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STUDIES ON THE TROPISM OF CANINE ADENOVIRUS TYPE I

By

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ABSTRACT

Adenoviruses have been extensively researched for use as vectors for both gene therapy and vaccination. Gene therapy is the delivery of transgenes in order to treat genetic disease, whereas vaccination with viral vectors is primarily to induce immune responses to transgenes and in some cases the vector itself. While immune reactions against the adenovirus backbone can be desirable in vaccination strategies, this has proved to be a particular problem in gene therapy settings. The most fully investigated human adenoviruses (HAds) are serotypes 2 and 5, which are endemic in the population with most people harbouring neutralising antibodies to them. Quantifying the levels of neutralising antibodies to adenoviral vectors in patients is important as these may impede vector efficiency upon administration. Recent research has shown that this can be overcome by the use of alternative vectors based on less prevalent HAds and adenoviruses from heterologous species. However, before such alternatives can be used it is first necessary to investigate whether these viruses can express genes in cells of the target species. For development as a live vaccine vector their ability to infect cells of the target species is of interest.

Canine adenovirus type 1 (CAV-1) is a candidate vector for both gene therapy and vaccination. In its canine host CAV-1 causes a number of diseases including hepatitis. It has the ability to infect a wide range of tissues in the dog and cells from several animal species (Koptopoulos & Cornwell, 1981). In addition, limited investigations have shown CAV-1 capable of infecting human cells (Gehle & Smith, 1969). One of the aims of the presented work was to investigate CAV-1 infection in cells of two candidate species: cats and humans, using sensitive modern techniques including real-time PCR and RT-PCR. This would determine the suitability of CAV-1 as a vector in these species, primarily for vaccination in cats and gene therapy in humans.

In feline cells CAV-1 infection was productive with growth kinetics similar to CAV-1 infection of canine cells. In addition, transgene expression was demonstrated from a replication competent CAV-1 vector. DNA replication and transcription were demonstrated in human cells, as was transgene expression. Classical adenoviral cytopathic effect was not observed in human cells, however, in A549 cells CAV-1 infection resulted in the limited production of *de novo* infectious virus. These findings indicate that CAV-1 has potential for development as a vector for use in both species.

One of the features that initially attracted researchers to the development of adenoviral vectors was their ability to infect a wide variety of tissues. However, the promiscuous tropism of these vectors can lead to limited transgene expression in target tissues. Untargeted vectors can cause a number of problems such as toxicity in non-target tissues and high dosage requirements, which can lead to harmful immune responses. The fibre mediates initial adenovirus attachment to host cells and research

has shown that adenoviruses can be targeted through modification of this protein. A second aim of this work was proof-of-principle experiments to investigate whether CAV-1 could tolerate the fibre of another adenovirus serotype. CAV-1 vectors that were pseudotyped with CAV-2 and HAd5 were created that grew to high titres and displayed altered tropism in preliminary investigations. As such, a vector system was developed that will facilitate targeting through exploitation of existed targeted HAd5 fibres in their entirety or through knob exchange. In addition, fibre deleted vectors were developed. Infection of MDCK cells with these viruses did not result in cytotoxicity. However, fibre expressing cell lines allowed productive infection.

Finally, it is important to determine the levels of pre-existing antibodies in target species and therefore the neutralisation of CAV-1 by human and feline serum samples was determined. None of the feline samples had neutralising activity against CAV-1. Only 22% of human serum samples were able to neutralise CAV-1 compared with the neutralisation of HAd5 by 46% of samples. The same human samples were investigated for the ability to neutralise CAV-1 vector pseudotyped with the HAd5 fibre. The results showed that neutralisation of this vector by human sera was related to that of CAV-1 and HAd5.

In conclusion these results demonstrate that CAV-1 can express transgenes in feline and human cells, that there is potential to modify its existing tropism and that there are low (or no) neutralising antibodies to it in both species investigated.

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DECLARATION

The work described in this thesis took place at the Department of Veterinary Pathology at The University of Glasgow Veterinary School. Practical work was carried out between December 1999 and December 2002. The author was personally responsible for all the practical work completed.

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CHAPTER 1

Introduction

1.1 INTRODUCTION

Gene therapy in its broadest sense is the amelioration of disease by delivery of genes to target cells and tissues. There are over 4000 known genetic disorders, of which only a few can be treated by conventional medicine and where treatments exist they are often inefficient. The aim of therapeutic gene therapy is to deliver and express transgenes without causing further disease. Another application of gene therapy is in the treatment of cancer either by targeting genes or lytic viruses to tumour cells, which will lead to their destruction, or by stimulating the immune system to reject tumours. Another example of gene therapy working with the immune system is in the administration of vaccine vectors.

Current research explores the delivery of genes to patients by both viral and non-viral processes. Non-viral gene therapy involves briefly disrupting the cell membrane by either chemical or physical methods in order to allow direct delivery of the therapeutic DNA to the cell, where the transgene is expressed. In contrast, virus based gene therapy utilises the natural ability of viruses to target specific cells in the body, cross the plasma membrane and enter the cell, where, integration into the host chromosome or genomic persistence occurs and viral gene expression occurs. Manipulation of virus genomes to accommodate heterologous genes allows the delivery and expression of these transgenes in host cells.

Non-viral vectors are usually only capable of low levels of gene expression, which is typically transient. For the treatment of certain genetic diseases and forms of cancer, short-term gene expression is sufficient, however, other diseases demand long-term expression. Transient gene expression is a feature of some viral vectors, however, some viruses integrate their genome into that of their host cells or maintain episomal copies part of their normal life-cycle and are maintained for the lifetime of the cell. This attribute is being exploited in the development of vectors for the treatment of patients that require stable expression from a therapeutic gene. However, immune responses against virus vectors continue to cause problems and the search for improved gene delivery

systems is ongoing. Both non-viral and viral vectors have a great deal to offer gene therapy and the choice of vector is determined by the nature of the disease. This chapter investigates some examples of these vectors and their applications.

1.2 NON-VIRAL GENE THERAPY

Around 20% of gene therapy trials to date have used non-viral vectors (Journal of Gene Medicine 2001). In general, non-viral vectors have a high safety profile, but low efficacy, in comparison with viral vectors. The various methods include chemical transfection, injection of purified DNA, DNA transfer mediated by carrier lipids, electroporation, ultrasound and gene gun.

Chemical transfection is a relatively simple method of gene delivery, achieved by complexing DNA with a chemical such as calcium phosphate. The complex causes disruptions in the cell membrane and enters the cell, where expression of the gene takes place (Loyter *et al.,* 1982). Results by Werner *et al.,* in 1990 showed that DNA could be transfected into, not only dividing, but also non-dividing primary postmitotic chick embryo cells *in vivo*. This treatment did not affect cell survival but it was found that the metabolic activity of the cells was reduced. In contrast, another group demonstrated that transfection efficiency was significantly affected by the growth rate of cells (Kjer & Fallon, 1991). Transfection by this method leads to transient gene expression of low efficiency and has a high degree of variability between experiments (Werner *et al.,* 1990).

Injection of naked plasmid DNA directly into skeletal muscle, liver, tumours and epidermis results in efficient gene expression (Wolff *et al.,* 1990, Hickman *et al.,* 1994). However, this expression is transient and there are areas of the body that cannot be reached directly and as such are untreatable by this method. With this in mind a non-viral vector that could be administered either orally or intravenously may be considered an improvement for the treatment of diseases with distant target sites. Coupling DNA with cationic lipids was hoped to provide a method for achieving this. Cationic lipids have a polar head group which binds DNA, and a lipid portion that both protects the DNA from degradation in plasma and binds to the cell membrane (Felgner *et al.,* 1987, Monck *et al.,* 2000). Although DNA delivery via cationic lipids can be achieved, expression is transient and proteins have been shown to clear as quickly as a week after administration (Song *et al.,* 1997). It has also been reported that

cationic liposomes are toxic to certain cells when administered orally (Filion & Phillips, 1997). Alternatives to cationic lipids are polymers such as poly-L-lysine (Wu & Wu, 1987) and monoclonal antibodies (Schachtschabel *et al.*, 1996). Like cationic lipids, these polymers prevent the degradation of DNA but they too are rapidly cleared from serum by plasma proteins (Dash *et al.*, 1999).

