enzyme *Kpn*I. pWAP-BAGgal was also digested with the same enzyme in parallel. The *Kpn*I recognition site is present in the R region of both LTRs. After digestion with *Kpn*I two fragments of 7 kb and ~4 kb could be seen in the recloned plasmid. In pWAP-BAGgal bands of 7 kb und 0.7 kb were expected. In the recloned plasmid both the 7 kb as well as the 4 kb fragment should contain the WAP promoter if promoter conversion has taken place. In the plasmid pWAP- BAGgal the U3 region of the WAP promoter is only present in the 3'LTR and should only be detected in the 7 kb fragment. The gel was blotted and hybridised to an WAP specific probe. As expected both the 7 kb and the 4 kb band from the recloned plasmid hybridised to the probe gave a hybridisation signal (lane 1), and only the 7 kb product in pWAP- BAGgal was seen to be positive.

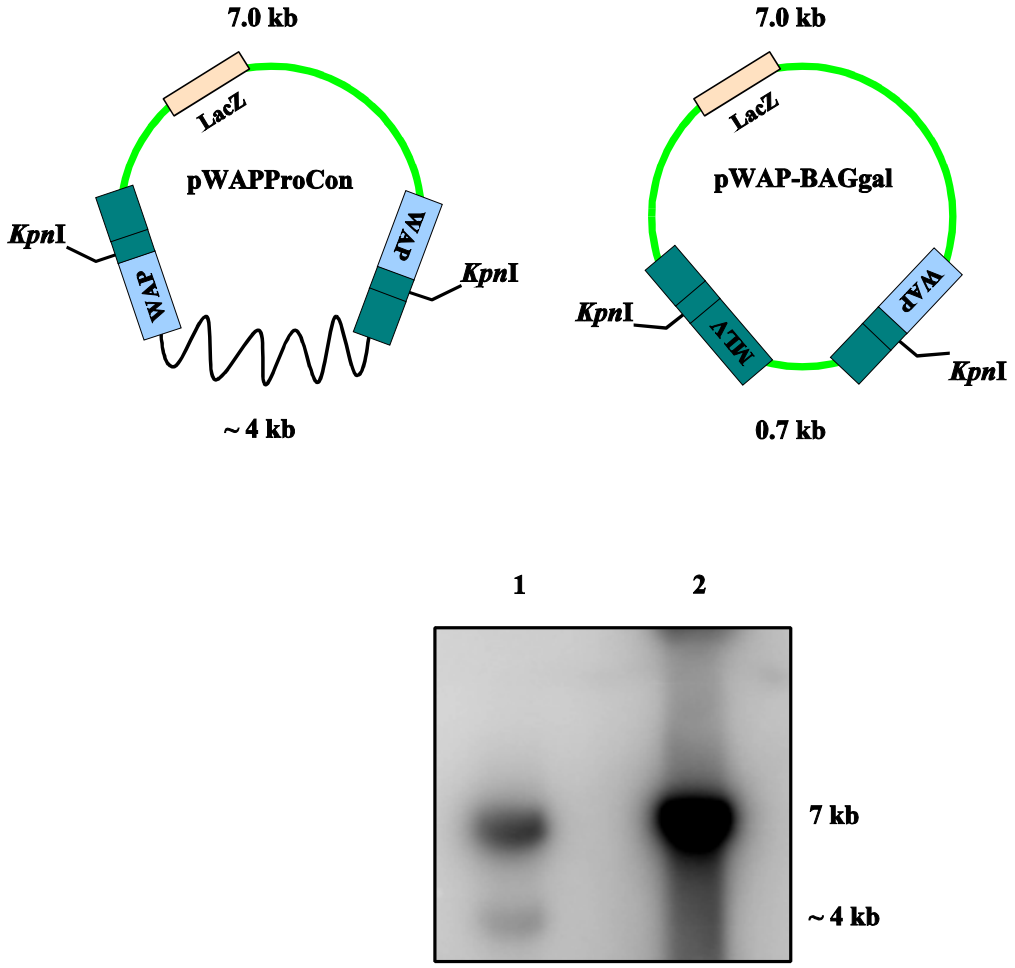

Figure 3.55: Southern Blot

1 µg plasmid DNA was digested with the restriction enzyme *Kpn*I. This enzyme was chosen as it could be used to show the presence of the WAP promoter in the 5' LTR after hybridisation to WAP specific probe. The *Kpn*I digest shows a 7 kb fragment and approximately 4 kb fragment in the recloned plasmid. Here it was expected that the WAP promoter would be present in both fragments after reverse transcription. The 4 kb fragment represents the remaining parts of the 5' and 3' LTRs as well as genomic DNA from the integration site. pWAP-BAGgal gave fragments of 7 kb and 0.7 kb as expected. The recloned plasmid is in lane 1 and pWAP-BAGgal in lane 2. After transfer to a nylon membrane, the DNA was hybridised to a radioactively labelled probe. In lane 1 the recloned, promoter converted plasmid shows two hybridised bands corresponding to the 7 and 4 kb fragments from the KpnI digest. In lane 2, pWAP-BAGgal shows only the 7 kb hybridised band as expected.

3.2.9.2 PCR Analysis of the Recloned Vector

PCR was used to further characterise the recloned plasmid. The primers used can be seen in figure 3.56. In the first primer pair the downstream primer binds at the beginning of the WAP promoter region in the 5' LTR and the other binding to the packaging signal of the vector. In a second pair, the downstream primer binds in the middle of the WAP promoter region in the 5' LTR, the complementary upstream primer binding in the ß-galactosidase gene. If the WAP promoter region has been duplicated and translocated from the 3' LTR to the 5' LTR 0.9 kb and 2.1 kb bands would be expected from the first and second primer pairs respectively. As expected the 0.9 kb PCR product can be seen in lane 1 and a 2.1 kb PCR product in lane 2 from the recloned plasmid. A 1.6 kb product in lane 5 and a 2.8 kb product in lane 6 arising from the parental plasmid control pWAP-BAGgal as WAP NRE is only present in the U3 of the 3' LTR. The resulting gel was then blotted to a nylon membrane and hybridised to a 0.32 kb radioactively labelled probe isolated from the WAP promoter in order to check the specificity of the bands present. After hybridisation a strong band could be seen at the expected sizes (Fig. 3.56). This shows the presence of the WAP promoter region in both the 5' and 3' LTR's of the recloned plasmid.

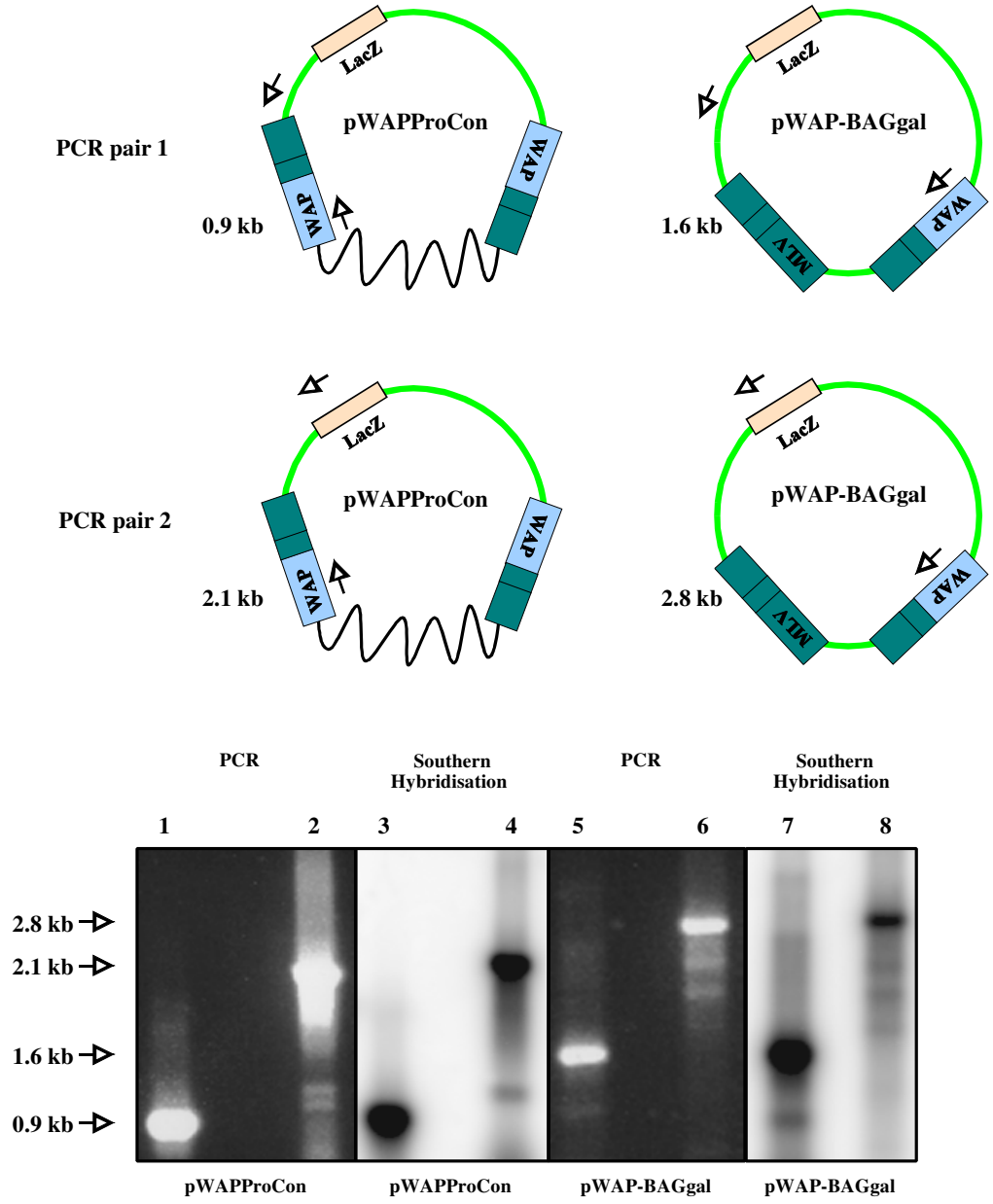

Figure 3.56: PCR Analysis of the Recloned Vector

1 ng of plasmid DNA was amplified by PCR using 40 pmol of each primer. In the first primer pair, one primer is specific for the beginning of the WAP promoter region (Wapanf, 5'-AGATGTAGCCACGAACTC-3') and the other primer specific for the MLV packaging region (125posc2, 5'-GGTCGGCCAGATACAGAGCTAGTTA-3'). In the second primer pair one primer binds in the middle of the WAP promoter (Wapmit, 5'-GTGTGGCCAAGAAGGAAGTG-3'), the complementary primer being specific for the ß-galactosidase gene (Baggal2, 5'-TTCATCCACCACATACAGGC-3'). The PCR was performed under the following reaction conditions: denaturating for 1 min at 94°C, annealing for 2 mins at 51°C (P1-P2) or 55°C (P3-P4), and elongation for 3 mins at 68°C; 35 cycles were made. The primer pair P1-P2 gave a 0.9 kb PCR product (Lane 1) and P3-P4 gave 2.1 kb (Lane 2) from the recloned plasmid (pWAPProCon) whereas 1.6 and 2.1 kb PCR products (Lane 5 and 6) arose from pWAP-BAGgal. 10 µl of the PCR product was separated on a 0.8% agarose gel and transferred to a nylon membrane. The membrane was then hybridised against an α^{32}P-labelled 0.32 kb WAP specific *Xho*I/*Xba*I fragment.

3.2.9.3 Sequencing of the Recloned Plasmid

Sequencing was used to show that the promoter conversion had taken place correctly at the level of DNA sequence and also to determine the sequence of the integration site. The recloned plasmid was digested with *Kpn*I, which recognises sites present in both LTRs, to separate the 5' and 3' LTRs and thus facilitate analysis of both LTRs individually. After promoter conversion it would be expected that the WAP promoter should be present in both LTRs. Two fragments were obtained, a 7 kb fragment and a 4 kb fragment. The 7 kb fragment contained the U3 region of WAP present in the 3' LTR and a part of the R region and the 4 kb fragment contained the WAP promoter region present in the 5' LTR and part of the R region. The 4 kb fragment was used for sequencing (see 2.2.5.1.3) as it contains the promoter converted LTR.

Two primers were chosen for the sequencing reactions, one of which was complementary to the U3 region of WAP (S1) and the other which was complementary to the R region of MLV (S2). Sequencing of the junction between the CrFK flanking sequence and at the 5'end of the provirus revealed that the *Sac*II restriction site and WAP NRE sequences inserted into this site were as expected intact.. The MLV IR was also present, but it had been shortened by 2 bp, as expected, due to processing during the integration event (Goff *et al.*, 1992). Similarly, the sequence at the border between the WAP promoter and MLV R regions in the 5' LTR revealed that the WAP promoter, *Mlu*I restriction site, and MLV R region were also intact (Fig. 3.57).

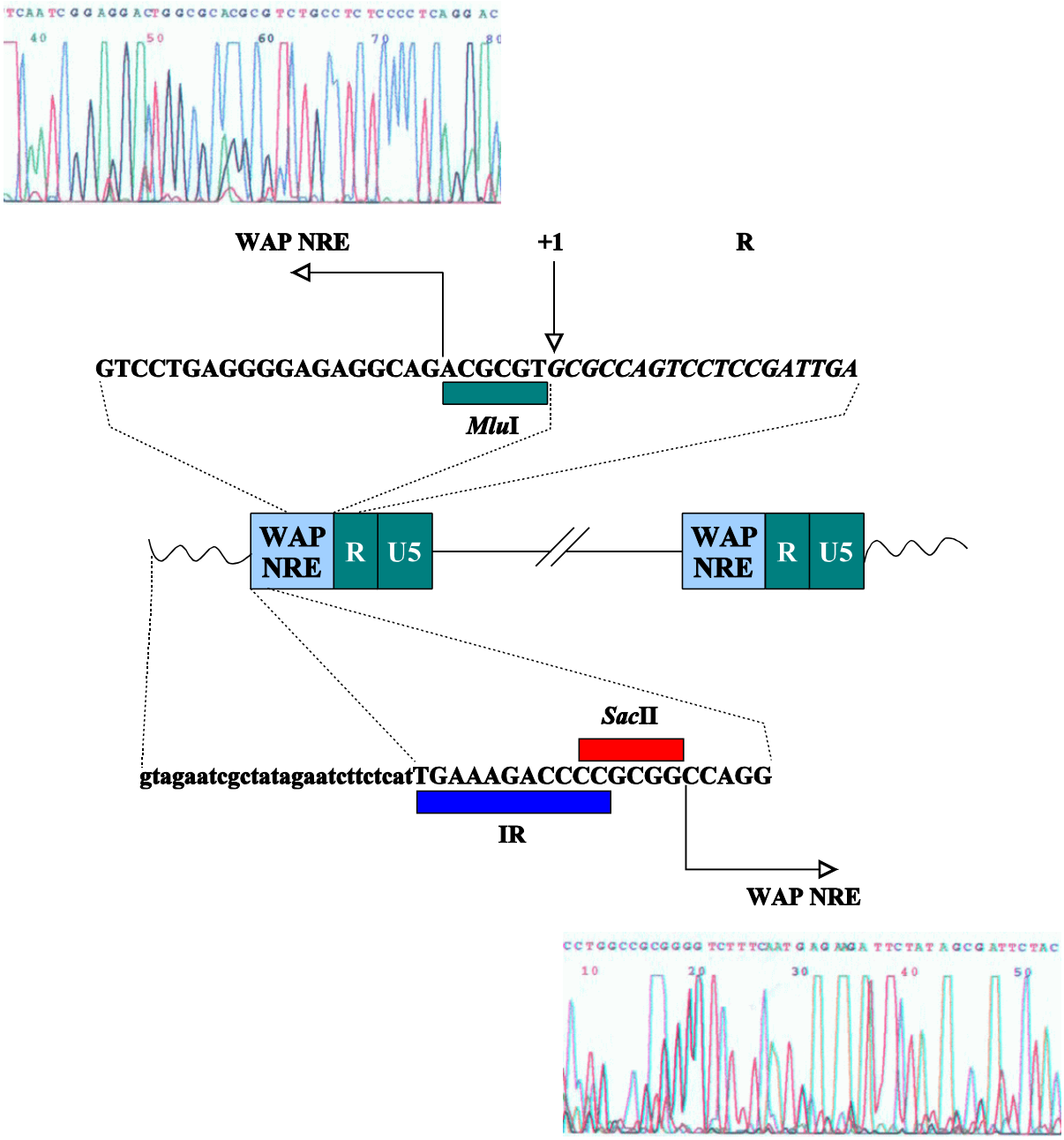

Figure 3.57: Sequencing of the Junction Regions of a Recloned Provirus

5 µg of plasmid DNA was digested with the restriction enzyme *Kpn*I. A 4 kb fragment, which contains the WAP promoter region present in the 5' LTR, was taken and extracted for sequencing.
Sequencing was carried out with an automatic sequencer (ABI 373a; Applied Biosystems). The reactions were performed with a dye terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions (see 2.2.5.1.3). PCR mixtures contained 1µg of plasmid DNA and 4pmol of primer, one of which was complementary to the WAP promoter (S1, 5'-AGTTCGTGGCTACATCTGAG-3') and the other of which was complementary to the R region of MLV (S2, 5'-CCACAAGTCGGATGCAACTG-3'). Shown are the sequences at the U3-R junction of the 5' LTR and the integration site-U3 junction of the 5' LTR. The sequence analysis reveals that (i) the borders between the WAP promoter and MLV R regions are intact after promoter conversion and (ii) the IR has been used for integration. The *Sac*II restriction enzyme cleavage site and the *Mlu*I site used to introduce the WAP promoter region are marked. +1 indicates the position of the classic CAP site. IR represents the position of the inverted repeat. The small letters represent the flanking sequence from the CrFK genome.

3.2.10 *In vitro* Infection and Expression Analysis

3.2.10.1 Infection of Primary Human Tumour Cells and Cell lines

After characterisation of the hybrid WAP-BAGgal vector, its' activity in human breast tumour cells was investigated. Different breast tumour cell lines as well as primary human breast cells that had been isolated from tumour biopsies were used for infection experiments and subsequent expression analyses. As has been previously observed for the WAP promoter, expression requires an intact three dimensional structure (Roskelley *et al.*, 1995). Therefore, the human tumour cell lines and primary breast tumour tissue were cultivated either in matrigel or on collagen (see 2.4.3.2.3 and 2.4.3.2.2) to try to ensure the correct cellular environment for the differentiation of many cell types. Interaction with a basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant cells (Li *et al*., 1989). In mammary epithelium the matrix of the matrigel as well as lactogenic hormones and glucocorticoids cooperate to direct accurate tissue-specific gene expression. It has also been shown that these hormones are sufficient to induce differentiation of established mammary epithelial cells *in vitro* (Danielson *et al.,* 1984*;* Doppler *et al.,* 1989*;* Reichmann *et al.,* 1989). The lactogenic hormone prolactin (3 µg/ml) as well as insulin (3 µg/ml) and dexamethasone (10^{-6} M) were used to stimulate the cells before expression analysis.