Electroporation *in situ* provides another means of transducing tissues with DNA. When DNA is injected into target tissue and electric fields are applied, the cell membranes become permeable (Neumann & Rosenheck, 1972) and DNA gains access to the cells. This technique is very sensitive; varying the pulse strength and duration slightly, and changing the number of repetitions can cause large variations in the levels and duration of expression and therefore must be optimised for each individual application (Rols & Teissie, 1990, Rols & Teissie, 1998). Gene therapy provided by electroporation has proven to be successful in experimental rats and mice in a variety of dividing and non-dividing tissues, including tumour masses (Suzuki *et al.*, 1998, Heller *et al.*, 2000, Nishi *et al.*, 1996, Wells *et al.*, 2000, Yoshizato *et al.*, 2000, Goto *et al.*, 2000). Transgene expression can be maintained for periods of over 9 months and readministration is possible due to the lack of an immune response to the vector (Mir *et al.*, 1999, Rizzuto *et al.*, 1999).

Low intensity ultrasound co-administered with echo contrast microbubble (Optison) also increases the transfection of DNA to target cells when applied after the injection of DNA (Lawrie *et al.*, 2000, Taniyama *et al.*, 2002 (a), Taniyama *et al.*, 2002 (b)). Optison causes permeability of the cell membrane and is usually used as an ultrasound contrast agent as it enhances the image produced by ultrasound equipment. It consists of gas-filled microspheres surrounded by a shell of denatured human albumin. Spreading of these microbubbles over tissues is thought to induce transient holes in the cell membrane. Injection of this complexed with DNA, followed by ultrasound treatment leads to transduction of cells. This method showed a 300-fold increase in transgene activity in vascular smooth muscle cells *in vitro* compared with injection of plasmid DNA alone (Lawrie *et al.*, 2000). *In vivo* transfection of rabbit muscles with the human angiogenic growth factor, hepatocyte growth

factor (HGF), in rabbit ischaemic models was demonstrated by this method (Taniyama *et al.*, 2002 (a)). When compared with HGF delivered by plasmid alone, transfection resulted in angiogenesis characterised by increased capillary density and blood flow to these tissues. Ultrasound can be applied to almost any tissue of the body and this technique shows great promise for the treatment of genetic disease. It was also found that reduced levels of DNA could be used if coupled with Optison and low intensity ultrasound, in such cases, and no toxicity could be detected.

The gene gun is a helium propellant that delivers DNA-coated gold or tungsten particles to target cells. There have been recent reports of transduction of heart, liver and corneal tissues *in situ* (Kuriyama *et al.*, 2000, Tanelian *et al.*, 1997). When Kuriyama *et al.*, demonstrated the delivery of a plasmid carrying the lacZ gene with the gene gun, they found that there was a broad distribution of transgene expression with expression evident at sites relatively distant from the site of administration. Two days after administration, Kuriyama compared the efficiency of gene gun delivery with that of injected plasmid DNA and electroporated DNA and found that expression was significantly higher in mice that had been treated by gene gun. However, by 28 days expression had fallen approximately 5-fold and readministration would be required for longer periods of expression.

The lack of immunogenicity and toxicity induced by non-viral vectors is a major benefit to their use in gene therapy. Although the expression of transgenes is transient, short-term expression has applications in the treatment of certain diseases such as ischaemia.

1.3 VIRAL GENE THERAPY VECTORS

Viruses make efficient use of cellular processes to express genes and replicate. We can exploit viral vectors for the delivery of therapeutic genes to cells. Each family of viral vectors in development offers different potential benefits. Each varies in its potential insert capacity, target cell tropism and duration of expression within the host cell. Stable, long-term, gene expression is achieved with vectors that are capable of maintaining genome expression in the host cell i.e. by integration into the host chromosome or autonomous replication as episomes. Otherwise, expression is transient, the vector DNA being eventually degraded by cellular factors or lost through cell division. However, each virus also carries a degree of risk with it, for example oncogenicity or pathogenicity. Viral vector research aims to enhance the benefits offered and eradicate the risks conferred by these viruses to optimise them for the therapy of genetic disease.

The treatment of most genetic diseases requires the use of replication deficient viral vectors, for safety purposes. This can be achieved by deleting essential genes, which has the potential benefit of creating space for the insertion of transgenes. For the treatment of cancer, the use of viral vectors that can specifically infect and replicate in tumours (conditionally replicative viruses) has been shown to lead to the regression of tumours. In some cases, the induction of immune responses against these vectors has aided this regression (see sections 1.3.1, 1.3.3, 1.4.3.5).

Humoral and cellular immune responses induced by viral infections, inactivate viruses and are cytotoxic to infected host cells. Although these responses can be utilised for the treatment of cancer, they must be diminished in the development of viral vectors for replacement therapies. It is therefore vital that the immune responses against candidate viruses are understood when developing a gene therapy vector.

1.3.1 The Immune Response to Viruses

The following information is reviewed in greater depth by Whitton & Oldstone, 2001, and Roitt, 1997. The immune system can be functionally divided into non-specific (innate) and antigen-specific (adaptive) immune responses. Antigen-specific immune responses involve the action of B and T lymphocytes that both destroy microbes and provide memory in order to prevent future attack. Non-specific responses kill pathogens and also direct the specific immune effectors to the area of infection. Although these are classified separately, they work together closely in order to strengthen the defence against infectious agents.

The innate immune response is the body's first line of defence against microbes and it becomes active within minutes of infection. There are various branches of the innate immune system, including interferon (IFN), macrophages and natural killer cells (NK cells). IFN inhibits viral replication by inhibiting protein synthesis and degrading viral RNA. It upregulates cellular proteins that are involved in the immune response e.g. major histocompatibility complexes (MHC) (see later in this section) and activates other cytokines and cells of the immune system such as NK cells and macrophages. Macrophages can limit viral spread either by killing virus infected cells or by inducing molecules such as IFN or TNFα, which selectively lyses infected cells. NK cells either kill viruses by perforin induced cytolysis or by the induction of cytokines. Later in infection, and in conjunction with the adaptive immune response, NK cells recognise IgG antibody-coated cells surfaces and kill these target cells.

The endocytotic uptake of virions by cells with antigen presenting potential, such as macrophages, dendritic cells or B cells, leads to the induction of the specific immune response. Virus containing endosomes fuse with lysosomes and are proteolysed. These vesicles then interact with major histocompatibility complex type II molecule (MHC-II) containing vesicles, which leads to the presentation of degraded viral antigens by MHC-II molecules at the cell surface. Antigen presenting MHC-II molecules are recognised by 'helper' CD4 T lymphocytes.

Clonal expansion of the interacting T cell follows and the helper T cells secrete cytokines which lead to the activation and differentiation of other immune cells such as CD8 T lymphocytes, NK cells, macrophages and B cells.

B cells, the main component of the humoral immune system, have immunoglobulins on their surface and act as receptors for antigens. The formation of antigen-immunoglobulin complexes leads to the clonal expansion of B cells and the formation of specific antibody producing memory cells. In addition, the binding of antibodies to the surface of viruses prevent virus-cell interactions and when complement proteins bind to antibody-antigen complexes the complement cascade is activated (see later in this section).

A proportion of the proteins within every cell are degraded into peptides of approximately 9 amino acids in length. These are transported across the endoplasmic reticulum where they encounter major histocompatibility complex type I molecules (MHC-I), which take them to the plasma membrane for immune surveillance. When a virus has infected the host cell, the viral proteins produced by translation are also degraded and presented in this way. CD8 T lymphocytes, otherwise known as 'killer' T cells or cytotoxic T lymphocytes (CTLs), recognise peptides presented by MHC-I molecules and either destroy the presenting cells by releasing perforin, a protein which forms pores in the target cell, inducing apoptosis, or through the release of cytokines such as IFN and TNF.

As mentioned previously in this section, antigen-antibody complexes may activate the complement system. Inactive complement proteins are found circulating the bloodstream at all times. There are over 25 complement proteins that work in conjunction with antibodies to destroy pathogens. The binding of the first complement molecule, C1, to antigen-antibody complexes triggers off a 'complement cascade' that results in the attack of infected cell membranes, allowing the flow of molecules and fluids in and out of the cell, which as a result swells and bursts. There are other consequences of the complement system, for example, target cells are coated with component C3b, which is recognised by phagocytes and the cells are therefore engulfed and destroyed by phagocytosis.

These immune functions are beneficial to the host during wild-type viral infection, however, when they are induced against viral gene therapy vectors they may lead to destruction of the vector. Understanding of the immune response that patients mount against a vector backbone is therefore vital.

The immune responses to adenovirus vectors are discussed in Chapter 6.

1.3.2 Retroviral Vectors

The retroviruses are a diverse family of viruses that have been isolated from most species of vertebrates and invertebrates (see review by Goff 2001). The first human retrovirus to be discovered was Human T cell leukaemia virus (HTLV). In general, the effects of retroviral disease are mild, with infection resulting in life-long low-level viraemia. However, there is a small subgroup of retroviruses that causes significant disease. This includes human immunodeficiency virus (HIV) (section 1.3.3), and the transforming retroviruses, which potentially induce oncogenesis in host cells and consequently the formation of tumours.

Retroviruses have a diploid RNA genome of between 8 and 11kb that is encased, along with reverse transcriptase and other regulatory proteins, in a nucleocapsid surrounded by a lipid envelope impregnated with glycoproteins. They gain entry to host cells via interactions between the viral glycoproteins and cellular receptors. Within the cell cytoplasm the RNA genome disassociates from the capsid and envelope and is converted into double stranded DNA (provirus) by reverse transcriptase. The provirus DNA then migrates to the nucleus and is integrated into the host genome. The integrated retroviral DNA is maintained for the lifetime of the cell and expressed along with the host genome. The major genes of the retrovirus genome are the *gag*, *pol* and *env* genes, which are flanked by long terminal repeats (LTRs) that contain regulatory transcriptional elements and a packaging sequence (Ψ) at the 5'end. *Gag* and *pol* encode proteins essential for encapsidation and reverse transcription, respectively. The *env* protein encodes glycoproteins are present in the viral envelope. The LTRs are required for integration into the host genome and expression of the viral genes, acting as promoter-enhancer elements.

The most commonly researched virus for gene therapy is Moloney murine leukaemia virus (MoMLV), which has been used in almost 70% of gene therapy trials (Journal of Gene Medicine 2001). Replication defective retrovirus vectors are created by engineering plasmids which contain the desired therapeutic gene,

along with LTRs and the Ψ. These plasmids are transfected into cell-lines that provide *gag*, *pol* and *env* genes, which are essential for packaging *in trans* (packaging cell-line) (Markowitz *et al.*, 1988). The deletion of these genes from the genome allows a transgene capacity of approximately 7kb and since they have been separated from Ψ and the LTRs, only the chimeric genome can be packaged, reducing the risk of recombination events leading to replication competent viruses (Takahara *et al.*, 1992). The newly assembled viral vector can now infect cells and integrate into the host genome. In theory, the vector DNA will be maintained for the lifetime of the host cell and will be expressed along with the host DNA to produce therapeutic proteins.

Although the chromosomal insertion of transgenes is the major benefit to the use of retroviruses for gene therapy, insertion is irreversible and as such the long-term mutation of host genes is a consequence that must be considered. Also, integration of retroviral proviruses is random (Seiki *et al.*, 1984) and creates a different insertional mutation in every cell the viral vector infects, so it is possible that oncogenes will be activated and essential genes such as tumour suppresser genes will be inactivated (Hansen *et al.*, 2000, reviewed by Gray 1991). An area of retroviral research that must be expanded is the development of retroviral vectors that mediate site-specific integration into the host genome. This is likely to be achieved by creating chimeric vectors that encode for sequences known to integrate into specific areas of the host chromosome. Therapeutic genes targeted to and integrated at their natural sites are more likely to function normally. The integration site also determines the level of transcription of the transgene.

Most retroviruses can only infect cells that are dividing at the time of infection and as such cannot infect tissues such as muscle, brain, lung and liver (Miller *et al.*, 1992). However, non-dividing cells can be transfected *ex vivo* and transplanted back into the animal (Dai *et al.*, 1992). Although the *ex vivo* approach has been problematic, in that transplanted cells may not graft to tissues of the animal and the expression of therapeutic proteins is often shut off soon after transplantation (Palmer *et al.*, 1991), there has been a degree of success

with this approach (Pruchnic *et al.*, 2002). For example, Suzuki *et al.*, demonstrated expression of a green fluorescent protein (GFP) transgene for at least 8 weeks from retrovirus transduced neuronal progenitor cells grafted onto the brains of mice (Suzuki *et al.*, 2002).

The selective replication of retroviral vectors in dividing cells has been used to provide a treatment of tumours in non-proliferating tissues. A vector based on MoMLV was developed which delivered drug susceptibility genes (herpes simplex thymidine kinase [HS-TK]) to brain tumours in rats (Ram *et al.*, 1993). Upon administration of ganciclovir (normally non-toxic) to the HS-TK expressing brain tumours, the thymidine kinase phosphorylated the ganciclovir. Phosphorylation of ganciclovir results in a deoxyguanosine triphosphate (dGTP) analogue which inhibits the incorporation of dGTP, terminating DNA elongation. In this study, HS-TK expressing cells died upon ganciclovir administration and the tumours regressed. Alternatively, treatments for cancer could include the use of retroviral vectors that are targeted specifically to tumour cells and either induce apoptosis or provide tumour suppressor genes (reviewed in Nielsen & Maneval, 1998).

Retrovirus based gene therapy has great prospects for a range of genetic diseases, including cancer. Nevertheless, in 2002 retrovirus based gene therapy suffered a serious set back (Marshall, 2003, Kaiser, 2003). A research group in France had previously restored the immune systems of 9 out of 11 X-linked severe combined immunodeficiency (X-SCID) sufferers using a retroviral vector. However, within 4 months of each other, 2 patients were diagnosed as suffering from leukaemia. In both patients the vector had inserted within or close to a gene, LMO2, which has been associated with leukaemia when abnormally expressed. Such events highlight the need for development of retroviral vectors with an improved safety profile, perhaps through targeting of integration site, and for appropriate risk-benefit analyses.

1.3.3 Lentiviral Vectors

Viruses from the lentivirus subclass of the retrovirus family have the potential to infect both dividing and non-dividing cells. The best known of these is the human immunodeficiency virus (HIV) (reviewed by Freed & Martin, 2001). HIV infects cells expressing the CD4 epitope, which is present on two cell types of the immune system: CD4 helper cells and monocytes. It replicates in these cells and kills them, compromising both arms of the immune system and leaving the host vulnerable to infection. This eventuality is preceded by an asymptomatic carrier state that can last for 10 years or longer before acute disease occurs, known as acquired immune deficiency syndrome (AIDS).

Like other retroviruses, lentiviruses have *gag*, *pol* and *env* genes, LTRs defining the ends of the genome, and a similar replication cycle. In addition, they have six accessory genes termed *tat*, *rev*, *vpr*, *vpu*, *nef* and *vif*, which augment viral infectivity through modulation of the host cell and the enhancement of viral expression, assembly and release. The full range of function for each of these accessory genes is yet to be elucidated (see Klimatcheva *et al.*, 1999 for a review).

The development of lentiviral vectors is difficult as a result of the complexity of the lentivirus genome. For example, it is essential that the *vpr* gene is provided *in trans* for the propagation of *vpr* deleted vectors. However, *vpr* gene products are cytotoxic to cells and initial attempts to develop packaging cell-lines were therefore problematic (Planelles *et al.*, 1995). Nevertheless, the development of inducible cell-lines that expressed all HIV-1 proteins except the envelope polyprotein overcame this problem and modified HIV vectors were produced to relatively high titres (Kafri *et al.*, 1999).