3.2.10.2 Titre on Human Breast Tumour Cells

To investigate the viral titre of the recombinant retroviral vectors, T-47D cells were infected with the retroviral vectors. Virus supernatant harvested from a population of either WAP-BAGgal or the parental BAGgal producing cells were used to determine the titre. The infections were performed was made in a similar manner to the infection of NIH 3T3 cells described previously. NIH 3T3 cells were also infected in parallel with the same supernatant to check whether or not the infection experiment had worked technically. After approximately 14 days, the mock-infected cells were seen to be dead, the number of resistant colonies was counted and the virus titre calculated as colony forming units per millilitre supernatant used.

Here it could also be seen that the T-47D cells are less efficiently infected than NIH 3T3 cells in both the parental and hybrid vectors as was previously observed (see 3.1.10.1). This indicates that the lack of infection is due to the fact that the T-47D cells are more difficult to infect than the NIH 3T3 cells (Table 3.7). The titre on T-47D cells being on average 4.5 times lower than those seen in NIH 3T3 cells.

Construct	Cell line	
	T-47D	NIH 3T3
	cfu/ml	
BAGgal	9×10^2	5.1×10^3
WAP-BAGgal	5×10^2	2.2×10^3
LXSNEGFP	7.9×10^3	2.3×10^4
LXPCWAPEGFP	2.8×10^3	1.5×10^4

Table 3.7: Titre on T-47D Cells
Both T-47D cells and NIH 3T3 cells were used as a target cells for the infection with WAP-BAGgal and BAGgal virus from producing populations. T-47D and NIH 3T3 cells were infected with supernatant from the same virus-producing cells. 24 hours after infection the target cells were diluted into 10 cm plates and a further 24 hours later G418 was added to the medium (NIH 3T3, 400 µg/ml; T-47D, 200 µg/ml). Approximately 14 days later, as soon as the mock-infected cells had died, the surviving colonies were counted and the titre calculated in colony forming units per millilitre infectious medium used (cfu/ml). The virus titre was calculated from three independent experiments.

3.2.10.3 Infection of Primary Human Breast Tumour Cells

Human breast tumour tissue that had been obtained after a biopsy was used for these experiments. The tissue was first cultivated in a monolayer system (see 2.4.3.2.1), infected (see 2.4.8.3.2) and then further cultivated on collagen or in matrigel to further three dimensional (3D) growth.

3 days after the tissue had been taken into cell culture, primary cells were infected with either one of the hybrid WAP vectors (WAP-BAGgal or LXPCWAPEGFP) or the corresponding parental vector as a control (BAGgal or LXSNEGFP).

For the histological x-gal assay, ß-galactosidase activity was analysed (see 2.5.3.2.1) in cells transiently infected with either the hybrid WAP-BAGgal or the parental BAGgal vector. The infected cells were plated out on collagen and stimulated for 3 days [with prolactin (3 µg/ml), insulin (3 µg/ml) and dexamethasone (10^{-6} M)]. If the primary cells had been successfully infected and the promoters controlling the expression of the transgene are active then a ß-galactosidase expression should be seen. In this case ß-galactosidase expression could be seen in a few cells infected with WAP-BAGgal after hormone stimulation, as well as in BAGgal infected cells (Fig. 3.58). This may be due to a low transduction efficiency of the primary cells. Treatment with lactogenic hormones is essential for induction of WAP. The maintenance of WAP gene expression in primary breast cells is dependent on the presence of all these

hormones. They also allow the breast or breast tumour cells to retain their original morphology to a certain extent.

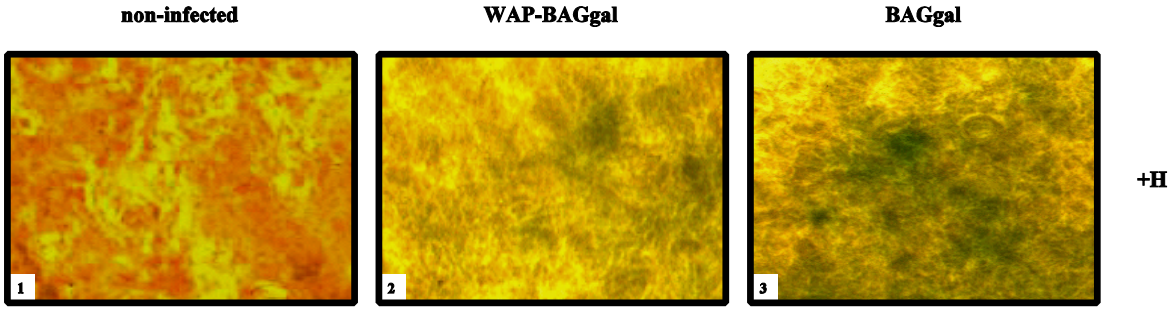

Figure 3.58: ß-Galactosidase Expression in Infected Primary Human Breast Cancer Cells
Primary human breast tumour cells isolated from a biopsy were first cultivated in a monolayer system (see 2.4.3.2.1), infected and then further cultivated on collagen to further three-dimensional growth. 3 days after the tissue had been taken into cell culture, primary cells were infected with either one of the hybrid WAP vectors (WAP -BAGgal or LXPCWAPEGFP) or the corresponding parental vector as a control (BAGgal or LXSNEGFP). Cells were then stimulated for 3 days with hormones. The cells were then fixed in 2% paraformaldehyde (see 2.5.3.2.1) and the expression of ß-galactosidase visualised by staining with x-gal (4-chrome-5-bromo-3-indoyl-ß-D-galactosidase). Non-infected primary human tumour cells with stimulation (panel 1). WAP BAGgal infected cells with stimulation show expressing cells (panel 2). Primary human tumour cells infected with the parental BAGgal vector shows some ß-galactosidase expressing in cells with stimulation (panel 3). 400x magnification.

In parallel, primary human breast tumour cells infected with either the hybrid LXPCWAPEGFP or the parental LXSNEGFP were selected in G418 (400 µl/ml). After approximately 2 weeks the mock-infected cells were found to be dead and the surviving infected clones were pooled to form populations (approximately 30 clones/population in this case). Infected and non-infected cells were plated out on collagen and either stimulated for 3 days [with prolactin (3 µg/ml), insulin (3 µg/ml) and dexamethasone (10^{-6} M)] or not stimulated at all.

The expression of the enhanced form of the green fluorescent protein (EGFP) was examined using fluorescence microscopy. The LXPCWAPEGFP infected cells exhibited higher levels of expression when stimulated (Fig. 3.59). LXSNEGFP infected cells showed expression of EGFP regardless of the state of hormone stimulation.

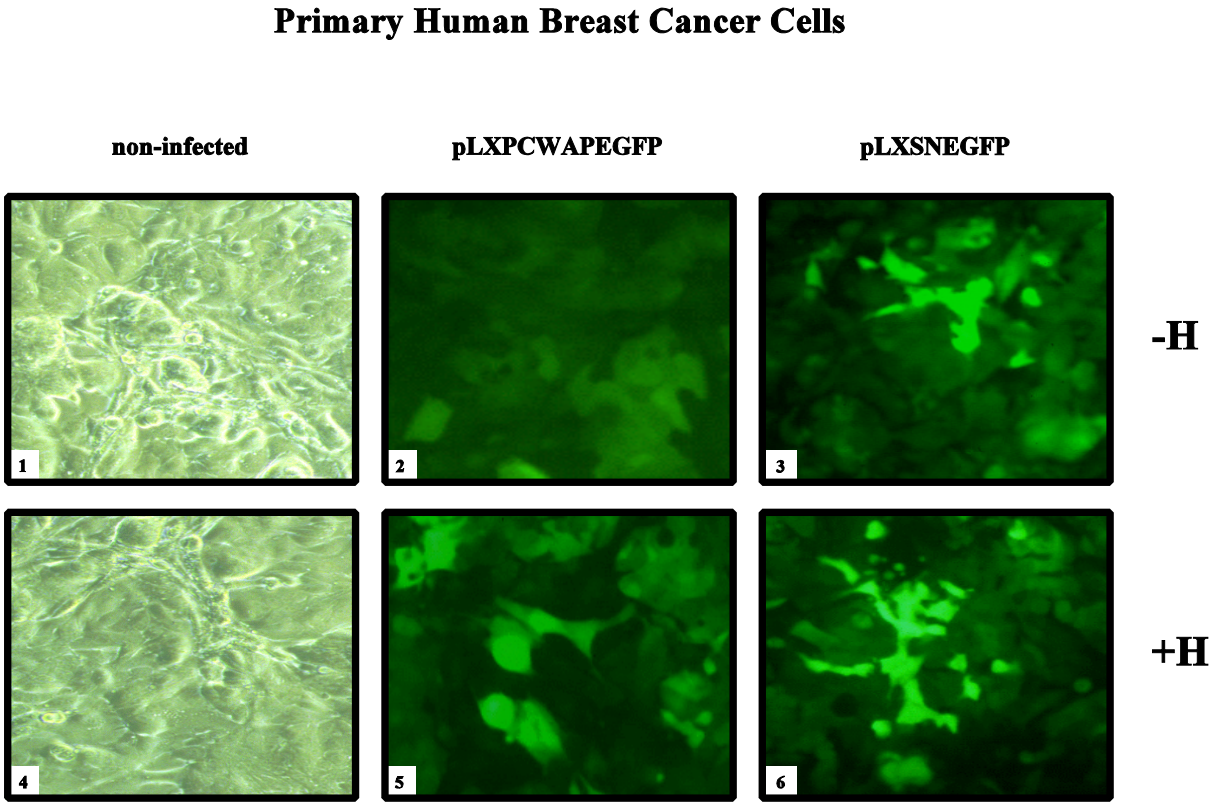

Figure 3.59: EGFP Expression in Infected Primary Human Breast Tumour Cells
Primary human breast tumour cells were stably infected with either the hybrid LXPCWAPEGFP or the parental LXSNEGFP vector and cell populations established. Infected cells that had been cultured on collagen were examined using fluorescent microscopy after 3 days +/- hormone treatment [prolactin (3 µg/ml), insulin (3 µg/ml) dexamethasone (10^{-6} M)].
Non-infected primary human tumour cells with stimulation (panel 1) show no green EGFP expressing cells. LXPCWAPEGFP infected cells with stimulation (panel 2) show the strong expression of the vector with many cells exhibiting strong EGFP expression. Primary human tumour cells infected with the parental LXSNEGFP vector after stimulation with dexamethasone (panel 3) show strongly expressing EGFP cells. 200x magnification.

3.2.10.4 Infection of Estrogen Receptor Positive Human Breast Tumour Cell Lines

The human breast tumour cell lines MCF7, T-47D and ZR-75-1 were used for the infection experiments and expression analysis.

The cells were cultured in a monolayer system and then infected with either WAP-BAGgal or BAGgal. As these cells are more difficult to infect than the NIH 3T3 cell line, the virus particles were pseudotyped with the G protein from the vesicular stomatitis virus in order to obtain sufficient infected clones to allow the creation of a cell population (normally >50 clones). This was performed in a similar manner to described in 3.2.6.1.

To examine the expression of ß-galactosidase with or without hormone stimulation in WAP BAGgal infected cells, enzyme activity was determined by either using a photometric quantitative ß-galactosidase assay (see 2.5.3.1) or qualitatively using histochemical staining.

For the quantitative assay, ß-galactosidase activity was analysed in cells (MCF7, T-47D and ZR-

75-1) stably infected with either the hybrid WAP-BAGgal or the parental BAGgal vector. $3x10^5$ cells were plated out in matrigel and stimulated for 3 days [with prolactin (3 µg/ml), insulin (3 µg/ml) and dexamethasone (10^{-6} M)]. The protein lysate was prepared from hormone stimulated infected or non-infected cell populations.

All of the tested infected cell populations showed expression of ß-galactosidase from the WAP promoter. Levels of expression in the presence of hormones were between 3.7 and 38.2 fold the expression seen in uninfected cells, depending on the cell type; the best expression was observed in T-47D cells (Fig. 3.60). The parental BAGgal vector was expressed in all cell types analysed. Levels of expression were 1.9 fold more than that seen in the WAP- BAGgal in MCF7 cells, comparable to those seen in ZR-75-1 cells, but 2.3 fold lower in T-47D cells.

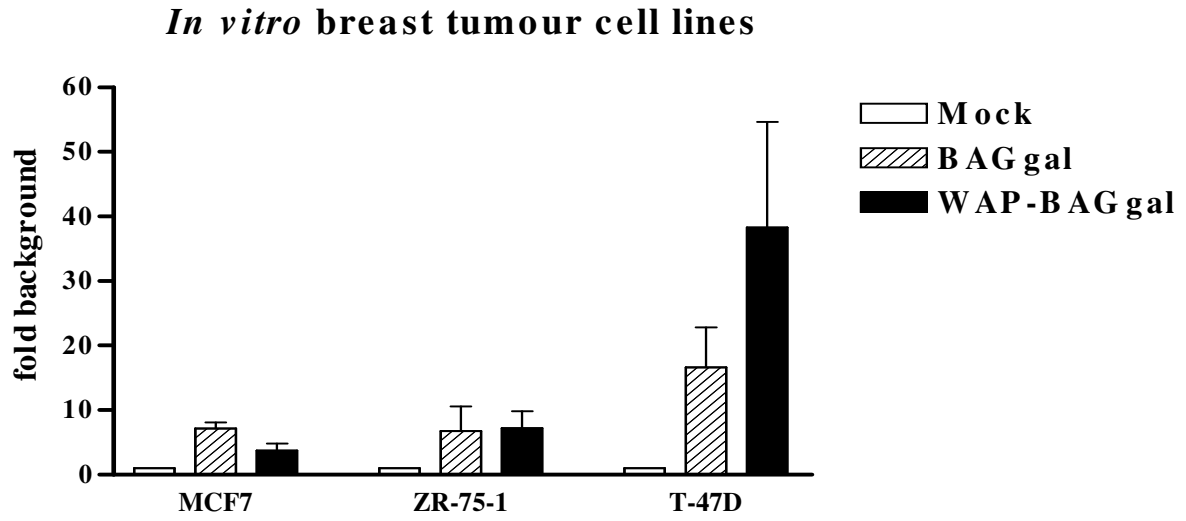

Figure 3.60: Hormone Inducible Expression of ß-galactosidase in Transduced Human Breast Tumour Cells Lines

The breast tumour cell lines MCF7, T-47D and ZR-75-1 were stably infected with either the hybrid WAP- BAGgal vector or the parental BAGgal vector and cell populations established. $3x10^5$ infected cells that had been cultured in matrigel were lysed and total cell protein extracted after 3 days hormone treatment [prolactin (3 µg/ml), insulin (3 µg/ml) dexamethasone (10^{-6} M)]. 10 µg protein was analysed for ß-galactosidase enzymatic activity using the galactolight kit (Perkin Elmer) (see 2.5.3.1). The expression of ß-galactosidase is measured in relative light units after a chemoluminescent substrate is added. The background ß-galactosidase activity was set to a value of 1 and the expression of the vectors then calculated in relationship to the background. The best expression was observed in WAP- BAGgal infected T-47D cells; levels of 38.2 fold background being observed. Expression could also be seen in WAP- BAGgal infected MCF7 and ZR-75-1 cells, being 3.7 and 6.7 fold background respectively. The fold background expression observed from the MLV promoter was maximally 16.6 fold in T-47D cells. The experiment was repeated 3 times and mean with error bars is shown (standard error of the mean).

Hormone induced ß-galactosidase expression was also analysed in a qualitative assay using histochemical x-gal staining (4-chrome-5-bromo-3-indoyl-ß-D-galactosidase). The cells were treated in the same manner as described above for the chemiluminescent assay. After 3 days the cells were fixed and subsequently stained with an x-gal solution. 24 hours later the cells were examined using light microscopy. In the presence of hormones expression of the ß-galactosidase enzyme from the WAP NRE promoter could be observed in all infected cells. The expression of ß-galactosidase could clearly be seen best in T-47D cells, less in ZR-75-1 and the least in MCF7 cells. Thus, the expression observed when using this assay (Fig. 3.61) correlates with the results seen in the quantitative ß-galactosidase assay (Fig. 3.6.1). Expression of the ß-galactosidase

enzyme from the parental vector BAGgal could also be observed in all cell types examined. Here too, the results correlated with those seen in the quantitative ß-galactosidase assay.

T-47D Cells

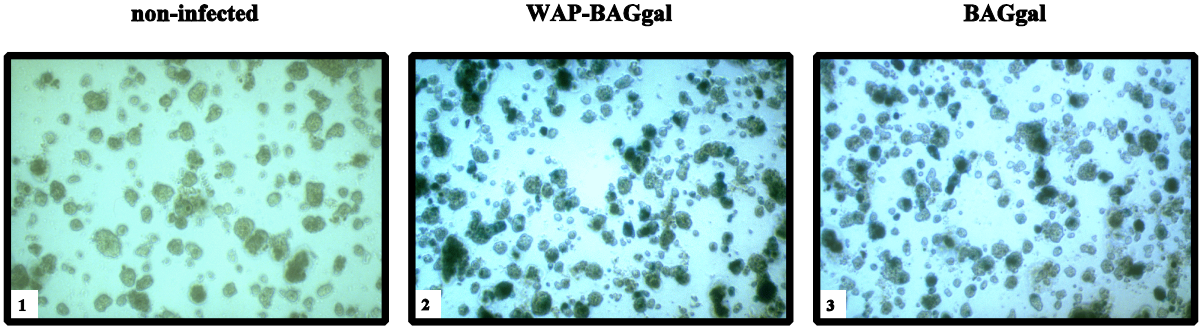

Figure 3.61: ß-galactosidase Expression in Infected Human Breast Tumour Cell Lines
Expression of ß-galactosidase was determined histologically by x-gal staining stably infected T-47D, ZR-75-1 or MCF7 cells. Cells were seeded out in duplicate and stimulated for 3 days with hormones [prolactin (3 µg/ml), insulin (3 µg/ml) and dexamethasone (10^{-6} M)]. The cells were then fixed in 2% paraformaldehyde and the expression of ß-galactosidase visualised by staining with x-gal (4-chrome-5-bromo-3-indoyl-ß-D-galactosidase). Non-infected T-47D cells with stimulation (panel 1) show no blue ß-galactosidase expressing cells. WAP-BAGgal infected cells with stimulation (panel 2) show high levels of expression from the WAP promoter, with dark blue, strongly expressing ß-galactosidase cells visible. T-47D cells infected with the parental BAGgal vector with stimulation (panel 3) also show ß-galactosidase expressing cells (panel 3). Similar results were observed in both ZR-75-1 and MCF7 cells. 100x magnification.

3.2.10.5 Infection of Estrogen Receptor Negative Human Breast Tumour Cell Lines

An estrogen receptor negative human breast tumour cell line, MDA-MB-435S, was used for further *in vitro* analysis experiments. Here, the pLXSN based pLXPCWAPEGFP retroviral vector was used. The reporter gene in this vector is EGFP instead of ß-galactosidase and carries a similar modification of the 3' LTR as in pWAP-BAGgal. It should also undergo reverse transcription in a similar manner leading to expression being controlled by the WAP NRE after infection of target cells. As it is well known (Lesoon-Wood *et al*., 1995) that the MDA-MB-435S cells are highly metastatic, the use of EGFP as a marker gene should allow easier detection of expression in an *in vivo* mouse model where these cells have been transplanted and metastases allowed to develop.

MDA-MB-435S as well as T-47D breast tumour cells were infected with either pLXPCWAPEGFP or pLXSNEGFP. As it had already been shown that the WAP NRE was active in T-47D cells they were used as a positive control in this series of experiments.

MDA-MB-435S and T-47D cells were cultured in a monolayer system and then infected with with either the hybrid LXPCWAPEGFP or the parental LXSNEGFP vector. As in the infection experiments that have been previously described, the recombinant retroviral particles were here also pseudotyped with the VSV-G protein in order to obtain enough clones to establish a population (>50 clones) after G418 selection (MDA-MB-435S, 400 µg/ml: T-47D, 200 µg/ml). The stably infected cells were then seeded out in a 3D cell culture system and stimulated for 3 days with prolactin (3 µg/ml), insulin (3 µg/ml) and dexamethasone (10^{-6} M). The expression of

the enhanced form of the green fluorescent protein (EGFP) was examined using fluorescence microscopy. Expression from the WAP promoter could be seen in both infected cell lines after hormone stimulation. Here, the expression from the BAGgal vector was seen to be much stronger than the WAP expression levels in MDA-MB-435S cells (Fig. 3.62). It was also observed that infected MDA-MB-435S cells that had grown to high confluency exhibited higher levels of expression than non-confluent cells. This supports the theory that a complex structure of cells is important for the expression from the WAP promoter.

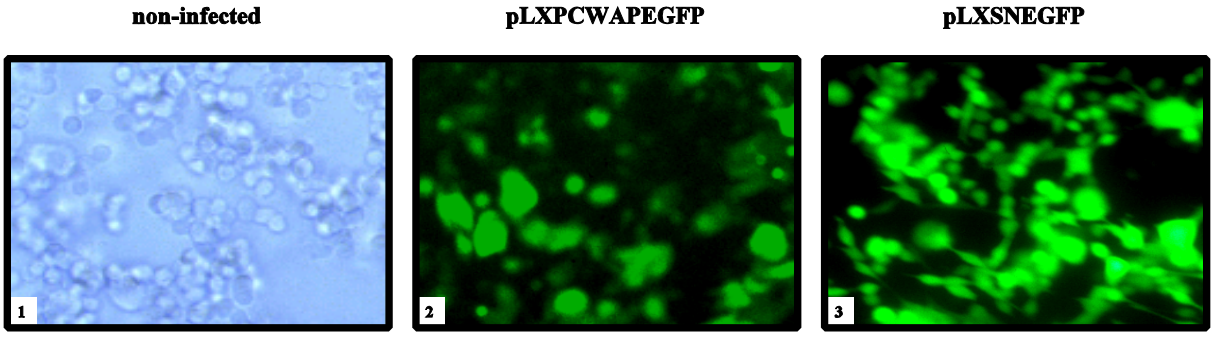

Figure 3.62: EFGP Expression in Transduced MDA-MB-435S Human Breast Tumour Cell Lines

The breast tumour cell lines MDA-MB-435S and T-47D were stably infected with either the hybrid LXPCWAPEGFP or the parental LXSNEGFP vector and cell populations established. Infected cells that had been cultured in matrigel were examined using fluorescent microscopy after 3 days hormone treatment [prolactin (3 µg/ml), insulin (3 µg/ml) and dexamethasone (10^{-6} M)].

Non-infected MDA-MB-435S cells with stimulation (panel 1) show no green EGFP expressing cells. LXPCWAPEGFP infected cells with stimulation (panel 2) show many green, EGFP expressing cells. MDA-MB-435S cells infected with the parental LXSNEGFP vector show strong EGFP expressing cells (panel 3). 400x magnification.

3.2.10.6 S1-Analysis

To accurately quantitate at the transcriptional level expression from the WAP-BAGgal infected cells grown under different conditions, S1 analyses performed.

Total RNA was prepared from stably infected T-47D and ZR-75-1 human breast cell lines that had either been grown on collagen and stimulated for 3 days or grown in monolayer and not stimulated at all. Non-infected T-47D and ZR-75-1 cells were also treated in the same manner and RNA isolated for use as a negative control. Primary human breast tumour cells obtained from a biopsy were also examined in this experiment. Both infected and uninfected cells were stimulated for 3 days before the RNA extraction was made. These RNAs were then hybridised to a γ^{32}P-end-labelled *Bsa*I DNA fragment from the recloned hybrid MMTV-BAGgal vector (pMMTVProCon). The same probe was used here as was used to determine the transcriptional start site of the MMTV-BAGgal recloned vector. However, as the sequences upstream of the R region differ between the two vectors, no conclusion can be made here as to the start site of the WAP-BAGgal transcript.

A clear induction of the signal could be observed in RNA derived from cells grown on collagen in the presence of hormones (lanes 1, 2 and 7) compared to that from cells grown in monolayer in the absence of the hormones (lane 8). This also shows that the start of transcription is located in the 5' LTR, presumably under the control of the WAP promoter.

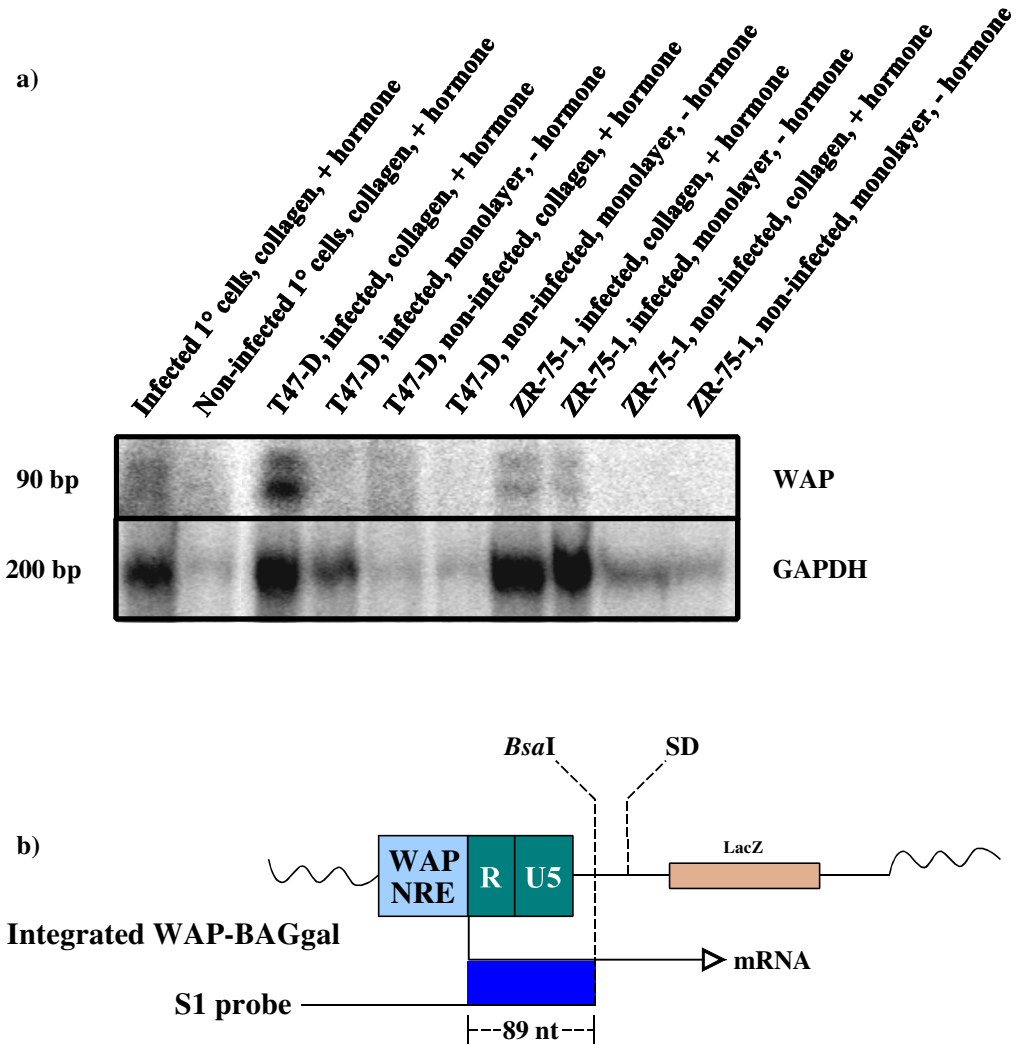

Figure 3.63: S1 Nuclease Protection Analysis

a) Diagram of the S1 probe used. The probe was isolated after a *Bsa*I digest of the recloned hybrid MMTV-BAGgal plasmid pMMTVProCon. The protected fragment after S1 digestion was 90 nucleotides.

b) Total RNA was isolated from a stably infected population of hybrid WAP-BAGgal infected cells grown on collagen and stimulated with hormones for 3 days before RNA isolation. 40 µg total RNA was used per reaction. RNA was hybridised against a γ^{32}P-end-labelled *Bsa*I LTR DNA fragment as previously described (Günzburg *et al*., 1986). After S1 digestion, a protected fragment of a 90 bp was obtained, as determined with Phosphor imager software (Molecular Dynamics). RNA from WAP-BAGgal infected 1° human breast tumour cells grown on collagen in the presence of hormones (lane 1) or non-infected 1° cells (lane 2). The RNA of infected, hormone treated T-47D cells (lane 3) gives a stronger signal than that of infected, hormone treated ZR-75-1 cells (lane 7) and infected, non-stimulated cells T-47D and ZR-75-1 cells (lanes 4 and 8 respectively). No signal was obtained either from the RNA derived from non-infected cells cultured on collagen with hormonal stimulation or from monolayer culture in the absence of hormones (lanes 2, 5, 6, 9 and 10). *Hae*III-digested pBR322 was used as a marker.

3.2.10.7 Infection of Other Cell Types

After it had been shown that the hybrid WAP-BAGgal vector was active in human breast tumour cell lines it was important to analyse the activity of this vector in human non-mammary tumour cells as well as some other non human cells. NIH 3T3, CrFK, Panc-1 as well as EJ cells were used (see 2.4.1).

All these cells were infected in monolayer cell culture with either the hybrid WAP-BAGgal or the parental BAGgal vector in an identical manner to the infections of human breast tumour cell lines and selected for approximately 2 weeks with G418 (Panc-1, 400 µg/ml; EJ, 800 µg/ml; NIH 3T3, 400 µg/ml; CrFK, 400 µg/ml).

The stably infected cells were then seeded out in matrigel culture and stimulated for 3 days with hormones. The cells were treated in exactly the same manner as the breast tumour cells lines to ensure that any differences observed in the expression was not due to the cultivation conditions.

To examine the expression of ß-galactosidase in WAP-BAGgal infected cells, enzyme activity was determined by either using a photometric quantitative ß-galactosidase assay or qualitatively using histochemical staining.

Infected/non-infected cells were grown for 3 days stimulated with hormones before being lysed and a total protein extract being made. 10 µg was used for a quantitative ß-galactosidase assay using the galactolight kit (Perkin Elmer). This experiment was repeated 3 times. Expression of the ß-galactosidase could not be observed in cells infected with the WAP-BAGgal vector, whereas the parental BAGgal vector shows high levels of expression in EJ, CrFK and NIH 3T3 cells being 62.2, 76.6 and 87.6 fold background levels respectively. Very low levels (1.2 fold background) were observed in Panc-1 cells (Fig. 3.64a and b). This seems to indicate that the WAP-BAGgal vector is not active in either non-human non-breast tumour cell lines or other non-breast derived cell lines.

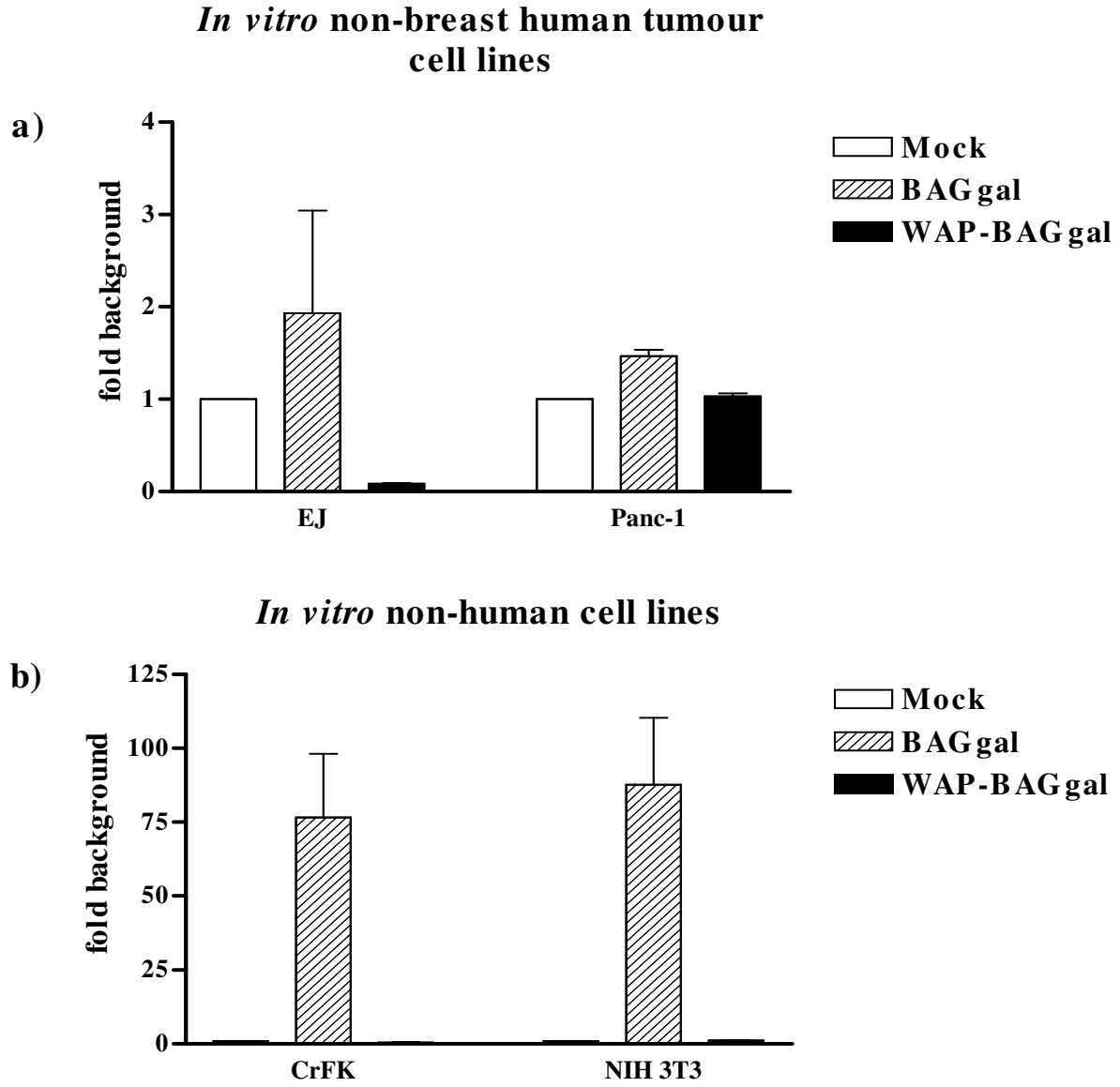

Figure 3.64: Expression of ß-galactosidase in Human and Non-Human Cell Lines
The human non-breast tumour cell lines Panc-1 and EJ as well as the non-human cell lines NIH 3T3 and CrFK were stably infected with either the hybrid WAP-BAGgal vector or the parental BAGgal vector and cell populations established. $3x10^5$ infected cells that had been cultured in matrigel were lysed and total cell protein extracted after 3 days hormone treatment [prolactin (3 µg/ml), insulin (3 µg/ml) and dexamethasone (10^{-6} M)]. 10 µg protein was analysed for ß-galactosidase enzymatic activity using the galactolight kit (Perkin Elmer). The expression of ß-galactosidase is measured in relative light units after a chemoluminescent substrate is added. The background ß-galactosidase activity was set to a value of 1 and the expression of the vectors then calculated in relationship to the background. Expression was observed only in BAGgal infected cells. No expression could be seen in hybrid WAP-BAGgal infected cells. The results are shown as the mean of three independent experiments (error bars show the standart error of the mean).

ß-galactosidase expression was also analysed in a qualitative assay using histochemical x-gal staining (4-chrome-5-bromo-3-indoyl-ß-D-galactosidase). The cells were treated in the same manner as described above for the chemiluminescent assay. After 3 days the cells were fixed and subsequently stained with an x-gal solution. 24 hours later the cells were examined. The expression of ß-galactosidase could clearly be seen in BAGgal infected cells (Fig. 3.65) whereas

no expression could be seen in the WAP-BAGgal infected cells.