HIV has all the advantages of the other retroviruses, such as insert capacity, and long-term expression of genes through integration, and additionally it can replicate in non-dividing cells. Although the natural tropism of HIV is limited to CD4 positive cells, it can be modified by replacing the *env* glycoprotein with that

of another virus, providing the vector with an extended range of infectivity. This is generally performed in trans and is called pseudotyping. Expression of β-gal from an HIV-based vector pseudotyped with the neurotrophic vesicular stomatitis virus (VSV) envelope glycoprotein was demonstrated in rat brains *in vivo*. This expression was maintained for over 3 months after a single administration (Naldini *et al.*, 1996a, Naldini *et al.*, 1996b), highlighting the retargeting of this vector, the transduction of non-dividing cells and maintenance of expression.

As with all viral vectors, a major concern with the use of lentiviruses for gene therapy is the risk of development of replication-competent recombinants or regeneration of wild-type virus. The LTRs are necessary for both the integration and transcription of lentiviruses and as such it is also necessary to provide the LTR for vector integration. Self-inactivating vectors have been developed which have a partial deletion in the LTR that prevents transcription from the LTR without affecting integration of the vector (Zufferey *et al.*, 1998, Deglon *et al.*, 2000), therefore preventing the generation of recombinant replicative viruses.

Considering the highly pathogenic nature of wild-type lentiviruses, the safety of vectors based on these viruses must be ensured. When this safety is achieved with confidence, it is expected that lentivirus vectors will be powerful tools for long term therapeutic gene therapy.

1.3.4 Herpesvirus Vectors

Herpesviruses are DNA viruses that primarily infect the nervous system but also have the ability to infect other cells such as muscle, liver, lung and tumour cells (reviewed by Roizman & Knipe, 2001, Roizman & Pellet, 2001). The herpesvirus family includes herpes simplex viruses (HSV), varicella-zoster virus (VSV), epstein-barr virus (EBV) and cytomegalovirus (CMV). These viruses have complex genomes of 100 to 250kb, expressing approximately 80 genes that are wound around a protein core and are enclosed in an icosahedral capsid. The capsid is surrounded by a proteinaceous tegument, which is in turn encompassed by an envelope embedded with glycoprotein spikes.

The most intensively researched herpesvirus for gene therapy is HSV-1. Many of the herpesvirus genes are nonessential and can therefore be replaced with transgenes. As such herpesvirus vectors have potentially large insert capacities. HSV-1 has a capacity of approximately 150 kbp, which allows expression of multiple transgenes from the same vector (Krisky *et al.*, 1998a, Krisky *et al.*, 1998b). The two main types of HSV-1-based vectors developed are recombinant virus vectors and amplicon vectors. Recombinant HSV-1 vectors can be either replication-conditional or replication-deficient. Replication-conditional HSV-1 vectors, which replicate only in tumour cells and destroy them, have been designed and replication in brain tumours without associated toxicity was demonstrated (Rampling *et al.*, 2000, Papanastassiou *et al.*, 2002). On the other hand, replication-deficient vectors have had essential functions deleted with the purpose of stably expressing therapeutic transgenes in target cells in the absence of cytotoxicity (Krisky *et al.*, 1998a, Samaniego *et al.*, 1998). However, it has proven difficult to eliminate all viral cytotoxicity (Toda *et al.*, 1999, Todryk *et al.*, 1999) and this has led to the development of HSV-1 amplicon vectors. These are plasmids that carry the HSV-1 origin of replication and packaging signal along with a transgene. Propagation of amplicon vectors requires cotransfection of these plasmids with helper plasmids that lack the packaging signal but contain essential HSV-1 genes (Fraefel *et al.*, 1996, Saeki *et al.*, 1998, Stavrapoulos & Strathdee, 1998). However, cytopathic effects from the viral packaging proteins

are still difficult to remove completely (Fraefel *et al.*, 1996) and as such strong immune responses are developed against amplicon vectors (Wood *et al.*, 1994).

HSV has two separate life cycles, latent and lytic. During the latent cycle only the latency (LAT) promoter is expressed. The LAT promoter is a useful site for insertion of a transgene to ensure its stable expression during latency. Expression of transgenes under the control of the LAT promoter in a herpes simplex virus type I (HSV-I) vector has been shown to last for up to 18 months (Carpenter & Stevens, 1996). The lytic life cycle produces many virus particles that leave the cell by budding, enveloping the particles with the cellular plasma membrane. This budding eventually leads to death of the cell. The lytic cycle may be utilised in gene therapy for the destruction of tumour tissue (Toda *et al.*, 1999, Todryk *et al.*, 1999), although this requires the specific transduction of tumour cells.

Herpesviruses attach to host cells via glycoproteins embedded in their lipid envelope (Rajcani & Vojvoda, 1998). Like retroviruses, vectors based on herpesviruses can be targeted by replacing the cell surface glycoproteins with ligands attracted to different receptors or surface determinants from other viruses (Laquerre *et al.*, 1998).

The replication of herpesviruses within neurones makes them particularly suitable for the treatment of neurological diseases. Their very large insert capacity and the possibility of long-term gene expression are also advantages. However, these are hampered by the inherent cytotoxicity of herpesvirus proteins and the induction of powerful immune responses. Further development of these vectors is required in order to optimise them for use in gene therapy.

1.3.5 Adeno-Associated Virus Vectors

Adeno-associated viruses (AAVs) are the sole members of the genus Dependovirus of the parvovirus family (reviewed by Muzyczka & Berns, 2001). Those that infect humans have not yet been associated with disease, although other parvoviruses are able to cause significant infection in the human host. For example, parvovirus B19 causes the predominantly childhood illness commonly known as fifth disease that usually causes a mild rash and is often preceded by a fever and malaise. However, infections with B19 are more problematic in immunosuprossed patients, pregnant women or people suffering with haematological diseases such as chronic anaemia or sickle cell disease (reviewed by Katta, 2002). Despite the lack of AAV pathogenesis in humans, 87% of the population have anti-AAV type 2 antibodies and the less common AAVs range from 20-60% in prevalence (unpublished data Li & Samulski [Walsh & Chao, 2002]). AAVs possess a single stranded DNA genome of approximately 4.7 kb, which has 2 open reading frames (ORFs) and is encapsidated by three structural proteins, VP1, VP2 and VP3. The left-hand ORF, *rep*, encodes for proteins involved in replication, gene expression and integration; the right-hand ORF, *cap*, encodes for the capsid proteins, all of which are essential for formation of virus particles (Hermonat *et al.*, 1984). These ORFs are flanked by ITRs containing a packaging sequence. AAVs are capable of replication in both dividing and non-dividing cells (Flotte *et al.*, 1994). However, they are unable to replicate independently and require factors from a helper virus such as adenovirus or herpes simplex virus. In the presence of a helper virus, AAV infection results in a productive infection. The lack of a helper virus leads to integration of the AAV genome specifically into human chromosome 19 where a latent infection is established. If the cell is subsequently infected by a helper virus the integrated genome is recovered and AAV replication takes place. As such the host range of AAV is determined by that of the helper virus. .

AAV vectors can be produced by providing the ITRs and Ψ *in cis* and the *cap* and *rep* genes *in trans* (Samulski *et al.*, 1989). The production of AAV vectors

also requires a helper virus that is attenuated for safety purposes e.g. E1 deleted adenovirus (Samulski *et al.,* 1989) that must be removed before administration. AAV genomes have packaging limitations of up to 108%, and therefore have a potential capacity of only 5 kb (Dong *et al.,* 1996, Rolling & Samulski, 1995). This 5 kb includes the ITR, packaging sequence and any chosen promoter and enhancer elements, limiting the possible applications of an AAV vector for gene therapy e.g. the coding sequence of the cystic fibrosis transmembrane conductance regulator (CFTR) approaches 4.5 kb, with additional essential sequences the limit of 5kb will be exceeded.