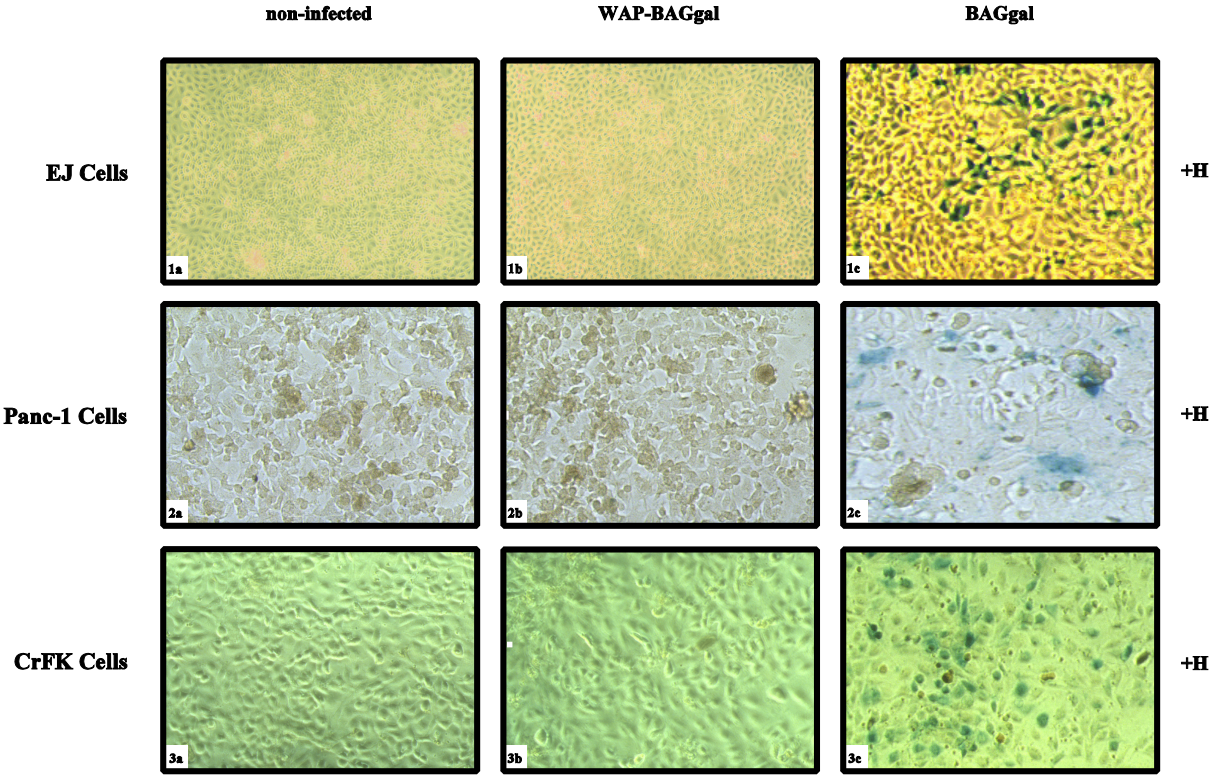

Figure 3.65: Histological ß-galactosidase Expression in Infected Human Non-Breast Tumour Cell Lines and Non-Human Cell Lines

Expression of ß-galactosidase was determined histologically by x-gal staining stably infected Panc-1 and EJ cells. Cells were seeded out in duplicate and stimulated for 3 days with hormones. The cells were then fixed in 2% paraformaldehyde and the expression of ß-galactosidase visualised by staining with x-gal (4-chrome-5-bromo-3-indoyl-ß-D-galactosidase). Non-infected Panc-1 cells with stimulation (panel 2a) shows no blue ß-galactosidase expressing cells. Stimulated hybrid WAP-BAGgal infected Panc-1 cells also show no blue ß-galactosidase expressing cells (panel 2b), whereas a few Panc-1 cells infected with the parental BAGgal vector show expression after hormone stimulation (panel 2c). Stimulated WAP-BAGgal infected and non-infected EJ cells (panels 1a and 1b respectively) show no blue ß-galactosidase expressing cells. EJ cells infected with the parental BAGgal vector show high levels of ß-galactosidase expression (panel 1c). Stimulated WAP-BAGgal infected and non-infected CrFK cells (panels 3a and 3b respectively) show no blue ß-galactosidase expressing cells. CrFK cells infected with the parental BAGgal vector show high levels of ß-galactosidase expression (panel 3c). 100x magnification

3.2.10.8 RNA Expression Analysis of Hybrid WAP- BAGgal Infected Cells *in vitro*

Northern blot analysis was undertaken to show the expression levels of ß-galactosidase *in vitro* from human or non-human breast (ZR-75-1, T-47D, MCF7 and GR cells) and non-breast (EJ, Panc-1, CrFK and NIH 3T3 cells) derived cells. Northern blot analysis not only indicates the levels of expression but also allows the length of the transcript to be visualised. Total RNA was isolated from stably infected cells (see 2.2.2.2.1) and on collagen cultivated cells after 3 days hormonal stimulation. 20 µg RNA from these cells was run over a denaturing formaldehyde gel (see 2.2.2.3.2) and, after being transferred to a nylon membrane via capillary blotting (see 2.2.5.2.4), was hybridised to a radioactively labelled 0.4 kb ß-galactosidase specific fragment (see 2.2.6.1 and 2.2.7.2) The 7 kb fragment that could be seen in all human breast cells as well

as in mouse mammary tumour derived GR cells (Fig. 3.70) corresponds to the full length vector transcript. The strongest ß-galactosidase expression could be observed in those human breast cells infected with the hybrid WAP- BAGgal vector (T-47D and ZR-75-1, MCF7). Expression was also seen to a lesser extent in GR cells. The ß-galactosidase expression could not be detected in the two human non-mammary tumour cell lines examined (EJ and Panc-1) or in either feline kidney cells or mouse fibroblast cells (CrFK and NIH 3T3). None of the non-infected cells showed any ß-galactosidase expression as expected. The same membrane was then stripped and hybridised to a radioactively labelled 1.2 kb ß-actin specific fragment. It shows the presence of similar quantities of RNA in each lane.

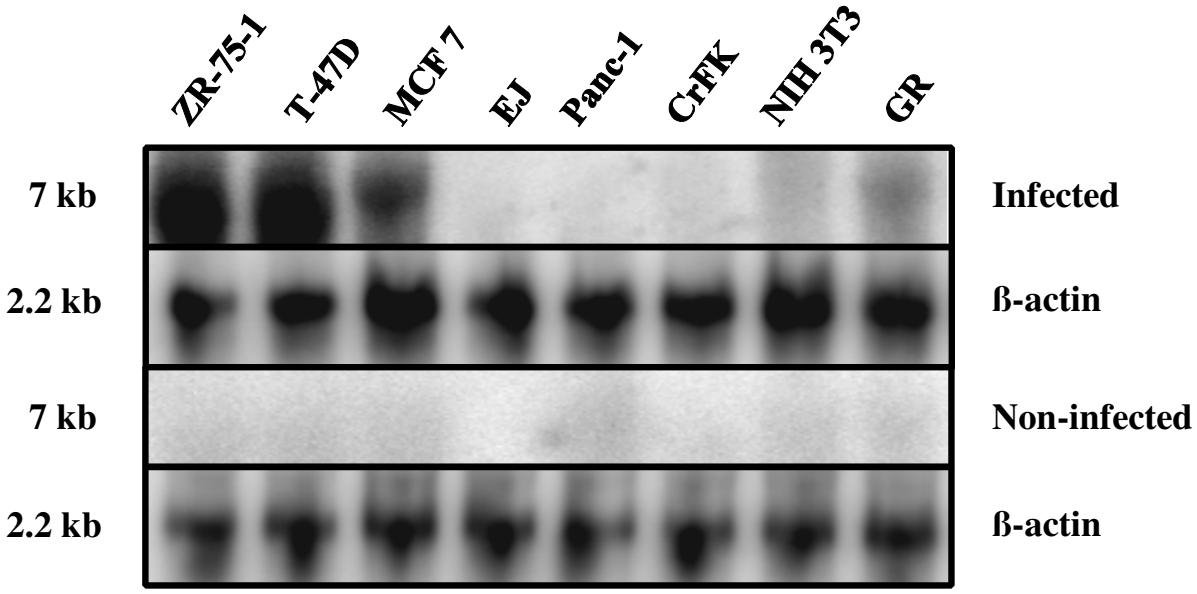

Figure 3.66:Northern Blot Analysis of Infected Human/non-Human Breast and non-Breast Cells

20 µg of total RNA was separated on a denaturing formaldehyde gel (see 2.2.2.3.2) and transferred to a nylon membrane using capillary blotting (see 2.2.5.2.4). The membranes were then hybridised (see 2.2.6.1 and 2.2.7.2) to an α^{32}P labelled 0.4 kb ß-galactosidase specific fragment (from pBAGgal after *Mlu*I digestion) and, following documentation and stripping, also to an α^{32}P labelled 1.2 kb ß-actin fragment [from pAL41 (Alonso *et al*., 1986) after *Pst* I digestion]. Lane 1, RNA from WAP-BAGgal infected ZR-75-1cells; lane 2, RNA from WAP-BAGgal infected T-47D cells; lane 3, RNA from WAP-BAGgal infected MCF7 cells; lane 4, RNA from WAP-BAGgal infected EJ cells; lane 5, RNA from WAP-BAGgal infected Panc-1cells; Lane 6, RNA from WAP-BAGgal infected CrFK cells; lane 7, RNA from a WAP-BAGgal infected NIH 3T3 cells; lane 8, RNA from WAP-BAGgal infected GR cells. No ß-galactosidase expression could be observed in the non-infected cells as expected (panel 3, loaded as in panel 1).

3.2.11 *In vivo* Expression Analysis of Severe Combined Immunodeficiency Mice (SCID) Xenografts

Xenotransplantation of WAP-BAGgal infected cells derived from both breast and non-breast cells into SCID mice was performed. To determine whether or not the *in vitro* ß-galactosidase/EGFP expression of the WAP-BAGgal hybrid vector is mirrored in an *in vivo* setting, human tumour cells that had been stably infected with the hybrid vector were taken up in matrigel before being injected into SCID mice. The use of matrigel embedded cells has been described as being particularly useful when transplanting human breast tumours cells. It has been observed that especially breast tumour cells injected in the presence of matrigel exhibited close histopathalogical resemblances to surgical breast specimens after the tumours that arose had been recovered. Co-injection with matrigel can increase the incidence of tumours and enhanced the tumour growth. Estrogen supplementation was also found to increase the growth rate of such tumours (Rae-Venter *et al.*, 1980; Osborne *et al.*, 1985). An estrogen pellet (1.7 mg) was therefore transplanted into the SCID/bg mice 24 hours before the transplantation took place (see 2.6.1). Such pellets release estrogen for a period of approximately 90 days.

3.2.11.1 Transplantation of Infected Breast and Non-Breast Cells into SCID/bg Mice

Human breast or non-breast tumours cells that had previously been infected and selected with either the WAP- BAGgal hybrid vector or BAGgal were cultivated in a monolayer cell culture system. $1x10^7$ cells per SCID mouse were prepared for the implantation (Fig. 3.67). These cells were mixed together with Matrigel on ice and immediately injected into the mammary fad pad (mfp) of SCID/bg mice. 4 mice were injected per construct and cell line. The mice were then examined 2 times per week and any tumours measured. 5 to 8 weeks later tumours could be observed in all treated mice, the time depending on the cell line used. The mice were sacrificed when the tumour had reached a size of 10 to 15 mm. The tumour was then removed from the surrounding tissue and both RNA and protein prepared for further analysis.

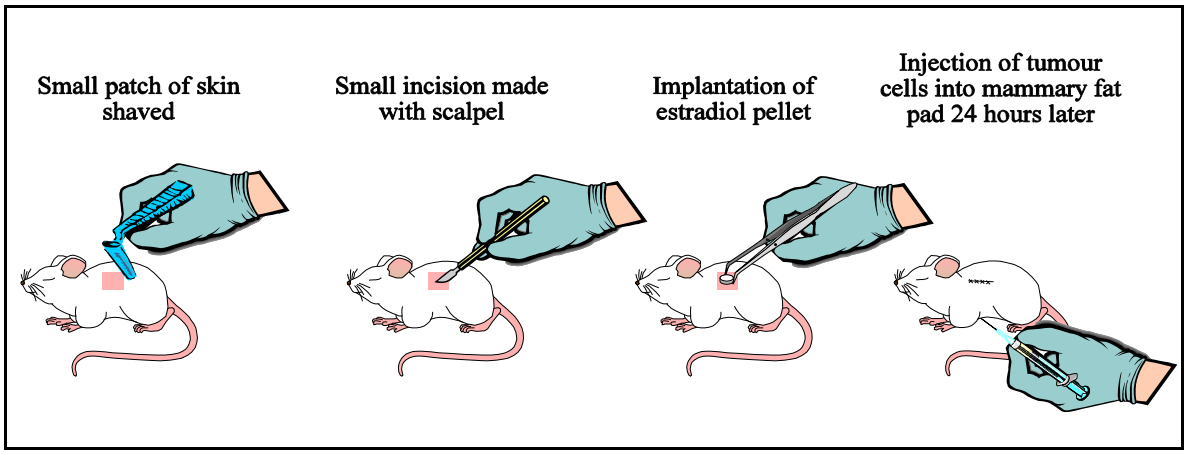

Figure 3.67: Transplantation of Infected Breast and non Breast Cells into SCID/bg Mice
Mice were housed in microisolator cages, fed only autoclaved food and water, and handled only with gloves. Female mice that weighed between 17 and 20 g were anaesthetised with ketamin (Ketalar 1%, 0.2 ml/20 g mouse i.p). Antibiotic (Borgal^{R} 24%) was added to the drinking water on the day of implantation. For the implantation, a small area of skin was shaved (~2x2 cm) and disinfected. A small incision was made in the skin and an oestrogen pellet pushed into it. Depending on the size of the wound, the cut was either sewn together (by a veterinarian) or simply pushed together. 24 hours later $1x10^{7}$ cells that had been previously mixed with Matrigel were injected into the mammary fat pad. After a 5-8 week incubation period, the mice were killed and the tumour immediately removed from the surrounding tissue.

3.2.11.2 Protein Expression Analysis

Protein was extracted from the tumours of 3 mice per tumour type. Tumours were homogenised on ice in lysis buffer. The tumour extracts were then treated with a cocktail of protease inhibitors [PMSF (0.2 mM) and leupeptin (5 µg/ml)] in conjunction with a heat inactivation step, just before analysis, in order to reduce high levels of endogenous ß-galactosidase activity that had been previously observed in tissue samples (Shaper *et al.*, 1994). Every human breast tumour showed high levels of ß-galactosidase expression from the WAP-BAGgal hybrid vector when compared to the parental BAGgal (Fig. 3.68).

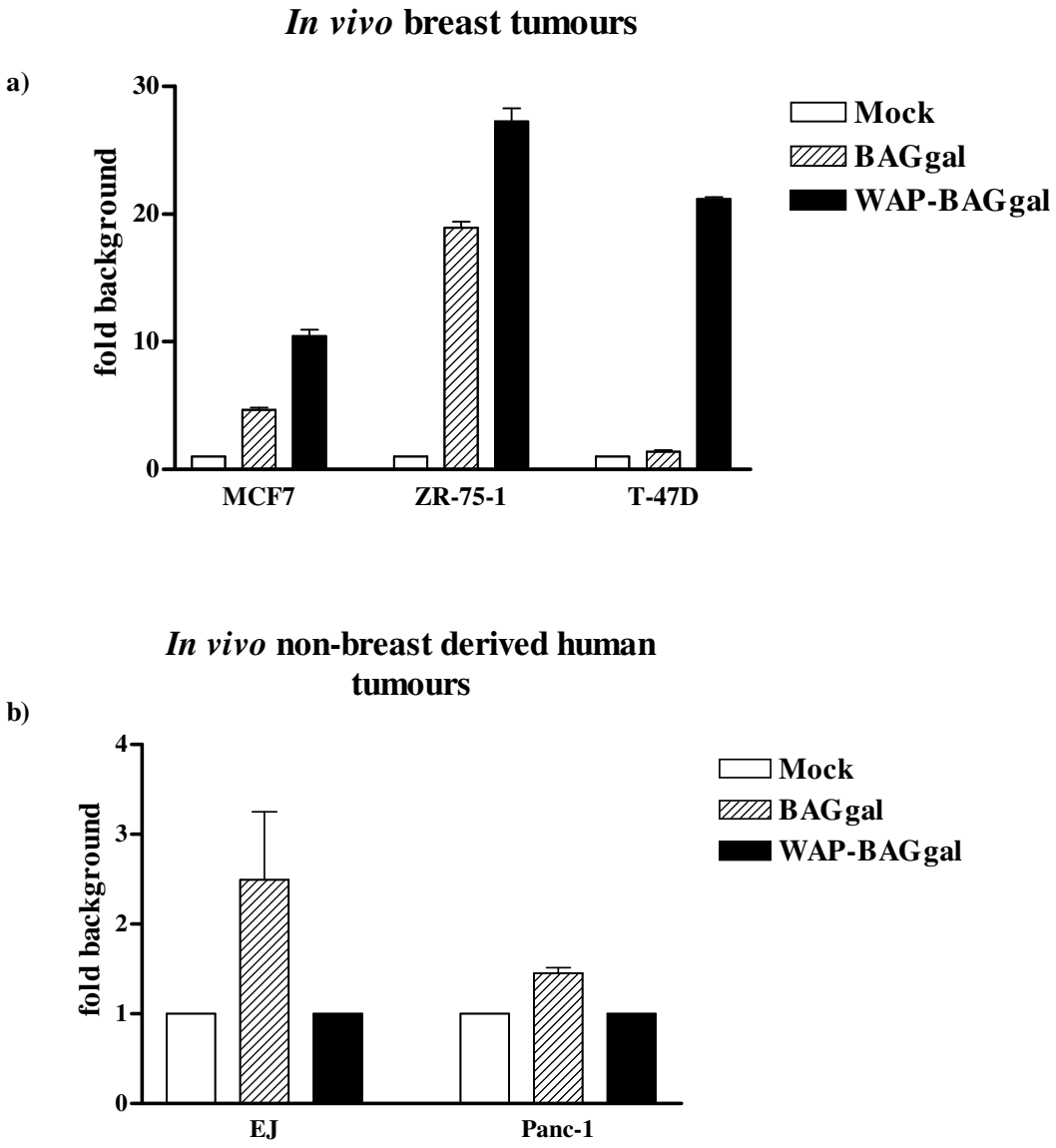

Figure 3.68: Analysis of ß-galactosidase Expression in Human Breast and non-Breast Tumour Cell Xenografts in SCID/bg Mice

a) Expression of ß-galactosidase in human breast cell derived tumours in SCID/bg mice.
10 µg of protein that had been previously been extracted from tumours that had arisen after implantation of tumour cells was used for a quantitative ß-galactosidase assay (Galactolight, Perkin Elmer). The expression of ß-galactosidase is measured in relative light units after a chemoluminescent substrate is added. The background ß-galactosidase activity was set to a value of 1 and the expression of the vectors then calculated in relationship to the background. High levels of expression could be seen from the hybrid WAP-BAGgal construct in MCF7, ZR-75-1 and T-47D cells. Expression from the BAGgal vector was lower by comparison, with no expression being observed in T-47D cells.

b) Expression of ß-galactosidase in human non-breast cell derived tumours in SCID/bg mice
The experiment was performed in the same manner as described above for human breast cell derived tumours. Expression of the hybrid WAP-BAGgal vector could not be seen in both non-breast tumour cell lines examined. High levels of expression from the parental BAGgal vector could be seen in EJ cell derived tumours. No significant expression could be seen in Panc-1 cell derived tumours. The results are shown as the mean of three independent experiments (error bars show the the standard error of the mean).

The T-47D cells showed expression levels from the WAP-BAGgal hybrid vector 21.2 times that of the background, whereas the BAGgal levels were only 1.4 times that of the empty cells. Expression could also be seen in both ZR-75-1 as well as MCF7 cells to a lesser exetent, being 27.2 and 10.4 fold background respectively. Expression from the parental BAGgal vector could also be seen in MCF7 and ZR-75-1 cells, but was always at lower levels than those observed for the hybrid vector. The high levels of expression from WAP-BAGgal could also be shown histologically in both T-47D (Fig. 3.69a) and ZR-75-1 tumours after fixation and staining (see 2.5.3.2.2).

In the non-breast tumours cell lines examined, EJ and Panc-1, no significant expression could be seen from the WAP- BAGgal hybrid vector. Low levels of expression were seen in EJ and Panc-1 cells infected with the parental BAGgal being 2.5 and 1.45 fold background respectively. Histological analysis confirmed these results (Fig. 3.69b)

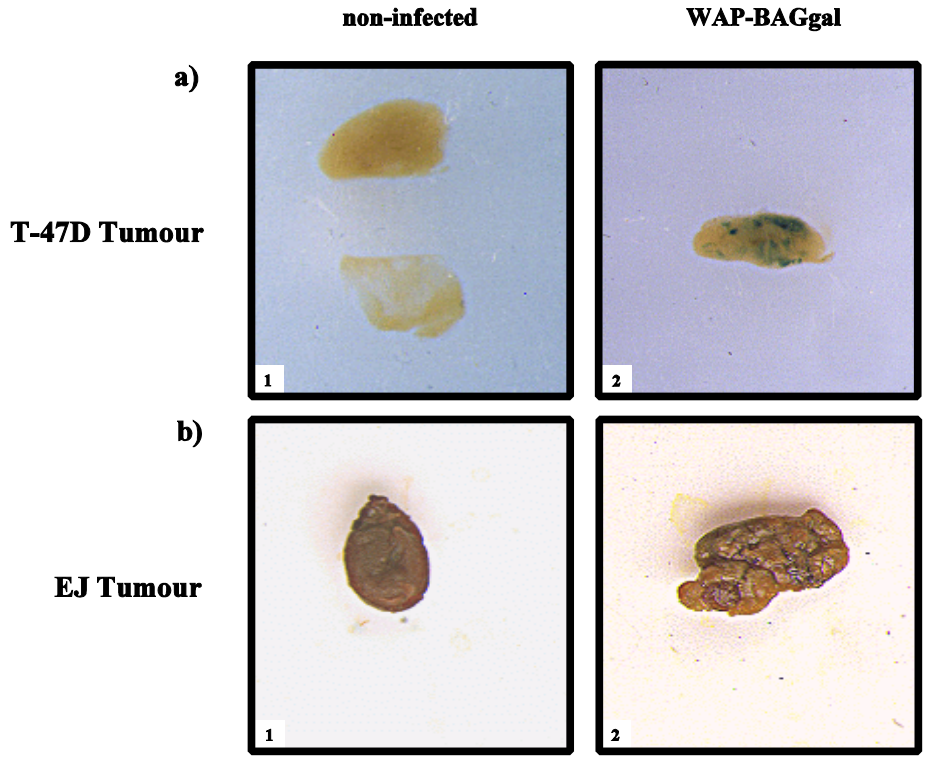

Figure 3.69: Histological Analysis of ß-galactosidase Expression in non-Breast Tumour Cell Xenografts in SCID/bg Mice

a) X-gal staining of breast cell derived tumours.
A tumour derived from implanted WAP- BAGgal infected T-47D cells was dissected and cut into small pieces before being fixed. The fixation was made in 4% paraformaldehyde for 24 hours at 4°C. The tissue was then stained with x-gal (2.5.3.2.2) before being embedded in paraffin and photographed. Blue staining indicates areas of ß-galactosidase expression. Similar results were also obtained for ZR-75-1 tumours (not shown).
b) X-gal staining of non-breast cell derived tumours.
The experiment was performed in the same manner as b). No expression, normally indicated by blue areas, can be seen in the EJ cell derived tumour.

3.2.11.3 RNA Expression Analysis

RNA analysis was made to show the expression levels of ß-galactosidase in the tumours arising from breast and non-breast derived cells. Northern blot analysis was performed as it not only indicates the levels of expression but also allows the length of the transcript to be determined. Total RNA was isolated from a tumour from 1 of the 4 mice per construct per cell and run over a denaturing formaldehyde gel (and, after being transferred to a nylon membrane via capillary blotting, was hybridised to a radioactively labelled 0.4 kb ß-galactosidase specific fragment. The 7 kb fragment that could be seen in all human breast cell derived tumours in both the hybrid WAP- BAGgal as well as the parental BAGgal vector (Fig. 3.70, lanes 1, 2, 3, 6, 7 and 8) corresponds to the full length vector transcript. The strongest ß-galactosidase expression could be observed in those human breast cell derived tumours that had been infected with the hybrid WAP-BAGgal vector before implantation. The ß-galactosidase expression could not be detected in bladder carcinoma cell derived tumours (EJ, lane 4) where the MLV promoter showed higher levels of expression than the WAP promoter (EJ, lane 9). However, in pancreatic cell derived tumours (Panc-1, lanes 5 and 10), no expression could be detected from either the WAP-BAGgal or BAGgal vectors. There was no expression in non- infected tumours (lanes, 11, 12, 13, 14, and 15). The same membrane was then stripped and hybridised to a radioactively labelled 1.2 kb ß-actin specific fragment. It shows the presence of similar quantities of RNA in each lane.

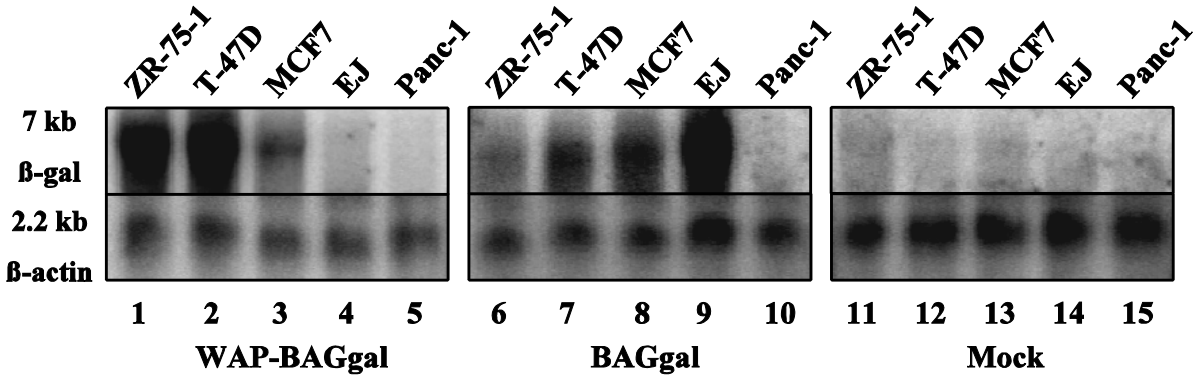

Figure 3.70: Northern Blot Analysis of Human Tumours from SCID/bg Mice

20 µg of total RNA was separated on a denaturing formaldehyde gel and transferred to a nylon membrane using capillary blotting. The membranes were then hybridised to an α^{32}P labelled 0.4 kb ß-gal specific fragment (from pBAGgal after *Mlu*I digestion) and, following documentation and stripping, also to an α^{32}P labelled 1.2 kb ß-actin fragment [from pAL41 (Alonso *et al*., 1986) after *Pst* I digestion]. Lane 1, RNA from a WAP-BAGgal infected ZR-75-1 tumour; lane 2, RNA from a WAP-BAGgal infected T-47D tumour; lane 3, RNA from a WAP-BAGgal infected MCF-7 tumour; lane 4, RNA from a WAP-BAGgal infected EJ tumour; lane 5, RNA from a WAP-BAGgal infected Panc-1 tumour; Lane 6, RNA from a BAGgal infected ZR-75-1 tumour; lane 7, RNA from a BAGgal infected T-47D tumour; lane 8, RNA from a BAGgal infected MCF-7 tumour; lane 9, RNA from a BAGgal infected EJ tumour; lane 10, RNA from an WAP-BAGgal infected Panc-1 tumour. Lane 11, RNA from non-infected infected ZR-75-1 tumour; lane 12, RNA from non- infected T-47D tumour; lane 13, RNA from a non-l infected MCF-7 tumour; lane 14, RNA from a non-l infected EJ tumour; lane 15, RNA from an non- infected Panc-1 tumour.

4. DISCUSSION

Breast cancer is the most common form of cancer amongst women in industrialised western countries (Kelsey and Berkowitz, 1988). Furthermore, the incidence of breast cancer appears to be continually increasing (Clarke *et al*., 1989), with an annual worldwide incidence of over one million predicted at the beginning of the 21st century (Miller *et al*., 1986).

Breast cancer is characterised by neoplastic changes in epithelial cells, with the majority being carcinomas, the malignant tumours of epithelia. Breast cancer patients usually do not die of their primary cancer; they die of subsequent metastatic disease. The technologies used to treat breast cancer (surgery, radiotherapy, chemotherapy and hormonal therapy) were developed decades ago and, with the exception of the development of new diagnostic methods, advances in these types of therapies have not helped treat metastases. Traditional treatments can only help one third of diagnosed patients to survive (O'Connell *et al*., 1994). The elucidation of the molecular basis of cancer allows for the possibility of specific intervention at the molecular level for therapeutic purposes, especially within the framework of gene therapy e.g. immunotherapy or molecular chemotherapy (enzyme-prodrug system). Gene therapy strategies with suicide genes (enzyme-prodrug systems) offer promising approaches toward the treatment of metastatic breast cancer. As with all types of gene therapy, one of major obstacles to successful clinical treatment is the development of a safe and effective delivery system. In the enzyme-prodrug system, it is important to make sure the suicide gene is only expressed in target cells. This could be achieved using one or more strategies, for example pseudotyping with molecules that allow tumour cell-specific binding; promoter/enhancer elements which target high levels of expression only in the appropriate cells. It is clear that the concept of targeting is central to this kind of therapeutic strategy and it is therefore important to both identify and characterise tissue or tumour specific promoter/enhancer elements. The use of heterologous viral or cellular promoter/enhancer elements should make targeted expression possible. Transcriptional promoters that are specifically functional in single tissues, are active in specific disease states or are induced by tumour specific conditions have been identified during basic research into cancer progression and could be utilised for targeted expression (e.g. tumour specific: MUC1, CEA, ERBB2; tissue specific: tyrosinase, HIV long terminal repeat (HIV-infected cells)).

Development of tissue specific retroviral vectors would make targeted gene delivery possible. Retroviral vectors, especially those derived from Moloney murine leukaemia (MoMLV) are an ideal means for targeting dividing tumour cells in post-mitotic tissues. Selective integration of the vector only into the chromosomes of replicating cells would, in fact, lead to almost exclusive expression of the transgene by growing neoplastic cells and, hopefully, to their selective elimination. Retroviral tropism is controlled at several different levels including the transcriptional activity of the viral long terminal repeat, making the design of tissue specific retroviral vectors possible.

Here, the ProCon system was used to construct tissue specific vectors containing either the mouse mammary tumour virus (MMTV) long terminal repeat (LTR) or the whey acidic protein proximal promoter, including the negative regulatory element (WAP NRE), which both direct breast tissue/breast tumour specific expression. The vectors were prepared and characterised at the molecular level and their targeting potential was tested in proof of principle experiments to demonstrate selective expression *in vivo* and *in vitro.*

4.1 Construction of Hybrid Retroviral Vectors

The U3 region of mouse mammary tumour virus (MMTV) was chosen as the heterologous viral promoter to be investigated. The MMTV long terminal repeat (LTR) is mainly active in mammary gland as well as in tumours of infected mice (Günzburg and Salmons, 1992). The regulation of MMTV expression is controlled by mammary specific factors, pregnancy hormones (Ringold, 1984) and steroid hormones (Groner *et al*., 1984; Rousseau, 1984; Toohey *et al*., 1990). Sequences responsible for mammary cell specific expression are located in an enhancer element in the extreme 5' end of the long terminal repeat region of this virus (Mink *et al*., 1990; Lefebvre *et al*., 1991; Yanagawa *et al*., 1991).

A Moloney murine leukæmia virus based (MoMLV) retroviral vector was used as the basis for construction of the tissue specific vectors. Retroviral vectors based on this virus are the most commonly used, particularly for gene therapeutic experiments in rodents and humans. Their efficiency relies upon the unique biological feature of retroviruses, *i.e.* the integration of the proviral genome into host chromosomes. This property guarantees that transduced somatic cells transfer the newly acquired genetic information to subsequent daughter cells. Genetic integration requires an active state of cell proliferation (Miller *et al*., 1990) and, although this requirement may be viewed as a limitation for purposes such as genetic transfer into resting progenitor cells, it could turn into a clear advantage if selective expression of the transgene is able to be achieved in actively proliferating neoplastic cells.

Retroviral vectors based on MMTV would be useful for inducible expression of transferred genes and preferential activity in organs such as the breast or in breast tumour tissue. However, even though such MMTV-based vector systems have been constructed (Salmons *et al*., 1985; Günzburg and Salmons, 1986; Shackleford and Varmus *et al*., 1988; Morris *et al*., 1989) they result in low titres, probably as a result of the complex regulation of MMTV (Günzburg and Salmons, 1992; Acha-Orbea and MacDonald, 1995).

A consequence of the reverse transcription of the retroviral genomic RNA into a double-stranded DNA molecule (see Fig. 9.1) in the infected cells is the duplication of the U3 region, located at the 3' end of the viral RNA, and the transfer of one of these copies to the 5' end of the genome (Luciw *et al*., 1992). The U3 region carries the retroviral promoter and enhancer elements, which control proviral gene expression. Yu and coworkers (Yu *et al*., 1986) used this phenomenon to construct retroviral vectors in which 299 bp of the murine leukæmia virus (MLV) U3 region, carrying the two 72 bp repeat enhancer elements, were deleted, creating self-inactivating vectors. Such vectors carry, after infection and reverse transcription, a single functioning internal heterologous promoter that controls the expression of a therapeutic gene. Double-copy vectors in which a promoter and a gene are inserted into the 3' LTR of the vector have also been constructed (Hantzopoulos *et al*., 1989).

A number of viral and cellular genes have been inserted in place of the virus enhancer, so that the expression of genes carried is placed under the transcriptional control of the heterologous promoter in the infected cell (Ferrari *et al*., 1995; Robinson *et al*., 1995; Vile *et al*., 1995; Cannon *et al.*, 1996).

The earliest attempt to use the MMTV promoter was the insertion of 335 bp of the U3 region (containing the MMTV glucocorticoid response element) into the 3'LTR U3 region of the MLV at the expense of the MLV enhancer. However, this modification was subsequently shown to be incompatible with recombinant virus production (Overhauser *et al.*, 1985). Only when part of the MMTV U3 region was additionally inserted into the 3' MLV U3 region could recombinant virus be obtained. However, this virus showed a serious reduction in infectivity and was not stable, undergoing frequent rearrangements. To overcome this problem, Overhauser and Fan had to insert MMTV U3 fragments into both the 3' and 5' MLV LTRs. Again, only constructs with additional MMTV sequences in both LTRs gave functional virus, and no virus could be recovered when the MLV enhancer sequences were replaced by MMTV sequences. This instability may have been due to the ability of this virus to undergo more than one round of infection.

In this study, almost all of the MLV vector U3 sequences, with the exception of the inverted repeat (IR) located at the 5' end of the U3 region, which is required for vector provirus integration (Goff *et al.*, 1992), were replaced with MMTV U3 LTR sequences. Following experiments that showed the system worked correctly, a cellular promoter element, the WAP-NRE, was also investigated.

The retroviral vectors BAG (Price *et al*., 1987) and LXSN (Miller and Rosman, 1989) were used as the basis for the hybrid vectors. The U3 region was deleted by a PCR mediated approach (see 3.1.2.1), ensuring that the inverted repeat at the 5' border of the U3 region was left intact. Additionally, unique *Sac*II and *Mlu*I sites were inserted to facilitate the introduction of the MMTV region. The complete 1.2 kb U3 region of MMTV was isolated by PCR carrying the same restriction enzyme cleavage sites and inserted into the 3' U3 deleted vectors. A reporter gene (ß-gal or EGFP) was introduced into the vector plasmid in such a way that after transfection it would be driven by the 5' MLV U3 promoter, whereas it would be under the transcriptional control of the MMTV U3 promoter in infected cells.

4.1.1 Testing the Infectivity of the Vectors

The hybrid retroviral vectors (pMMTV-BAGgal, pLXPCMMTVEGFP) as well as the parental vectors (pBAG, pLXSNEGFP), both of which carry an internal neomycin resistance gene constitutively expressed from an internal promoter (SV40), were introduced into the amphotropic packaging cell line PA317 (Miller and Buttimore, 1986). The transfection efficiencies showed that there was no significant difference between both types of vectors (see Tab. 3.1). Recombinant viruses produced from these cells were examined for their infectivity and titre.

Retroviral supernatants from G418-resistant PA317 virus-producing packaging cells (either populations or individual clones) were used to infect several cell lines including Rat-2, NIH 3T3, EJ and CrFK cells. Following infection, the target cells were split into selective medium and colonies arising were counted. Viral titre was calculated as colony forming units per millilitre (cfu/ml) supernatant used.

The titre from a population of MMTV-BAGgal hybrid vector virus producing cells was slightly reduced in comparison to that obtained from a population of parental BAGgal vector producing

cells when assayed on either Rat-2 or EJ cells but had similar titres when judged by the infection of CrFK and NIH 3T3 cells. The titre of several different virus producing clones was also examined, with all showing approximately the same results. Clonal differences could be seen, and it should be possible to select and screen clones for those producing high amounts of retrovirus if desired.

Generally speaking, the titre of the vectors was not observed to be particularly high, but this was not unexpected for the BAG based vectors. Here, the presence of a shortened packaging signal may result in a lower titre. This was confirmed when the LXSN based vectors, possessing a longer packaging signal, achieved titres of at least one log higher when compared to the BAG based vectors.

It was shown that the hybrid retroviral vectors were capable of producing infectious recombinant viral particles and that the replacement of the 3' U3 region did not dramatically affect the infection spectrum. In this case, the achieved titre would not be high enough to use in an *in vivo* gene therapy protocol. However, this was not due to the modification of the vectors. This phenomenon is a general problem that has also been observed by other groups. Low-level titres of between $4.1x10^4$ and $2.2x10^5$ have been reported when using pLXSN and PA317 cells in conjunction with one another (Pizzato *et al*., 1998). It is also known that low-level titres in amphotropic packaging lines may be due to the interaction between ecotropic *gag* and amphotropic *env* proteins not being as favourable as the interaction between ecotropic *gag* proteins and ecotropic *env* proteins (Markowitz *et al*., 1988). Another possibility is that amphotropic *env* proteins not being recognised by cell-surface receptors as efficiently as ecotropic *env* proteins (Markowitz *et al*., 1988).

Many methods have been developed to improve virus titre include ultrafiltration using low speed centrifugation and hollow fibre filtration (Paul *et al*., 1993; Chu *et al*., 1997), treating the producer cell line with sodium butyrate (NaB) (Tang and Taylor, 1992; Yeivin *et al*., 1992) and pseudotyping with the envelope of vesicular stomatitis virus (VSV-G) (Rose and Gallione, 1981; Burns *et al*., 1993).