A feature of AAVs is their site-specific integration (Kotin *et al.,* 1990), which requires expression of the *rep* protein (Kearns et al., 1996). Integration of gene therapy vectors at predetermined sites is highly desirable, however, inclusion of the *rep* gene significantly reduces the potential transgene capacity of AAVs. The limitation of small transgene capacity was overcome when Yang *et al.,* found that the ability of AAV genomes to form circular concatamers when transduced into some cells (Yang *et al.,* 1999) could be utilised to express genes that exceed the normal packaging limits (Yan *et al.,* 2000). This was carried out by transduction of two AAV2 genomes expressing separate portions of the erythropoietin gene (which alone exceeds the packaging limit of AAV2). The two genomes formed concatamers in cells and underwent intramolecular splicing. This splicing lead to the expression of the erythropoietin gene both *in vivo* and *in vitro* in muscle cells. Duan *et al.* also exploited this feature of AAV by the cotransduction of one AAV vector containing enhancer elements with a second containing the transgene of interest (luciferase) with or without the SV40 promoter (Duan *et al.,* 2000). The dual-vector without a promoter increased expression of luciferase 200-fold compared with a single AAV vector, and the dual-vector with the SV40 promoter increased expression 600-fold.

Although high proportions of the population have anti-AAV antibodies, neutralising antibodies to different AAV serotypes are non-cross-reactive (unpublished data Li & Samulski as referenced by Walsh & Chao, 2002). Therefore, immunological testing and the availability of a range of AAV serotype vectors are likely to limit the restrictions posed by pre-existing immune

responses. The site-specific integration of AAV vectors could result in long-term transgene expression without the risk associated with random integration provided that modifications were made to prevent mobilisation of the vector upon natural helper virus infection.

1.3.6 Other Viral Vectors

In recent years there has been increased interest in more diverse viruses. Examples of these include Kunjin virus, poliovirus and Semliki Forest virus from the flavivirus, picornavirus and alphavirus families, respectively (see Hewson 2000 for a review). As with the other viral vectors described in this chapter, each of these comes with its own advantages and disadvantages for gene therapy application. For example, Semliki Forest virus is an alphavirus with neurotropism. It has been found to express high levels of proteins in the host cell cytoplasm for relatively short periods of time and would therefore appear to be a suitable candidate for transient delivery of genes for the treatment of neural disorders. However, this virus induces a severe cytopathic effect that results in host cell death within days of infection and as such it is necessary that rigorous investigations and significant modifications to SFV are made in order to prevent unwarranted replication in patients. Nevertheless, this cytotoxicity could be used in the treatment of tumours. Replication-deficient SFV vectors which have had their structural genes removed (packaged using helper viruses) have been shown to be efficient in causing the regression of tumours (Murphy *et al.,* 2000).

As with other viral vectors, detailed characterisation of these viruses is necessary prior to their use as gene therapy vectors. It essential that the life-cycles of any new candidate viral vectors are well understood and that essential and pathogenic genes are identified in order to ensure safety before they can be used as gene therapy vectors.

1.4 ADENOVIRUS VECTORS

There are over 100 different serotypes of adenovirus classified in either Aviadenoviridae or Mastadenoviridae genera of the Adenoviridae family. They have a broad range of hosts with new members being discovered continually. Natural adenovirus hosts include humans, dogs and farm animals such as sheep, pigs, goats, cattle, chickens and turkeys; they have also been found to have simian, murine and reptilian hosts (McFerran *et al.*, 1971, Kawamura *et al.*, 1972, Lehmkuhl & Cutlip, 1999, Darbyshire *et al.*, 1965, Sarma *et al.*, 1965, Simmons *et al.*, 1976, Fong *et al.*, 1965, Missal *et al.*, 1969, Clark *et al.*, 1973). Of the adenoviruses isolated, 51 are human adenoviruses (HAds) that have low pathogenicity, causing primarily mild respiratory distress and occasionally ocular, gastrointestinal and urinary tract diseases. Of these the most intensively researched have been human adenoviruses types 2 and 5 (HAd2 and HAd5) and from this work there is now a wealth of information about the adenovirus structure and life cycle that is applicable to many other serotypes.

1.4.1 Structure of Adenoviruses

Adenoviruses are non-enveloped viruses that have a linear double stranded DNA genome, which ranges in size between serotypes. The frog adenovirus genome is the smallest known, approximately 26 kb (Davison *et al.*, 2000), compared with chicken embryo lethal orphan virus (CELO), the largest known adenovirus with a genome of approximately 44 kb (Chiocca *et al.*, 1996). HAd genomes are approximately 36 kb and are packaged in an icosahedral capsid approximately 80nm in diameter. This capsid is made up of 252 protein capsomeres: 240 hexons and 12 pentons that are found at the 12 vertices of the virion (see Figure 1.1). A trimeric fibre protein extends from each penton base that consists of a tail domain at the N-terminus, a long shaft domain made up of repeating beta sheet structures (van Raiij *et al.*, 1999) and ends in a globular head domain at the C-terminus. The role of the fibre in infection is the binding of the appropriate

cellular receptor. There are also four minor cement proteins (VI, VIII, IX and IIIa) present in the capsid shell that stabilise the capsid through associations with the hexons, pentons and core proteins. Within the capsid the viral DNA is covalently attached to terminal proteins at its 5' ends and is also associated with core proteins V and VII in a chromatin like structure. A protein that has unknown functions, Mu, and has a molecular weight of approximately 4000, is also found within the capsid (Hosokawa & Sung, 1976).

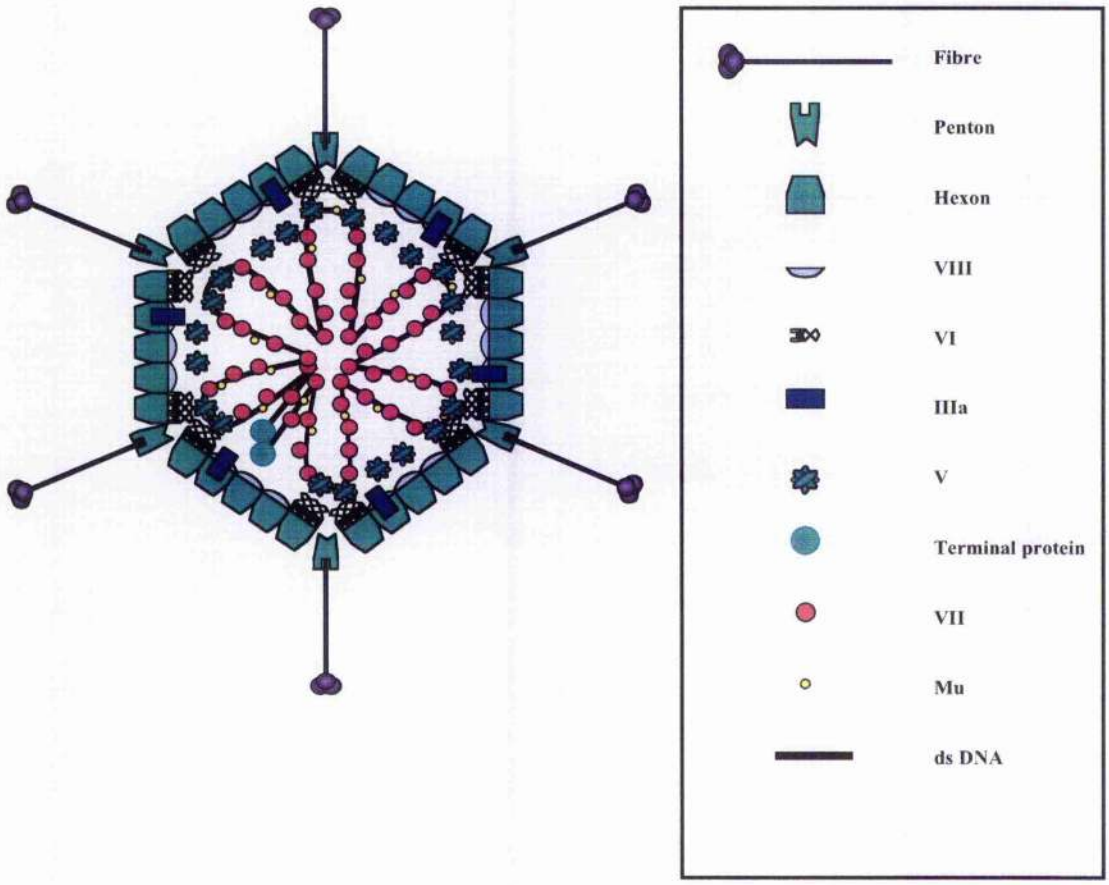


Figure 1.1 Structure of adenovirus (derived from Russell, 2000). Within the virus core, the double stranded DNA genome forms a chromatin like structure with proteins V, VII and Mu. It also has a 55Kda terminal protein (TP) located at the 5' end of each strand. The core is connected to the viral capsid via protein VI interactions with hexon proteins. The hexon, penton and fibre proteins are the major capsid proteins; they are stabilised through associations with minor capsid proteins IIIa, VIII and IX (not shown).