Although the main aim of this work was not making high titre producing cells, an attempt was made to do so, to show that this was indeed possible with the modified vectors. In order to do this, the promising system of pseudotyping with VSV-G was tested. The G protein of VSV-G (Rose and Gallione 1981; Rose and Bergmann, 1983) efficiently forms pseudotyped virions with genome and core components derived from MuLV (Emi *et al*., 1991; Burns *et al*., 1993; Yee *et al*., 1994). The VSV-G pseudotyped vectors have a very broad host range and can reach titres of 10^5-10^6 cfu/ml and, following ultracentrifugation, can be concentrated to titres of greater than 10^9 cfu/ml (Burns *et al*., 1993). It is thought that VSV uses a universal memmbrane compenent to enter cells, possibly a phosholipid, giving a wide infection spectrum. The presence of the protein on the surface of the retrovirus is thought to confer this ability (Schlegel et al., 1982).

The generation of vector particles by transient expression of the VSV-G gene is a cumbersome and labour intensive method of vector production, requiring precise preparation. An appropriate amount of the plasmid pHCMV.6 was transfected into virus producing cells 48 hours prior to infection (Fig. 3.7). 24 hours later the cells were washed and fresh medium was added. After a further 24 hours, supernatants were titred on NIH 3T3 cells. Viral titres were calculated after

G418 selection was complete.

The titre achieved from a virus producing population, after pseudotyping with VSV-G, was seen to increase from 10^3 to10^4 cfu/ml for BAGgal and from 10^4 to 10^5 cfu/ml for LXSNEGFP. A similar increase could also be observed in the pseudotyped MMTV-BAGgal and LXPCMTVEGFP modified viruses. Two virus producing clones per construct were also analysed, with the results being seen to be comparable to those seen in the virus producing populations tested. It could clearly be shown that the modified vectors could be pseudotyped with VSV-G and the titre was increased. It would be possible to achieve a much higher titre with optimisation of this system, for example by ultracentrifugation of the viral supernatant from the best virus producer clone. Unfortunately, the VSV-G protein is toxic to cells in which it is expressed and therefore can only be used in a transient expression system. This system could be developed to create producer cell lines in which a silent VSV-G gene is integrated in a form that permits reproducible, high-level and inducible expression (Yang *et al*., 1995; Chen *et al*., 1996)

Due to the fact that the aim of this work was not the production of high titre retroviral producing cell lines, but was to investigate the infectivity of the modified vectors, the optimisation of this system was not continued. Here it was demonstrated that it is possible to modify the 3' U3 region of Mo-MLV based retroviral vectors without dramatically affecting their titre whilst achieving acceptable levels of virus production.

4.1.2 Molecular Characterisation of MMTV Hybrid Vector

To confirm that the transduced cells had acquired the vectors and to verify that the MMTV promoter was now present in the 5' LTR of the provirus in those infected cells, DNA was prepared from either infected G418 resistant or non-infected cells. PCR and Southern blotting were used to confirm the integrity of the proviral structure.

Primers specific for either the MMTV U3 (see Fig. 3.10-3.11) or MLV R sequences in combination with a second primer complementary to either the packaging region or the β-gal gene present in all three constructs were used. The PCR products were hybridised to both MMTV U3 and MLV specific probes. DNA prepared from MMTV BAGgal infected cells gave PCR products of 1.2 and 1.6 kb following amplification with the P1-P2 and P3-P2 primer pairs, respectively. In contrast, the primer pair P4-P5 gave a PCR product of 1.8 kb that hybridised to MLV probe but not to the MMTV U3 probe. Taken together, these data are consistent with the occurrence of promoter conversion.

In order to characterise the structure of the hybrid provirus (named MMTVProCon), the integrated proviruses were recloned from infected cells. The MMTV-BAGgal hybrid vector carries a prokaryotic origin of replication (see Fig. 3.1), allowing the recloning of the integrated provirus from genomic DNA. Genomic DNA was isolated from a population of MMTV-BAGgal infected Rat-2 cells, digested with the restriction enzyme *Asp700* I, which does not cleave within the vector provirus, ligated and transformed into bacteria (*E. coli*). After selection on kanamycin-containing medium, a number of colonies were obtained and one of them taken for the following molecular characterisation which was also important for the further *in vivo* experiments.

Restriction enzyme mapping combined with Southern blotting and PCR confirmed that the MMTV U3 region had replaced that of MLV. The restriction enzyme *Kpn* I was chosen for the Southern blotting strategy as it has recognition sites in the R regions of both LTRs. After digestion with *Kpn* I, two fragments of 7.67 kb and c. 3 kb could be seen in the recloned MMTVProCon, whereas pMMTV-BAGgal displayed 7.67 kb and 0.7 kb fragments. In the MMTVProCon plasmid both the 7.67 kb as well as the 3 kb fragment should contain the U3 region of MMTV, if promoter conversion has taken place without any rearrangements. To confirm this, hybridisation was made using an MMTV U3 specific probe. As expected, both the 7.67 and 3 kb bands from the MMTVProCon hybridised (Fig. 3.13). Further characterisation was also made using PCR and, following hybridisation, also confirmed promoter conversion had taken place in the infected cells.

Sequencing of the junctions between the rat flanking sequence at the 5' end of the provirus revealed that the *Sac* II restriction site and the MMTV U3 sequences inserted into this site were intact. The MLV IR was also present, but it had been shortened by 2 bp, as expected, due to processing during the integration event (Goff *et al*., 1992). Similarly, the sequence at the border between the MMTV U3 and MLV R regions in the 5' LTR revealed that the MMTV U3, *Mlu* I restriction site and MLV R sequences were also intact. As the LTRs of retroviral vectors contain enhancer and promoter elements, the random integration of a vector near a cellular proto-oncogene can activate that oncogene and result in a malignant cell. Therefore sequencing from the 5' U3 and 3' U5 regions into the bordering rat genomic DNA was made and aligned with online databases to see if any well-known sequences (like any cellular proto-oncogenes) were nearby. This was found not to be the case. Here, only one clone was analysed, but in future work it would be interesting to analyse multiple clones in order to gain an insight into the frequency of integration near to any proto-oncogenes, also to determine whether all or most ProCon vectors have the expected structure.

In summary, here it was demonstrated that the replacement of the U3 region of the 3' LTR with a heterologous viral promoter resulted in a vector that was still able to produce viral particles without a drastic change in the titre being observed. This vector also behaves as expected at the molecular level throughout the whole transduction cycle. Moreover, using a different packaging protocol and pseudotyping with the vesicular stomatitis virus G envelope protein, it is possible to generate relatively high titres which would be suitable for direct *in vivo* delivery. In addition, to overcome the limitations of titre, it may be possible to engineer replication competent retroviral vectors incorporating heterologous tissue-specific regulatory elements into their LTRs. The use of replication competent vectors has already been described (Schmidt and Rethwilum, 1995; Jespersen *et al.,* 1999; Barsov *et al*., 2001). Although development of such targeted replicating vectors remains a prospect for the future, the use of replicating vectors in the gene therapy of cancer is already being used in clinical trials with, for example, an adenoviral system (Bischoff *et al*., 1996).

4.1.3 *In vitro* Infection and Expression Analyses

The mouse mammary tumour virus long terminal repeat (MMTV LTR) is mainly active in the mammary gland of infected mice as well as in mammary tumours of mice (Günzburg and Salmons, 1992). Previous studies showed that the tissue specific expression of MMTV is governed by regulatory elements located in the LTR. These known elements include a hormone responsive element (HRE) (Beato *et al*., 1990), several negative regulatory elements (NRE; Bramblett *et al*., 1995), a mammary gland enhancer, nuclear factor 1 (NF-1), Oct-1 and TFIID binding sites (Bruggemeier *et al*., 1991; Mink *et al*., 1992; Zhu *et al*., 2000). Glucocorticoids, progesterone and androgens strongly stimulate the rate of MMTV transcription through the binding of hormone receptor complexes in the HRE region of the LTR.

The effect of glucocortoid hormones on β-galactosidase expression in MMTV-BAGgal infected cells was examined either quantitatively, by using a light emitting substrate in a photometric assay, or in a qualitative assay using histochemical staining which is less sensitive. The β-galactosidase activity was set to a value of one and the expression of the vectors then calculated in relationship to the background. Stable populations of Rat-2, CrFK and NIH 3T3 cells that had already been infected with either hybrid MMTV-BAGgal or parental BAGgal vectors were used. Cells from each of the lines were plated out and either stimulated for 3 days with the glucocorticoid hormone dexamethasone or not stimulated at all.

β-galactosidase expression was seen in both stimulated and non-stimulated populations. However, the addition of dexamethasone resulted, as expected, in enhanced expression from the MMTV promoter. Levels of induction were between 3 and 40 fold, depending on the cell type; the best induction being observed in CrFK cells. In contrast, the levels of induction from the MLV promoter were maximally 1.5 fold. This low level glucorticoid induction of murine C-type promoters has been previously reported (Miksicek *et al*., 1986).

After the biological function of the MMTV-BAGgal vector had been shown using reporter gene expression analysis, experiments were performed to determine whether or not the initiation site of RNA transcribed from the MMTV promoter carried in the U3 region was maintained after insertion of the *Mlu* I restriction site used for cloning. It is known that the spacing between the TATA box in the promoter and the transcription initiation site is critical. The RNA initiation site was determined by S1 analysis. Total RNA from infected or non-infected cells, either stimulated by dexamethasone or non-stimulated, was prepared and hybridised to a ^{32}P end-labelled *Bsa* I DNA fragment of the recloned vector. The probe protected a fragment of 90 nucleotides, placing the site of transcription initiation 1 bp upstream of the classic CAP site. This may be a result of the introduction of the *Mlu* I restriction site, which increases the spacing between the TATA box within the promoter and the classical R-U5 border by 6 bp. Clear induction of the signal in RNA derived from dexamethasone stimulated cells compared to that from nonstimulated cells could be observed. This result confirmed data obtained from an enzymatic assay for ß-gal.

These experiments show that the hybrid vector functions in non-human cell lines. Experiments were then performed to test the ability to infect primary human breast tumour cells, human breast tumour or non breast tumour cell lines and examine the expression levels in these cells. Previous work has demonstrated the ability of a solo MMTV LTR in several breast tumour cell lines (Cato *et al*., 1986; Bansal and Latchman, 1990; Yanagawa *et al*., 1991). However, none of

these experiments assessed the activity of the LTR in the context of a MMTV-BAGgal hybrid retroviral vector in either breast tumour cell lines or primary human breast tumours.

Primary human breast tumours as well as breast and non-breast tumour cell lines could successfully be infected with the MMTV-BAGgal hybrid vector. Titre experiments were performed using T-47D cells, a human breast tumour cell line, and with NIH 3T3 control cells which had previously been shown to be infectable. Infection using both the parental and hybrid vectors showed that the titre was lower in T-47D cells than in NIH 3T3 cells. The low level of infection indicates that the T-47D cells are more difficult to infect than the NIH 3T3 cells as support by titres of the parental BAGgal vector. The reduction in titre when infecting T-47D cells could be explained by a down regulation of the amphotropic receptor in breast tumour cells, though this was not examined, difficulties with G418 selection or both. Further experiments using either other breast tumour cell lines or different virus-producing cell lines (e.g AM 12, etc.) could have been made. However, the main objective was to see whether or not the hybrid retrovirus could infect human breast tumour cells.

Due to the fact that these cells are more difficult to infect, the virus particles were pseudotyped with the G protein from the vesicular stomatitis virus in order to obtain sufficient infected clones to allow the creation of a cell population, which was then used for expression analyses. Two types of human breast tumour cell lines were investigated: estrogen receptor positive and negative cells were used. The infections were carried out using on cells in a monolayer culture, but were cultivated either on collagen or in matrigel, which makes three-dimensional growth possible, prior to the expression analyses. This type of growth is not necessary for MMTV promoter activity but it has been shown that mammary epithelial cells have the ability to differentiate into alveoli structures and that breast tumour cell lines also degrade and invade ECM gels, which mimics metastatic behaviour in culture (Thompson *et al*., 1991; Bae *et al*., 1993). These conditions would be more ideal to test MMTV LTR tissue specificity. The infected cells were stimulated not only with dexamethasone but also insulin and prolactin, which are not required for transcriptional induction of the MMTV promoter but stimulate the proliferation of mammary epithelial cells (Vonderhaar, 1988) as well as human breast cancer cell lines *in vitro* (Shiu, 1979; Manni *et al*., 1986; Biswas and Vonderhaar, 1987).

The expression of ß-galactosidase from infected stable populations was analysed in the same manner as in non-human cells. All of the analysed cell populations showed ß-galactosidase expression in the absence of hormonal stimulation. However, addition of hormones resulted in enhanced levels of expression. Levels of hormone induction were between 9.1 and 1270 fold. The best induction was observed in T-47D cells, followed by ZR-75-1 and MCF7. Low levels of hormone induction were also observed in cells infected with the parental BAGgal vector, the induction being approximately 3 fold. The histochemical examination correlates with the results seen in a quantitative ß-galactosidase assay. The histological assay showed that the level of expression in stimulated BAGgal infected cells was not greatly different from non-stimulated ones. This was most probably due to the fact that histological detection of ß-galactosidase expression is not as sensitive as quantitative detection is.

MDA-MB-435 S cells were chosen as the estrogen receptor negative human breast tumour cell line, this cell line being very aggressive and causing metastases *in vivo* in xenotransplanted mice. These cells were infected with the LXPCMMTVEGFP vector, which has EGFP as the

reporter gene. EGFP detection is technically much easier in both cell culture as well as in tissues, and because of the need to do *in vivo* experiments it was preferred as it could be more easily detected in metastases than ß-galactosidase. In the absence of hormones a basal level of expression was observed whereas addition of hormones led to higher expression levels. Although a quantitative examination of EGFP expression is technically difficult, microscopic examination allowed the inducible expression to be seen clearly, whereas in cells infected with parental vector expression of EGFP could be seen regardless of the state of hormone stimulation.

It could be shown that the MMTV promoter gives high levels of inducible expression in the context of the MMTVProCon system in both human breast tumour cell lines as well as in primary tumour cultures. What about with other human tumour cell lines? To answer this question, the activity of MMTV was analysed in other human non-breast cell lines such a Panc-1 and EJ. The cells were treated in exactly the same manner as the human breast tumour cell lines to ensure that any differences observed in the expression was not due to the cultivation conditions. Hormone inducible ß-galactosidase expression was also analysed here in both quantitative and qualitative assays. In MMTV-BAGgal infected Panc-1 cells no expression could be detected, with expression after BAGgal infection being at a very low level, whereas MMTV-BAGgal infected EJ cells showed inducible expression. The inducibilty of the MMTV promoter was not as high as had been observed in breast tumour cells and the absolute expression levels were lower, as previously described (Mrochen *et al*., 1997).

Primary human breast tumour culture was established from patient biopsies. In order to test their infectability with MMTV-BAGgal virus and to determine the activity of the MMTV promoter in different individual tumour cells, primary human breast tumour cells were cultivated in similar cell culture conditions to the other cell lines investigated. Hormone stimulated infected cells were analysed using a histological assay only. A lack of primary cells meant that only one type of assay could be made. MMTV-BAGgal infected cells that had not been treated with hormones showed low levels of expression that increased greatly after hormones were added. BAGgal infected cells showed expression that was independent of hormone treatment. The expression levels in primary cells were lower than those of established cell lines due to the fact that this assay was performed 3 days after infection whereas in established cell lines all assays were made using selected populations. It was then decided to see if selection of the infected cells was possible and, if so, it would allow further experiments to be made. LXPCMMTVEGFP infected primary cells were put into G418 selection. In fact, 2-3 weeks later several clones had grown and they all were pooled together. The selected cells were then cultivated in the same way as the other cell lines and the EGFP expression was observed using fluorescent microscopy. This now allowed the highly inducible expression from the MMTV promoter to be seen well. Here it was possible to show expression from the MMTV promoter in both selected and long-term cultivated primary human tumour cells. Further experiments could have been made to both characterise the cell types present in the primary cultures as well as those cells present before and after selection. However, the main interest was to test the MMTV promoter in the context of the ProCon system. In future, systematic testing could be undertaken in order to describe and find out in which human primary tumour cells MMTV expression is both suitable and specific.

4.1.4 Mouse Models and *In vivo* Expression Analysis

An *in vivo* approach involving transplantation into SCID/bg mice was also utilised to determine whether or not the *in vitro* response is reflected *in vivo* and to complement the *in vitro* studies. Matrigel embedded human breast and non-breast tumour cells, infected with either MMTV-BAGgal/BAGgal or non-infected cells were transplanted into SCID/bg mice. $1x10^7$ cells per mouse were prepared, mixed with matrigel and immediately injected into the mammary fat pad (mfp) of SCID/bg mice. The appropriate organ for the implantation of breast cancer cells is the mfp and there is extensive literature showing that the mfp exerts growth-modulating effects on normal, preneoplastic and neoplastic mammary epithelium (De Ome *et al*., 1965). Co-injection of cells with matrigel has been shown to increase the incidence of tumours and to enhance the tumour rate (Lesoon-Wood *et al.*, 1995).

When the tumours had reached a size of 10-15 mm they were removed from the mouse and both RNA and protein lysates were prepared. Tumours arose from both non-infected as well as MMTV-BAGgal or BAGgal infected cells. Protein assays showed that in the breast tumours investigated all exhibited a high level of expression especially when compared to the parental BAGgal vector. MMTV-BAGgal infected EJ tumours also showed expression, but levels were not as high as those seen in breast tumours, and almost no expression was seen in Panc-1 derived tumours. Northern blot analysis was also made which not only indicates the level of expression but also allows the length of the transcript to be determined. A 7 kb fragment could be seen, which corresponds to a full-length transcript, in all breast tumours as well as in EJ tumours. Tumours arising from BAGgal infected EJ cells showed a stronger signal in the northern blot when compared to EJ cells that had been infected with MMTV-BAGgal but, after correlation with the ß-actin signal, it was clear that more RNA from the BAGgal was present. These could be due to technical problems. Very low-level ß-galactosidase expression could be detected in an enzymatic assay of BAGgal infected Panc-1 cells but no signal could be detected using northern blot.

LXPCMMTVEGFP and LXSNEGFP infected human tumour cells (MDA-MB-435 S and primary human tumour cells) were also simultaneously injected into the tail vein as this entry of cells into the circulatory system should enable them to travel to all areas of the body, including the liver and spleen, thus enhancing the chance of metastases developing. This work showed that the mice did indeed develop both tumours and metastases. However, a histological examination of the metastases for the expression from the MMTV promoter could not be completed. Completion of such experiments will give an insight into the level of expression from the MMTV promoter in metastases.