1.4.2 Adenovirus Life Cycle

Unless otherwise stated, the information that follows regarding the adenovirus lifecycle relates to either HAd2 or HAd5 (the wild-type adenovirus lifecycle is reviewed by Shenk 2001).

1.4.2.1 Attachment & Internalisation

The fibre facilitates attachment to the host cell membrane through high-affinity interactions between its knob domain (Henry *et al.*, 1994) and the appropriate host cell receptor. Internalisation is achieved via interactions between the penton base and a secondary receptor (Wickham *et al.*, 1993). These receptors differ between adenoviruses of different species and serotypes. In 1997 a 46 kDa cellular protein, which associated with the fibre in the attachment of HAds 2 and 5 to permissive HeLa cells was isolated and named the coxsackie and adenovirus receptor (CAR) as it is also the receptor for coxsackie B virus (Bergelson *et al.*, 1997). CAR consists of a 222-amino acid extracellular domain made up of two immunoglobulin like domains, a helical domain that spans the cell membrane and a 107-amino acid intracellular domain. It has been mapped to human chromosome 21 (Mayr & Friemuth, 1997, Bergelson *et al.*, 1997). Homologues of CAR have been identified in avian, bovine, canine, porcine, murine and rat samples, and in humans it is known to be a receptor for all HAds other than those of serotype B (Bergelson *et al.*, 1997, Roelvink *et al.*, 1998, Thoelen *et al.*, 2001). However, some of these serotypes also appear to recognise different receptors, for example, HAd41 has two different length fibres, of which only one binds CAR (Stevenson *et al.*, 1995).

In wild-type infection, after initial receptor attachment, many adenoviruses have been found to bind to cell surface integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ via an Arg-Gly-Asp (RGD) sequence found in the penton base (Mattias *et al.*, 1994). This leads to the internalisation of the virus through clathrin-coated pits that form endocytic vacuoles (Wickham *et al.*, 1993). RGD integrin binding has been hypothesised as

a common pathway for internalisation of adenoviruses since the RGD motif is conserved in most adenoviruses. Enteric adenoviruses of group F, which is comprised of HAd40 and HAd41, do not have an RGD motif, however, adsorption and internalisation of HAd41 is inefficient compared with HAds 2 and 5 (Davison *et al.*, 1993, Albinsson *et al.*, 1999). Following endocytosis the pH of the vacuole falls below 6 causing the endosome to rupture, releasing the virus into the cell cytoplasm. As adenoviruses enter host cells, their fibres are released and the virus capsid is destabilised by L3 viral protease mediated degradation of the structural protein VI, which connects the viral genome to the capsid (see Figure 1.1) (Greber *et al.*, 1993, Greber *et al.*, 1996). After entering the cytoplasm the virus particle is transported along microtubules (Suomalainen *et al.*, 1999, Leopold *et al.*, 1998) and binds to cellular receptors (fibril protein CAN/Nup214) on the nuclear pore complex (Trotman *et al.*, 2001). This induces the recruitment of nuclear histone H1, which attaches to the hexon and mediates further capsid disassembly (Trotman *et al.*, 2001). In addition, H1 is recognised by import factors that transport the viral DNA into the nucleus via the nuclear pores.

1.4.2.2 Early Transcription

Transcription of the adenovirus genome occurs in two main stages, and most of the adenovirus genome can therefore be divided into early and late transcription units i.e. E1-4 and L1-5 (Figure 1.2). There are also proteins expressed at intermediate times, between early and late transcription.

Early Region 1 (E1)

Within an hour of infection transcripts from early region 1 (E1) can be detected. The E1 transcription unit is divided into two functional regions, E1a and E1b. E1a and E1b proteins interact with a number of host cell factors to ensure a suitable environment for viral replication and the progression of the adenovirus life cycle.

The E1a gene products stimulate cell division and growth by several different methods, for example, they bind to cyclin dependent kinase inhibitors that

ordinarily inhibit cell growth (Keblusek *et al.*, 1999). The two major E1a mRNAs encode proteins of 289 (289R) and 243 (243R) amino acids in length, respectively. Although 243R, a 48kDa protein, has been found to be dispensable for viral replication in standard cell culture, it is necessary, together with 289R for adenovirus replication in growth arrested cells (Montell *et al.*, 1984). The E1a proteins also alter cellular transcription patterns through interactions with host proteins. For example, 289R binds cellular p105 (Retinoblastoma gene product) and inactivates it. p105 normally interacts with the cellular transcription factor E2F in suppressing growth of cells unless they are damaged, and activation of this transcription factor predisposes cells to transformation.