Future experiments could be made in which tumours could be established using a similar xenotransplantation protocol, after which a retroviral supernatant could be injected, either systemically or locally with respect to the tumour, into the mouse. This would give an insight into the efficiency of *in vivo* delivery and targeting of retroviral vectors to tumours and whether or not other organ/cell types would be infected. Subsequent analysis of the mouse organs would allow determination of whether or not infection had taken place and, if so, whether expression was present. These experiments could only be made after high titre retroviral stocks had been prepared which is currently a focus of research in the laboratory. Here it was shown that the MMTV expression in the context of the ProCon system behaved in a similar manner both *in*

vitro and *in vivo*. These results show that the MMTVProCon system, in conjunction with a suitable therapeutic gene, would be suitable for both further *in vivo* testing and potentially for an *in vivo* gene therapy treatment of breast cancer.

Transgenic mice were established as an alternative method to test the stringency of tissue specific of expression of the MMTVProCon constructs. Transgenic animals are useful tools because they give an *in vivo* insight into the ability and impact of foreign gene expression in a biological system. The promoter converted plasmid, pMMTVProCon, was linearised and introduced into fertilised mouse eggs. Genotyping was performed using PCR for both F_0 and F_1 generations. The animal breeding department had some technical problems during the establishment of the transgenic animals and unfortunately only one line was examined during this work.

Southern blot analysis was used to determine the copy number and integration pattern of MMTVProCon before expression analyses were made. The architecture of the transgene that is introduced into the germline of animals by microinjection plays an important role in the level of expression achieved. DNA is normally introduced randomly into the genome as head to tail concatemers. Due to the fact that the eukaryotic genome is organised into topologically constrained domains, random integration can lead to position effects in which transgene expression is influenced by the surrounding chromosomal sequences. Thus, in many cases, the level of transgene expression will vary over several logarithms, depending on the site of integration and expression.

Four trangenic mice were analysed and it was determined that 3 different sites of integration were present. After examining the intensity of the hybridised bands it appears that multiple head to tail or head to head integrations had taken place. The hybridised blot was then also analysed using a Phosphor Imager. Using the Fragment™ software it was possible to compare the strength of the bands with known DNA controls in order to estimate the copy number. This showed that, on average, 8 integrated copies were present.

There are several established transgenic mice lines described by others that utilise the MMTV promoter (Ross and Solter, 1985; Leder *et al*., 1986; Stewart *et al*., 1988; Ross *et al*., 1990; Mok *et al*., 1992), but none in the context of a MLV retroviral vector in a provirus form, as here using the recloned ProCon MMTVBAGgal. This approach allowed not only the tissue specificity to be tested but also enabled an insight to be gained into the long-term expression of retroviral vectors *in vivo*. Various organs were taken from either transgenic or non-transgenic mice. Results showed that the MMTV promoter preferentially directs the expression of linked genes to the mammary gland and spleen, but additional ectopic expression has also been observed in areas such as the brain and ovary in some, a finding that has also been shown by others (Ross *et al*., 1990; Rollini *et al*., 1992), but not all mice of the same line. It has been suggested that a member of the STAT family of transcription factors plays an important role in the tissue-specific expression of MMTV *in vivo* (Qin *et al*., 1999). The fact that STAT 5a and STAT5b are expressed in a wide variety of tissues and are activated by several cytokine and growth factor receptors (Wakao *et al*., 1992; Ihle *et al*., 1995; Schindler *et al*., 1995) may indicate that the hormonal state of the transgenic mice may allow transient, ectopic expression patterns to be observed.

In summary, it could be shown that the hybrid MMTV/MLV vector behaves as expected at the molecular level throughout the whole transduction cycle and, in the infected cell, expression of the integrated provirus is controlled by the newly inserted MMTV promoter rather than the original MLV promoter. The MMTV promoter is mainly active in human breast tumour cells *in vivo, in vitro* as well as in a few other cell types tested. This has been attributed to a mammary-specific regulatory region located in the MMTV U3 region between position -1166 and -799 (Günzburg and Salmons, 1992; Mink *et al*., 1992; Mok *et al*., 1992). This region was not included previously in MLV constructs with MMTV U3 sequences inserted in the 3' and 5' LTRs (Overhauser and Fan, 1985). Transgenic mice carrying the recloned MMTV-BAG provirus have been generated and analysed for expression of provirus specific RNA in various organs. Expression from the MMTV promoter was mainly restricted to the mammary gland and spleen, mirroring the expression pattern of MMTV. Limitations of the titre of retroviral systems could be alleviated using better virus preparation methods.

4.2 The Heterologous Cellular Whey Acidic Protein (WAP) Promoter

Whey acidic protein (WAP) is the major whey protein in the milk of mice (Hennighausen *et al*., 1982), rats (Campbell *et al*., 1984), rabbits (Devinoy *et al*., 1988), camels (Beg *et al*., 1986) and pigs (porcine) (Simpson *et al*., 1998), but a human homologue has yet to be identified. Nevertheless, it may be expected that the underlying regulatory mechanisms controlling the expression of many, if not all, milk genes are similar amongst all mammals. The regulatory mechanism is involved in high level, hormone dependent and tissue-specific expression.

The WAP promoter has been divided into two regions, one proximal and one distal to the transcription initiation site. The proximal region (-50 to -150) is equivalent to a DNase hypersensitive site (HSS II) identified in the rat WAP promoter whereas the distal region (-700 to 800 in the rat promoter) co-locates with a second hypersensitive site (HSSI) (Li and Rosen, 1994). These regions contain consensus-binding sequences for a number of transcription factors that have been associated with mediation of mammary specific gene expression. Detailed analysis of this region indicates that it contains several binding sites for the transcription nuclear factor I (NF-I) which plays a key role in the *in vivo* regulation of the WAP promoter (Li and Rosen, 1995). Surrounding the NF-I binding sites there are several specific glucocorticoid receptor (GR) binding sites present (Li and Rosen *et al*., 1995). Downstream from the GR and NF-1 binding sites, a consensus interferon γ-binding site has been identified (Li and Rosen *et al*., 1994). This site appears to mediate the prolactin responsiveness of the milk-protein genes by tyrosine phosphorylation of a unique member of the STAT (signal transduction and activation of transcription) family of transcription factors, designated mammary gland factor (MGF) or STAT5 (Wakao *et al*., 1994).

4.2.1 Establishment of WAP Transgenic Mice

It has been shown previously that transgenic mice harbouring either a 2.4 kb mouse WAP promoter fragment WAP2 (WAP2-hGH), containing both proximal and distal promoter elements, or WAP1 (WAP1-hGH) harbouring a 92 bp mouse WAP promoter which carries only part of the proximal promoter, show pregnancy specific expression in the mammary gland (Günzburg *et al*., 1991). This means that the distal region of the WAP promoter is not absolutely required for pregnancy dependent expression, although it may play a role in regulating the level

of expression. It has also been reported that the distal element of the rat WAP promoter is essential for tissue specific expression in mice (Li and Rosen, 1994) and yet a minimal mouse WAP promoter fragment containing only part of the proximal region is able to give low level, but pregnancy dependent, expression. Here, experiments were made in which the complete proximal promoter region of the WAP promoter was present, but the distal region was not. A 405 bp fragment of the WAP promoter including the complete proximal promoter (WAP3) was studied using transgenic mice. In order to be able compare the results with previous published data, the same hGH reporter gene was used in this study.

Two different mouse lines were generated and analysed for expression of the transgene. Southern blotting showed that the mice carried an estimated copy number of between 71 and 155 integrated copies of the transgene, respectively, integrated as concatamers. According to the intensity of the hybridised bands it was hypothesised that multiple head to tail or head to head integrations had taken place. This could happen when, after the linearisation of the plasmid with a restriction enzyme, the fragments formed concatamers before being incorporated into the genetic information of the fertilised ooyctes. The other additional bands are result of chimeric transgene-flanking mouse DNA at the borders of the concatamer. The stringency of tissue specificity of WAP3-hGH was analysed in various tissues of pregnant/non-pregnant female transgenic mice and also in either male transgenic or female non-transgenic mice using both RT-PCR as well as quantitative real time PCR. In examined mammary tissue, expression could be detected in all pregnant samples and some of the non-pregnant ones. Interestingly, the WAP-3hGH construct also displayed some expression in the cerebellum. Similar results were seen in the cerebellum of mice carrying the WAP2-hGH construct but not in the WAP1-hGH construct (Günzburg *et al*., 1991). Similar ectopic brain expression specifically from the WAP promoter has also been previously reported (Wall *et al*., 1996; Barash *et al*., 1999).

It has also been shown that mutation of only one of the two NF1 sites or the GAS element in the distal region of the rat WAP promoter is enough to severely affect or even abolish expression (Li and Rosen, 1994). In contrast, here a 405 bp fragment (WAP3) containing only the proximal region of the mouse WAP promoter is sufficient to direct pregnancy dependent expression of a coupled gene in transgenic mice. Nevertheless, the interpretation of this finding may be more complex, since a negative regulatory element (NRE) has also been mapped as being located between the proximal and distal regions (-93 to -411). This NRE limits expression from the promoter in cell culture (Kolb *et al*., 1994) and a factor binding to it has been characterised (Kolb *et al*., 1995). This NRE may contribute to suppression of expression in non-mammary organs. In this light, it is interesting that the NRE is present in all of the constructs used by Li and Rosen (Li and Rosen, 1994) as well as in the WAP2 (Günzburg *et al*., 1991) and WAP3 constructs used here that show mammary gland specific expression. These results also suggest that the sequences from the WAP promoter required to direct brain specific expression are located between -405 and -92, relative to the transcriptional initiation site. However, there has been no other experimental evidence corroborating this and brain specific WAP regulation was not further investigated here.

The ability of a small fragment of the WAP promoter to direct mammary specific expression raised the possibility of inserting this fragment (proximal promoter and NRE) into a retroviral vector in such a way as to replace the retroviral promoter with the WAP promoter in transduced cells. Such a vector may be useful for mammary tumour specific expression of genes. Here it

was also shown that the endogenous WAP promoter is activate in a significant number of primary rodents mammary tumours. This also raises the possibility that the promoter, in particular the WAP3 fragment used here, would be active in human mammary tumour cells for potential use in gene therapy.

4.2.2 Construction of a Hybrid Retroviral Vector with the Cellular WAP NRE Promoter and its Infectivity *in vitro*

A similar strategy as the one used for the creation of the MMTV-BAGgal hybrid vector was used. In this case, the 3' U3 region was replaced with a heterologous cellular WAP NRE promoter instead of a viral promoter. As the replacement was made with a cellular promoter, it was important to investigate whether or not any effect on virus production and infection efficiency had taken place.

The hybrid retroviral vectors (pWAPgal, pLXPCWAPEGFP) and the parental vectors (pBAG, pLXSNEGFP) were introduced into the amphotropic packaging cell line PA317 (Miller and Buttimore, 1986). The vectors showed no significant difference in transfection efficiency. Retroviral supernatants from G418-resistant virus producing cells (clones and populations) were used to infect target cells in order to test both virus production and infectivity. Titre experiments were made on NIH 3T3 cells, which are a commonly used target cell line. Following infection and subsequent selection, colonies were counted and the virus titre calculated as colony forming units per millilitre (cfu/ml) supernatant used.

The titre from the WAP-BAGgal virus producing population was, on average, $7.1x10^3$ cfu/ml compared to $8x10^3$ cfu/ml in the parental BAGgal vector. Similarly comparable values were obtained from the clones tested. LXPCWAPEGFP reached a titre of $1.9x10^4$ cfu/ml with parental vector achieving $3.5x10^4$ cfu/ml. As expected LXSN based vectors gave at least a 1 log higher titre. When comparing the vectors with one another, only a slight difference in titre could be observed. This demonstrated that the replacement of the U3 region of the 3'LTR by a cellular promoter had no drastic effect upon viral titre. This is noteworthy since, unlike the MMTV promoter, the WAP promoter has not evolved to function in the context of a retrovirus.

The titre achieved here is not particularly high and not enough for use *in vivo*. However, clones could be screened to obtain a high producer for *in vivo* use and combined with other, previously described, methods to achieve high titres. Work using the MMTV based retroviral vectors had already shown that pseudotyping of the hybrid vectors was possible and this was then tried with the WAP based vectors. Additionally, pseudotyping could also be utilised to enable further efficient infections of other cell types. Using the same packaging protocol in which MMTV hybrid retroviral particles were pseudotyped with the VSV-G envelope protein, WAP hybrid pseudotyped particles were generated. Viral titres were calculated following G418 selection after infections and limiting dilution experiments.

The virus titre from populations of either BAGgal or WAP-BAGgal pseudotyped retroviral supernatants showed at least a one fold increase from 10^3 to 10^4 cfu/ml and the titre of LXSNEGFP or LXPCWAPEGFP increased from 10^4 to 10^5 cfu/ml. Similar results could be seen in virus producing clones. Here, it was demonstrated that the hybrid vector containing a cellular promoter, irrespective of whether it was BAG or LXSN based, could be successfully

pseudotyped with VSV-G and that the modification of the U3 region of 3' LTR does not have a drastic effect upon virus production and infectivity after the incorporation of the cellular WAP NRE. It has been shown that by VSV-G pseudotyping with wild type vectors a much higher titre (10^8-10^9 cfu/ml) could be achieved (Yang *et al*., 1995). Although these retroviral particles had a titre of c. 10^5 cfu/ml, which in itself is not very low, better techniques could be used to achieve titres suitable for use in *in vivo* applications.

4.2.3 Molecular Characterisation of the WAP NRE Hybrid Vector

To confirm that the replacement of 3' U3 region did not affect the whole transduction cycle and that the WAP NRE was present in the 5' LTR of the provirus in those cells infected, DNA was isolated from populations of non-infected cells and from stably infected populations of CrFK, T-47D and MCF-7 cells.

Using a similar PCR strategy to that used in the characterisation of the MMTV hybrid retroviral vector, primers specific for the WAP NRE or MLV R region in combination with a second primer complementary to the ß-galactosidase were used to amplify fragments from the DNA which were then hybridise to either WAP or MLV specific probes. It was important to show that the promoter conversion had taken place correctly in infected cells before undertaking further experiments, especially those involving expression analysis in different cell lines. The results as a whole showed the presence of intact proviruses of the predicted structure.

In order to characterise the integrated provirus (WAPProCon), it was recloned from infected CrFK cells in a similar manner to that used for the recloning of the MMTVProCon. Characterisation of infected CrFK cells could also exclude viral rearrangement as the reason for a lack of expression following infection. The WAP-BAGgal hybrid vector carries a prokaryotic origin of replication, allowing the recloning of the integrated provirus from genomic DNA. DNA was digested with the restriction enzyme *BspH* I, ligated and subsequently transformed into bacteria. Colonies, which carried a plasmid-containing, promoter-converted recloned provirus, could be isolated after kanamycin selection.

One of the recloned proviruses was then chosen for further investigation. Following restriction enzyme mapping, the recloned plasmid was further characterised using both Southern blotting and PCR. The strategies for both PCR and Southern blot were similar to those used to characterise MMTVProCon. Of course, a WAP specific fragment was used as the probe for the Southern blot. Two different primer pairs were used to confirm that the plasmid contained a promoter converted provirus. Bands of 0.9 kb and 2.1 kb were seen in the recloned promoter converted plasmid whereas in the control plasmid pWAPgal bands of 1.6 kb and 2.8 kb were present seen. Both the PCR gel and the Southern blot both indicate the presence of the WAP promoter region in both 5' and 3' LTRs.

The recloned WAPProCon vector was then sequenced in order to certify that the promoter conversion had taken place correctly at the DNA level even though a non-viral promoter had been present. The sequencing of the junctions between the CrFK flanking sequence at the 5' end of the provirus revealed that the *Sac* II restriction site and the WAP NRE sequences inserted into this site were intact. The MLV IR was also present, but it had been shortened by 2 bp, as expected, due to processing during the integration event (Goff *et al*., 1992). Similarly, the

sequence at the border between the WAP NRE and MLV R regions in the 5' LTR revealed that the WAP NRE, *Mlu* I restriction site and MLV R sequences were also intact. Sequencing was also performed from the 5' U3 region into the flanking CrFK genomic DNA and the obtained sequences aligned with database in order to determine the presence/absence of any know genes (e.g. cellular proto-oncogenes), but this was not the case. This work demonstrates that a cellular promoter in conjunction with the ProCon system functions correctly at the DNA level both during reverse transcription and integration.

In summary, here it was demonstrated that cellular promoters can be used to replace the U3 region of the 3' LTR and such a modification leads to no drastic reduction in titre or infectivity.

4.2.4 *In vitro* Infection and Expression Analysis

After the modified vector had been characterised using several methods and shown that it functioned without any changes arising from the modifications, it was necessary to test the biological activity of the reporter gene being driven by the WAP NRE promoter in both human breast tumour and non-breast tumour cell lines as well as human primary breast tumour cells.

First, it was shown that it was possible to infect human breast and non-breast tumour cell lines as well as primary human tumour cells. The titre was determined on T-47D cells and both hybrid vectors (WAP-BAGgal and LXPCWAPEGFP) showed an average titre of 10^3-10^4 cfu/ml. This was approximately one log lower than the titre on NIH 3T3 cells, an observation that was also seen when using the MMTV-BAGgal vector. The reason for the reduction in titre might be due to the difficulty in infecting tumour cells. However, this is again due in general to the low level of titre of the retroviral vectors used, since if the reduction were due to the modifications, the difference in titre between the hybrid and the parental vectors would have been expected to be greater.

To study the tissue specificity and biological function of the WAP NRE in the context of a retroviral vector, a functionally relevant cell culture model was required which recognises the complexity of the normal mammary gland and neoplastic microenvironment. It has also been shown in previous studies that WAP promoter activity requires an intact three-dimensional structure (Roskelley *et al*., 1994 and 1995) and therefore the cell lines and primary human breast tumour cells used were cultivated in either matrigel or on collagen. Cell contacts in the mammary gland (Pitelka *et al*., 1973) and basement membrane interactions (Schmidhauser *et al*., 1990; Streuli *et al*., 1995) are important to obtain a differentiated phenotype. Full differentiation requires morphological changes that are triggered by the establishment of cell-cell contacts. These interactions are necessary for mammary epithelial cells to assume correct cellular polarity and form a luminal compartment. In fact, the presence of specific extracellular matrix components induces the formation of lobulo-alveolar structures and also a number of hormones promote mammary gland proliferation *in vitro*. In addition, several breast tumour cell lines degrade and invade ECM gels, mimicking metastatic behaviour in culture (Bae *et al*., 1993; Thompson *et al*., 1991). In culture insulin, prolactin and glucocorticoids (dexamethasone) are added, which allows the maintenance of mammary explants or cell lines in a state of lactation (Topper *et al*., 1975). These three hormones are sufficient to induce differentiation of mammary epithelial cells *in vitro* (Danielson *et al*., 1984; Doppler *et al*., 1989; Reichmann *et al*., 1989) and are also required for the induction of the WAP gene.