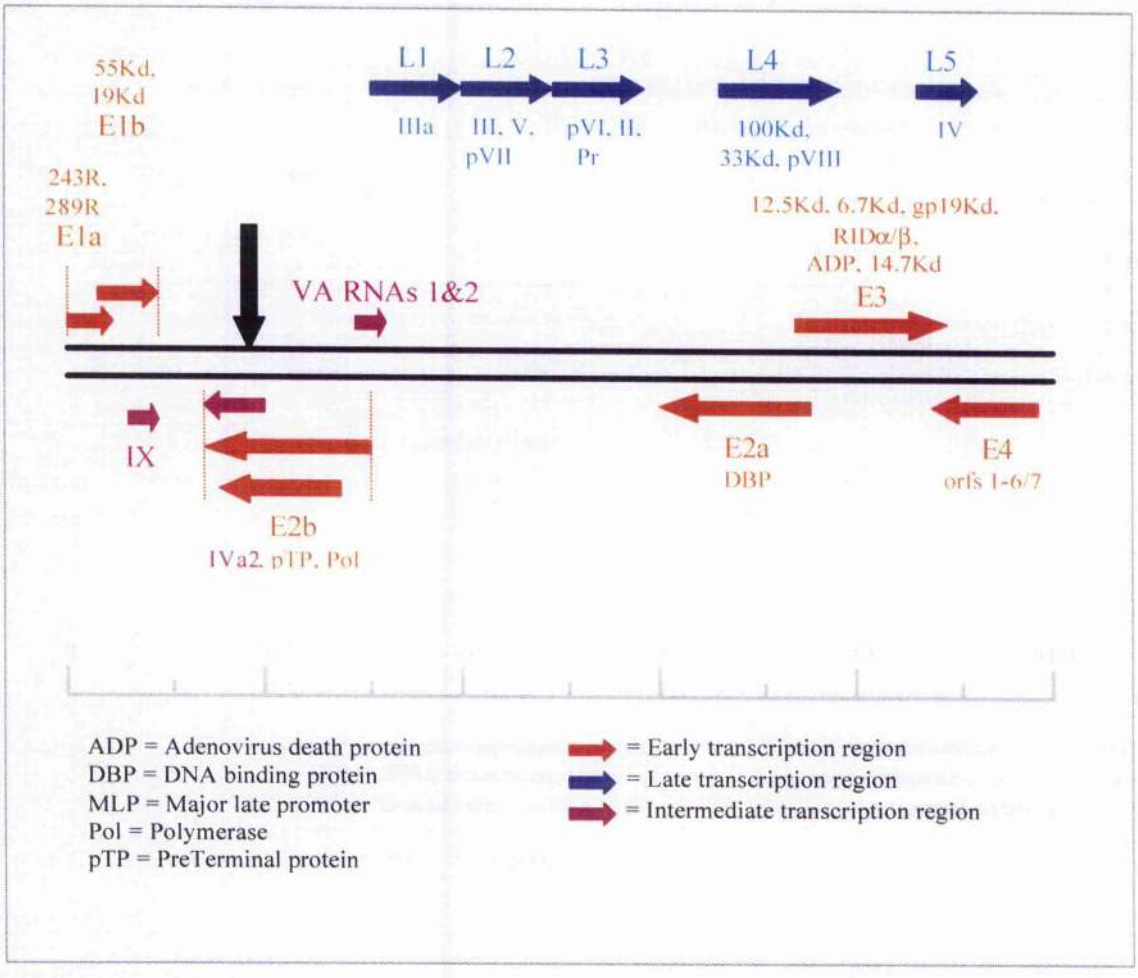


Figure 1.2 Transcription map of the human adenovirus genome (derived from Russell, 2000).

Another important function of 289R is the activation of transcription from E1b, E3 and E4 genes approximately two hours post infection (p.i.), which is closely followed by transcription of E2a and E2b and some cellular genes vital for adenovirus replication. The E1a region is also involved in the activation of late gene transcription, although it is not the sole determinant.

The E1b proteins facilitate the movement of viral mRNAs into the cell cytoplasm where translation of E2a and E2b transcripts on polyribosomes provides essential proteins for replication: the DNA binding protein (DBP), DNA polymerase and the DNA terminal protein (see 1.4.2.3). The E1b region codes for polypeptides of 19kDa and 55kDa. The 55kDa protein binds and inactivates the cellular p53 tumour suppressor protein and interacts with a 25kDa protein encoded in the E4 region to shutoff gene expression in the host cell (Babiss & Ginsberg, 1984). The 19kDa protein promotes cell survival by disabling the death-promoting *Bax* protein (Han *et al.*, 1996, Chinnadurai 1983). *Bax* normally induces both programmed cell death and necrosis in p53 defective cells, inactivation of this therefore induces cell transformation. The E1b proteins form a complex with the E4 34kDa protein in order to selectively export adenovirus mRNAs and inhibit the movement of cellular mRNAs out of the nucleus, which reduces host cell DNA transcription by more than 90% during viral infection (Pilder *et al.*, 1986, Bridge & Ketner, 1990). This is achieved by processes that are as yet unknown.

All HAds are able to transform cells in culture and human adenoviruses of serotypes A and B have oncogenic potential. The E1a region alone contains the genes responsible for immortalising host cells but the expression of both E1a and E1b is necessary for full transformation and, in certain serotypes, the formation of tumours in new-born hamsters (HAd transformation is reviewed by Akusjarvi 1986 and Graham 1984).

Early Region 2 (E2)

Transcription unit E2 is also divided into 2 regions, E2a and E2b. The E2a region encodes for the 72kDa DNA binding protein (DBP), a phosphoprotein with two domains. It has a nuclear localisation signal in the N-terminus domain (Cleghon *et al.*, 1989) and this domain has been found to play a role in the

determination of host range of different adenoviral serotypes (Anderson *et al.*, 1983, Brough *et al.*, 1985). The N-terminus of the DBP is also involved in late gene expression (Kruijer *et al.*, 1983). Both domains, although predominantly the C-terminal domain, are important for binding to both single stranded and double stranded DNA and RNA (Brough *et al.*, 1993). The C-terminal domain interacts with neighbouring DBP molecules to form a flexible chain that is required for DNA unwinding and as such DBP is essential in viral DNA replication (Dekker *et al.*, 1997, van Breuklen *et al.*, 2000). Viral DNA replication also requires the 80kDa precursor terminal protein (pTP) and 140 kDa DNA polymerase which are encoded by the E2B region. The pTP and DNA polymerase form a complex that is found covalently attached, via the pTP, to the 5'ends of the viral DNA (Challberg *et al.*, 1980, Lichy *et al.*, 1981). DNA polymerase has both 5' to 3' polymerase and 3' to 5' exonuclease activity (Field *et al.*, 1984). The action of these proteins in DNA replication is discussed in section 1.4.2.3. During virion assembly the pTP is processed, by an adenoviral protease, to the mature 55kDa terminal protein (TP), which remains attached at the 5' end of each DNA strand (Challberg & Kelly, 1981).

Early region 3 (E3)

In HAds 2 and 5, the E3 region has been found to be nonessential in tissue culture, although it encodes a number of proteins with functions that are important in immune modulation. These include a 19kDa transmembrane glycoprotein localised in the ER membrane (Cox *et al.*, 1991) that blocks the transport of major histocompatibility complex type I (MHC-I) to the host cell plasma membrane by binding both MHC-I and the transporter associated with antigen presenting (TAP) (Bennet *et al.*, 1999). Ordinarily, TAP is involved in the transfer of peptides to the MHC-I molecule; the binding of TAP and MHC-I prevents MHC-I migration from the ER to the cell surface, protecting the infected cell from CTL-mediated lysis. The receptor internalisation and degradation proteins (RID), RIDα (10.4kDa) and RIDβ (10.4kDa) proteins, together form a complex, which, in addition to the E1b 19KDa, E3 14.7kDa and E3 6.7kDa proteins, protect the infected cell from apoptosis and lysis by cellular antiviral defence proteins. For example, the E3 6.7KD and 14.7KDa proteins

and RID complex protect the cell from Fas induced apoptosis (Chen *et al.*, 1998, Shisler *et al.*, 1997, Elsing & Burgert, 1998, McNees *et al.*, 2002, Moise *et al.*, 2002). These E3 proteins and the E1B 19K protein also protect infected cells from the lytic activity of TNF (Gooding *et al.*, 1988, Gooding *et al.*, 1991a, Gooding *et al.*, 1991b, Moise *et al.*, 2002). The RID complex also stimulates endosome-mediated internalisation and degradation of the epidermal growth factor (EGF) receptor by the host cell (Tollefson *et al.*, 1991).

Another E3 protein, the 11.6kDa adenovirus death protein (ADP) (Wold *et al.*, 1984), is synthesised at both early and late stages of transcription (Tollefson *et al.*, 1992). Although ADP is transcribed from an early region of the genome it is synthesised most abundantly at late stages of infection (approximately 400-fold higher synthesis at 25 hours p.i. compared with that of early stages) (Tollefson *et al.*, 1992). Cells infected with ADP-mutants showed efficient viral replication; however, cell lysis was delayed by approximately 3 days compared with wild type HAds. ADP is clearly important for release of progeny virus particles via cell lysis (Tollefson *et al.*, 1996).

Early region 4

The E4 region is complex; it has 6 ORFs, which encode a range of proteins with a variety of function. ORFs 3 and 6 encode proteins of 11kDa and 34kDa, respectively. Both of these proteins bind cellular DNA-dependent protein kinase, preventing the activation of p53 and leading to the formation of viral DNA concatamers (Boyer *et al.*, 1999, Weiden & Ginsberg, 1994). The 34 kDa protein forms a complex with the E1b 55 kDa polypeptide that results in both the transportation of viral mRNAs to the cytoplasm (Weigel & Dobbelstein, 2000) and the binding of p53 leading to it's destabilisation and subsequent degradation (Boyer & Ketner, 2000). The E4 ORF 4 encodes a 14 kDa protein that has anticytolytic activity in conjunction with E3 proteins (Kaplan *et al.*, 1999). It has also been suggested that E4 may be involved in DNA replication and shut off of host macromolecular synthesis (Medghalchi *et al.*, 1997, Shenk 2001).