The fact that breast organoids are inclined to grow better three dimensionally on collagen or in matrigel, allowed the first experiments with primary human breast tumour tissue to be made from patient biopsies. Infections were made in a monolayer cell culture system, as the infectivity of the virus was not known in either collagen or matrigel. The cells were then grown either in matrigel or on collagen. After 3 days hormonal stimulation (prolactin, insulin and dexamethasone) of the cells the ß-galactosidase expression was analysed histologically. Here, the human primary breast cancer cells could be successfully infected, with ß-galactosidase expression being seen in both WAP-BAGgal infected cells as well as in cells infected with the parental vector. Due to the fact that biopsy material was at a premium, primary cells that had been infected with either LXPCWAPEGFP or the parental vector were put into G418 selection. The fact that colonies were obtained showed that it was possible to successfully select primary cells that had been infected with either LXPCWAPEGFP or the parental vector. Expression levels were analysed in the selected and pooled colonies after either 3 days stimulation or no stimulation at all. The EGFP expression could observed in LXPCWAPEGFP infected primary cells that had been intensively stimulated whereas the non-stimulated cells did not show any strong visible expression. Further experiments were then made in different types of human breast tumour cells, such as oestrogen receptor positive/negative cells, which are well known and have been characterised.

Due to the fact that these cells are more difficult to infect, the virus particles were pseudotyped with the G protein from the vesicular stomatitis virus in order to obtain sufficient infected clones to allow the creation of cell populations, which were used for expression analyses. Cells in 3-D culture conditions and after hormonal stimulation were examined either quantitatively, by using a light emitting substrate in a photometric assay, or in a qualitative assay using histological staining.

MCF-7, ZR-75-1 and T-47D cells infected with the WAP-BAGgal virus showed expression. The levels of induction were between 3.7 and 38.2 fold, depending on the cell type; the best induction was observed in T-47D cells. The histological examination correlated with the results seen in the quantitative ß-galactosidase assay.

MDA-MB-435 S cells, which are estrogen receptor negative, were then infected with the LXPCWAPEGFP virus. The stably infected cells were then cultivated in 3D culture and hormonally stimulated. A high level of EGFP expression could be observed in infected populations using fluorescent microscopy. Here, it was also observed that MDA-MB-435S cells that had grown to high confluency exhibited higher levels of expression than less confluent cells. This supports the theory that a complex structure of cells is important for expression from the WAP NRE.

S1 analysis confirmed that the WAPProCon provirus is expressed. The S1 probe, although not enabling the transcription start site to be accurately mapped, does allow the inducibility of the WAP NRE to be demonstrated. A clear induction of the signal was observed in RNA derived from cells grown on collagen in the presence of hormones when compared to that from cells grown in monolayer in the absence of the hormones.

The next investigation was to test the tissue specificity of the WAP NRE (WAP-BAGgal) in other types of human tumour cell lines and in non-human, non-tumour cell lines. These cells

were treated in exactly the same manner as human breast tumour cell lines to give the same 3D structural and hormonal conditions, although it was not clear if the hormones would have any effect on either cell differentiation or structure in these cells. The ß-galactosidase expression was analysed in both qualitative and quantitative assays. Panc-1, EJ, CrFK and NIH 3T3 cells all showed a lack of ß-galactosidase expression whereas BAGgal infected EJ, CrFK and NIH 3T3 cells showed very high levels of expression. Here Panc-1 cells showed very low levels of expression, with only a couple of weakly positive blue cells visible in the histological assay. This may even have been an artefact. The reason for the inability of the MLV to drive ß-galactosidase in Panc-1 cells is not clear.

The length of the transcript was confirmed by northern blot using RNA that had been extracted from these infected cell lines. A transcript of the expected size could be detected in all infected breast tumour cells examined, including the GR mouse tumour cell line. None of the other cell types showed expression. This correlated with results obtained in a parallel enzymatic assay.

Here it was demonstrated that the WAP NRE is indeed able to direct heterologous gene expression in retroviral vector transduced human mammary tumour derived cell lines as well as in primary cells in culture but not in other types of human tumour cells. This is unlikely to be an artefact of the cell culture system used since four independently established, human mammary tumour derived cell lines as well as primary cells showed similar expression. As has been previously observed for the WAP promoter, expression in the context of a retrovirus still requires an intact three-dimensional structure and hormonal stimulation *in vitro*, suggesting that a similar mechanism of expression control might be involved in both murine and human mammary tumours. The activity of the promoter in either the human mammary tumour cell lines or primary cells was not necessarily to be expected, particularly since a human WAP homologue has yet to be identified. Nevertheless, it seems likely that the underlying regulatory mechanisms controlling the expression of many, if not all, milk genes are similar and conserved amongst mammals.

4.2.5 Mouse Models and *In vivo* Expression Analysis

The tissue specificity of the WAP proximal promoter, including NRE, for mammary tumours was very encouraging and the next question was to ask whether this specificity of the WAP NRE would be maintained *in vivo*.
Stably infected breast tumour cells or non-breast tumour cells were transplanted mixed with matrigel (Petersen *et al*., 1992) into SCID/bg mice to grow and potentially to form metastases.

The most recommended organ for implantation of breast cancer cells is the mammary fatpad (mfp), with different routes of injection also being used to assess the ability of human cancer cells to form metastatic lesions (with MDA-MB-435 S cells). However, injection into the mfp has been shown to result in improved growth and metastasis, compared with subcutaneous (s.c) injections (Price *et al*., 1996). Here, the analysis was made using tumours that had arisen following mfp injection, as the microenvironmental conditions and presence of the stromal cells can influence the malignant phenotype of breast cancer cells (Price *et al*., 1996). It was shown that co-injection of human breast tumour cells with matrigel (into the mfp) increased the tumour incidence and the growth rate and may reflect a more physiologically relevant situation in view of the close histopathological resemblance. Estrogen is believed to be important in the

development of the progression of breast cancer (Rae-Venter *et al*., 1980; Osborne *et al*., 1985), therefore before human breast tumour cell transplantation an estrogen pellet was transplanted into the mice.

When the tumours, that had arisen from either WAP-BAGgal or parental vector infected cells, had reach an appropriate size for analysis, they were removed and processed for RNA, enzymatic and histological analysis. Three different protein lysates were used for ß-gal assay, which inherently allows for more accurate as well as more sensitive detection of expression in even small masses of tumour tissue that could be missed by histological x-gal staining of tissue or tissue sections. Here, the T-47D cells showed expression levels from the WAP-BAGgal hybrid vector that were 21.2 times that of the background, whereas the levels form the BAGgal vector were only 1.4 times that of the non-infected tumours. High levels of expression could also be seen in ZR-75-1 and MCF7 derived tumours being 27.2 and 10.4 fold background respectively. The parental vector also showed expression in ZR-75-1 and MCF7 derived tumours but was always at lower levels than those observed for the hybrid vector. The histological x-gal staining also confirmed these results. In the non-breast tumours no significant expression could be seen in WAP-BAGgal infected tumours, whereas BAGgal infected EJ and Panc-1 cells showed levels 2.5 and 1.45 fold background respectively. Histological analysis also confirmed these results.

Expression was also investigated on the RNA level using Northern blotting, which also allowed the size of the retroviral transcript to be seen. The 7 kb full-length transcript could be seen in all breast tumours irrespective of whether they had been infected with WAP-BAGgal or BAGgal infected. The non-breast tumours infected with WAP-BAGgal showed no expression whereas BAGgal infected EJ cells but not Panc-1 cells showed the transcript to be present. Despite this the Panc-1 cells did seem to show a low level of expression on the ß-gal assay.

In this *in vivo* mouse model, which more closely resembles a physiological situation, the WAP promoter was consistently more active than the promiscuous MLV promoter and was limited in expression to human breast tumours. The tissue specificity of the proximal WAP promoter plus NRE for mammary tumours was much more evident when the transduced human tumour cells were reintroduced into the mammary fat pad of SCID/bg mice.

Expression from the MMTV promoter was seen in the majority of mammary tumour cells. However, it was also observed in a range of cell types where the proximal WAP promoter was inactive. Although the WAP3hGH construct resulted in both brain- and mammary- specific expression this is not thought to be a barrier to mammary gland/tumour specific gene delivery. The retrovirus is unlikely to infect cells of the brain because the vector would have to cross the blood-brain barrier and infect nondividing cells. Concerning the latter, MLV is unable to efficiently infect nondividing cells (Miller *et al.*, 1990). The ability of the WAP promoter to function in the context of retroviral vectors is of interest for the potential use to drive therapeutic gene expression in gene therapy strategies directed against breast cancer.

Moreover, these experiments could be extended and combined with systematic delivery of modified retroviral vectors either locally or into the bloodstream instead of tranducing before transplantation into mice. This can be achieved following an improvement in vector titres, though even with extremely high titres it is to be expected that non-dividing cells can only at best be inefficiently infected.

5. SUMMARY

Metastatic breast cancer is commonly thought to be incurable, but gene therapy strategies with suicide genes are emerging as a potential treatment for metastatic breast cancer. Retroviral vectors are attractive candidates for such *in vivo* gene therapy applications. Most of these vectors contain viral promoters that are not tissue specific. In order to specifically target malignant cells while at the same time sparing normal tissue, cancer gene therapy will need to combine highly selective delivery with highly specific gene expression, specific gene product activity and, possibly, specific drug activation. Use of cell type or tissue specific promoters would allow specificity of gene expression to be achieved. It is therefore important to identify and characterise tissue/tumour specific promoter/enhancer elements in and combination with retroviral vector systems.

In this present study, the ProCon system was used to construct tissue specific vectors containing either the viral MMTV U3 region or the cellular WAP proximal promoter. It was important to characterise the vector system at the molecular level as well as investigating their targeted expression *in vitro* and *in vivo.* Validation of this system was first made with a hybrid MMTV-BAGgal vector. Here, it could be shown that the U3 from the 3' LTR could be replaced with the viral MMTV U3 region and that the hybrid vector behaves as expected at the molecular level throughout the whole transduction cycle. In transgenic mice, the ability of a small fragment of the WAP promoter to direct mammary specific expression was shown and this raised the possibility to replace the viral promoter with the WAP NRE. The replacement of viral U3 from the 3' LTR with the cellular WAP NRE showed no effect either at the molecular level or on the transduction cycle. These hybrid vectors were produced at titres ranging from 10^3 to 10^5 cfu/ml, showing that swapping of the U3 of 3' LTR with either heterologous viral or cellular sequences does not have an adverse affect on titre.

Following infection and reverse transcription, the expression of reporter gene should be under the control of either the WAP NRE or the MMTV U3 region. In order to determine the activity of the WAP NRE and MMTV U3 in the context of a retroviral vector *in vitro,* suitable cell culture conditions were established for both primary breast tumour cells as well as established tumour cell lines. Primary human breast tumour cells and breast tumour or non-breast tumour cell lines were infected in monolayer culture and were analysed in a three dimensional cell culture system for expression of the reporter gene. Here, it was demonstrated that the MMTV U3 was mainly active in human breast tumours. The WAP NRE was also able to direct heterologous gene expression in the context of a modified retroviral vector in human breast tumour cell lines as well as in primary cells in culture, but not in other types of human tumour cells. The activity of the WAP NRE in human breast tumour cells was not necessarily to be expected, particularly since a human WAP homologue has yet to be identified. An *in vivo* approach involving transplantation into SCID/bg mice allowed a determination of whether or not the *in vitro* response is reflected *in vivo* and to complement the *in vitro* studies. This work showed that mice did indeed develop both tumours and metastases. Both the MMTV U3 and WAP NRE in the context of the ProCon system behaved in a similar manner both *in vitro* and *in vivo.* Transgenic mice were also made in order to analyse the tissue specificity of the MMTV U3 region in the context of the ProCon system. A recloned provirus DNA was used for the establishment of the mice. Expression of the MMTVProCon was mainly restricted to the mammary gland and spleen, mirroring the expression pattern of MMTV.

The ability of the WAP proximal promoter and MMTV U3 region to function in the context of a retroviral vector is of interest for potential use to drive therapeutic gene expression in gene therapy strategies directed against breast cancer. Such genes could include those encoding toxic products, for example the diphtheria toxin gene or, alternatively, suicide genes allowing drug induced cell death with a bystander activity. Such strategies will be successful if ectopic expression can be avoided. In this study it was demonstrated that the proximal WAP promoter was more mammary tumour cell specific and it is therfore more suitable for use in gene therapy at this time. Whilst the MMTV U3 region, in the form used here, may exhibit ectopic expression, further experiments may identify regions or fragments that can be used without observing this phenomenon. Further, these experiments could be extended and combined with systematic delivery of hybrid retroviral vectors with a therapeutic gene either locally or into the bloodstream of xenotransplanted SCID/bg mice. In order to accomplish this, a high titre viral supernatant will be required and therefore efficient production systems will have to be developed and tested.

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7. ABBREVIATIONS

AAV	Adeno-associated virus
ADA	Adenosine deaminase
AFP	Alpha-fetoprotein
AMP	Adenosine monophosphate
Amp	Ampicillin resistance
APS	Ammonium persulfate
ATM	Ataxia telangiectasia mutation
ATP	Adenosine triphosphate
AV	Adeno-viruses
bp	Base pairs
BSA	Bovine serum albumin
CA	Capsid
CD	Cytosine deaminase
cfu	Colony forming units
CFTR	Cystic fibrosis transmemmbrane conductance regulator
Ci	Curie
CIP	Calf intestine phosphatase
CMV	Cytomegalovirus
CNS	Central nervous system
CPA	Cyclophosphamide
cpm	Counts per minute
dCTP	Deoxy-cytosine 5'-triphosphate
DMD	Duchenne muscular dystrophy
DEAE	Diethylaminoethyl
DEPC	Diethyl-pyrocarbonate
dGTP	Deoxy-guanosine 5'-triphosphate
DMEM	Dulbeccos modified eagle medium
DMFA	N, N'- Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTPs	Deoxyribonucleoside triphosphates
ds	Double-stranded
DT-A	Diphtheria-toxin A
DTT	Dithiothreitol
dTTP	Deoxy-thymidine 5' -triphosphate
EBV	Epstein barr virus
EDTA	Ethylendiaminetetra acetic acid
E. coli	*Escherichia coli*
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EtBr	Ethidium bromide
FCS	Foetal calf serum
g	Gravity constant
GCV	Ganciclovir

GFP	Green fluorescent protein
h	Hour
hGH	Human growth hormone
HIV	Human immunodeficiency virus
HSC	Haematopoietic stem cells
HSV	Herpes simplex virus
HPV	Human papilloma virus
HTLV	Human T cell leukemia virus
IgA	Immunoglobulin A
IL	Interleukin
IN	Integrase
IR	Inverted repeat
IRES	Internal ribosomal entry site
IRS	Internal repeat sequences
ITR	inverted terminal repeats
kb	Kilobase
kD	Kilodalton
LB	Luria-Bertani-Broth
LPS	Lipopolysaccharide
LTR	Long terminal repeat
lu	Light units
MA	Matrix
MHC	Major histocompatibility complex
Milli-Q	Double distilled water (aqua-bidest)
min	Minute
MEM	Minimum essential eagle medium
mfp	Mammary fat pad
MMTV	Mouse mammary tumour virus
MoMLV	Moloney murine leukaemia virus
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
NC	Nucleocapsid protein
NCI	National cancer institute
neo^r	Neomycin resistance
NLS	Nuclear localisation signal
NRE	Negative regulatory element
nt	Nucleotides
OD	Optical density
ori	Origin of replication
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PIPES	Piperazine-N,N‘-bis [2-ethane sulfonic acid]
rlu	Relative light units
PMSF	Phenylmethylsulfonylfluoride
RNA	Ribonucleic acid
RNAse	Ribonuclease

rpm	Revolutions per minute
RPMI	Roswell park memorial institute
RSV	Rous sarcoma virus
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
RV	Retroviruses
SCID	Severe combined immune deficiency
SCID/bg	Severe combined immune deficiency/beige mouse
sec	Second
SEER	Surveillance, epidemiology and results
SDS	Sodium dodecyl sulfate
ss	Single-stranded
SST	Serum separation tube
SV40	Simian virus 40
TAE	Tris-acetate
TAR	Trans activating response
taq	*Thermus aquaticus*
TBE	Tris-borate
TCA	Trichloroacetic acid
TE	Tris/EDTA buffer
TEMED	N,N,N‘,N-tetramethlylethylendiamine
tk	Thymidine kinase
TM	Transmembrane protein
TNE	Tris/NaCl/EDTA
TNF	Tumour necrosis factor
Tris	Tris (hydroxymethyl)-aminomethane
TSGR	Tumour suppressor gene replacement therapy
tTA	tetracycline-regulatable transcription factor
u	Units
UV	Ultraviolet
v/v	Volume/Volume
VZV	Varicella zoster virus
w/v	Weight/Volume
WAP	Whey acidic protein

8. PUBLICATIONS AND PRESENTATIONS

Öztürk, F., Saller, R.M., Salmons, B. And Günzburg, W.H. (1996). Tissue specific retroviral vectors for the treatment of human mammary carcinoma. 4th meeting of the European Working Group on Gene Therapy, Leiden, Netherlands.

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Renner, M., **Öztürk-Winder, F**., Gelbmann, W., Aichner, K., Müller, M., Salmons, B. and Günzburg, W.H. (1999) Cell therapy and tissue specific retroviral vectors for mammary carcinoma treatment. Keystone Symposia, Molecular and Cellular Biology of gene therapy, Salt Lake City, USA.

Renner, M., Öztürk-Winder, F., Hanak-Hammerl, A., Salmons, S., Günzburg, W.H.; Tissue- specific promoters in breast cancer gene therapy. 26 -28th November 1999, 7th Meeting of the European Society of Gene Therapy, München, Germany.

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9. CURRICULUM VITAE

Name: **ÖZTÜRK-WINDER**, Feride

Date of birth: 15th May 1967

Nationality: Turkish

Marital status: Married

Sex: Female

Education: September 1974 - June 1984, Primary and secondary education, Turkey

September 1984 - June 1988, University of Ankara, Ankara, Turkey.
Student (batchelor). Biology.

January 1989 - April 1990, Ludwig-Maximilians University, German language school for foreign students, Munich, Germany.
Learnt German to qualify to study at a German university.

April 1990 - August 1995, Ludwig-Maximilians University, Munich, Germany.
Student (Diplom student). Biology (major: human genetics.
minor: biochemistry & microbiology)

August 1994 - May 1995, Institute of Molecular Virology, GSF - National Research Centre for Environment and Health, Neuherberg, Munich, Germany.
Masters thesis (Diplomarbeit). Molecular retrovirology. "A model system for a gene therapy treatment for breast cancer using retroviral vectors"

September 1995 - January 2000, Institute of Molecular Virology, GSF - National Research Centre for Environment and Health, Neuherberg, Munich, Germany and Department of Virology, University of Veterinary Sciences, Vienna, Austria.
PhD student. "Development of a gene therapy approach for the treatment of human mammary carcinoma using tissue specific retroviral vectors"

January 2000 - date, Research associate (post.doc.candidate) at the University of Cambridge, Department of Medicine, England.
"The development of gene therapy for neurological storage disease: Niemann-Pick Type C as a Model System".

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