1.4.2.3 DNA Replication

Adenoviruses undergo two types of replication that begin within 6 to 8 hours of infection (Lechner & Kelly, 1977). Type I replication occurs on one strand of the parental DNA and type II replication takes place on the strand displaced during type I replication. It is hypothesised that the displaced strand is circularised through annealing of the ITRs at either end of the DNA (Leegwater *et al.,* 1988). For both types of replication the pTP and the DNA polymerase form a complex that binds, via the pTP, to the origin of replication in the ITR at the 5' end of the adenoviral DNA (Challberg *et al.,* 1980, Lichy *et al.,* 1981, Challberg & Rawlins, 1984, Temperley & Hay, 1992). Following this, the DNA polymerase recruits deoxycytidine monophosphate (dCMP), which binds to the hydroxy group of a serine residue with the pTP (Challberg *et al.,* 1982). This complex then 'jumps back' to the beginning of the template DNA strand and acts as a primer for DNA synthesis via strand displacement (King *et al.,* 1994). The DNA polymerase dissociates from pTP and elongates the strand and the pTP is later cleaved to the 55 kDa TP (King *et al.,* 1994, Challberg & Kelly, 1981). DBPs binds in a sequence independent fashion to ssDNA and form a chain that separates the annealed DNA templates during chain elongation (see section 1.4.2.2, Dekker *et al.,* 1997). DBPs also protect the displaced single stranded parent DNA from nuclease digestion during type I replication. In addition, DNA replication requires the involvement of cellular nuclear factors (NF) I and III; NFI binds to the pTP-polymerase complex, perhaps stabilising it, whereas NFIII makes contact with the pTP causing a conformational change (Mul *et al.,* 1990, Botting & Hay, 1999).

1.4.2.4 Intermediate transcription units

As well as early and late transcription units, the HAd genome has a range of intermediate transcription units, which encode virus associated (VA) RNAs I and II, and proteins IVa2 and IX. The VA genes are transcribed by RNA polymerase III and do not encode proteins (Weinmann *et al.,* 1976). VA RNA I binds to and inhibits protein kinase (PKR), which provides an important host defence against viral infections through the inhibition of translation by phosphorylation of eIR-2,

thereby preventing the shut down of protein synthesis (Pe'ery *et al.,* 1993, Ma & Mathews, 1996). The VA RNAs are also hypothesised to be involved in the regulation of splicing of cellular and viral mRNAs (Mathews, 1980, Naora & Deacon, 1981).

The IVa2 protein has a nuclear localisation signal and is found almost exclusively in the host cell nucleus (Lutz & Kedinger, 1996). It binds specifically to nucleotide motifs found in both the MLP and the Ψ (Lutz & Kedinger, 1996, Zhang & Imperiale, 2000). IVa2 is one of the adenovirus proteins involved in the activation of the major late promoter (MLP), which is responsible for late gene transcription (see section 1.4.2.5) (Lutz & Kedinger, 1996, Tribouley *et al.,* 1994). In addition IVa2 has been found to be essential for adenoviral packaging (Zhang & Imperiale, 2003) and interacts with Ψ in a serotype specific manner (Zhang *et al.,* 2001)

Expression of the IX protein (pIX) begins shortly after the onset of viral DNA replication, earlier than other capsid components (reviewed by Flint 1982). The N-terminal domain of the pIX protein is essential for its localisation in the capsid (Furcinitti *et al.,* 1989, Rosa-Calatrava *et al.,* 2001). The C-terminal domain activates transcription from both cellular and viral TATA-containing promoters (Lutz *et al.,* 1997, Rosa-Calatrava *et al.,* 2001). In addition, the pIX protein is involved in the nuclear reorganisation of adenovirus infected cells (Rosa-Calatrava *et al.,* 2001). It concentrates in nuclear inclusion bodies, however, the physiological roles of these inclusions are unknown.

1.4.2.5 Late transcription

Late transcription begins shortly after the initiation of DNA replication and is activated by a cascade of events that include protein IVa2 binding and E1a activation of the MLP. Only one RNA is transcribed, which encodes the products of the 5 late transcription units. This is subsequently spliced into at least 11 mRNAs, each encoding a single protein. All mRNAs issued from the MLP possess a 5' tripartite leader sequence, which gives them priority for translation over cellular mRNAs (Logan & Shenk, 1984) by eliminating the

requirement for the cap-binding complexes that provide binding sites for translation initiation factors (Dolph *et al.*, 1988). The late genes encode mostly viral structural proteins that self-assemble into empty capsids.

Once there is an excess of DNA molecules, interactions between viral proteins and the packaging sequence, ψ, found at the left end of the genome have been shown to initiate encapsidation of the viral genome (Grable & Hearing, 1992, Schmid & Hearing, 1998). This occurs within the host cell nucleus, on the nuclear matrix (Zhai *et al.*, 1987, Khittoo *et al.*, 1986). Progeny virions are then released by host cell lysis induced by the ADP (see section 1.4.2.2) (Tollefson *et al.*, 1996).

1.4.3 Adenovirus Vectors

HAds have been investigated as gene therapy vectors since the early 1990s (Boucher *et al.*, 1994, Bout *et al.*, 1994, Chen *et al.*, 1994, reviewed by Kozarsky & Wilson 1993). This research has provided a wealth of information regarding the host immune response towards adenovirus vectors and a wide range of transgenes. It is clear from this that there are several factors that influence transgene expression from adenovirus vectors and the immune response elicited against them. These factors include the nature of the transgene itself, choice of promoters and enhancing elements, dose of vector, delivery site and route of administration, strain/species of experimental animal used, genotype of the recipient (heterozygote or homozygote for genetic deficiency), age of recipient, immune status of the recipient and so on.

A major benefit to the use of adenoviruses for gene therapy is their large insert capacity. Human adenoviruses (HAds) have an insert capacity of approximately +5% (Bett *et al.*, 1993) and as such 1.8 kbp can be inserted into the HAd5 genome without making any deletions. In order to prevent replication of adenovirus based vectors the E1 region is usually deleted. The potential oncogenicity, if only in rodents, of the E1 region is another good reason for making this deletion.

The main obstacle to using the ubiquitous HAds 2 and 5 as gene therapy vectors is the immune response that the human body elicits against HAd based vectors (see section 5.1) (Harvey *et al.*, 1999, Yang *et al.*, 1995, Dai *et al.*, 1995). As well as increasing the insert capacity, multiply deleted adenovirus vectors have been developed that have had a degree of success in avoiding deleterious immune responses. However, suitable adenovirus gene therapy vectors that can completely evade the human immune system, provide optimal transgene expression and allow readministration are still in developmental stages.

1.4.3.1 First Generation Vectors

First generation vectors were mainly based on HAds 2 and 5, as these are the most prevalent human adenoviruses and their genomes and lifecycles have been thoroughly researched. The E1 region is deleted to render the virus replication deficient. As such, these vectors must be propagated in complementing cell-lines that express E1 gene product. The most common of these is the 293 cell-line, which was derived from human embryonic kidney cells transformed with the left 12% of the HAd5 genome and therefore contains the ITR, Ψ, and E1 regions (Graham *et al.*, 1977). Many first generation vectors also have deletions in the non-essential E3 region. Deletion of the both of these regions allows up to 8.5 kbp of insertion (Bett *et al.*, 1993).

Transgene expression from first generation HAd vectors has been demonstrated in a wide range of cell and tissue types including epithelial, renal, cardiac, and brain cells *in vivo* and *in vitro* (Rosenfeld *et al.*, 1994, Bout *et al.*, 1994, Herz & Gerard, 1993, Barr *et al.*, 1994, Ghadge *et al.*, 1995). Nevertheless, their effectiveness has been limited by short-term expression *in vivo*. In animal models, transient transgene expression has been shown to be due to strong immune responses that are elicited against the vector, which often result in liver toxicity (Herz & Gerard, 1993). Both arms of the immune system are activated in response to adenovirus vectors and administration to knockout mice lacking specific functions of the immune system has shown extended periods of transgene expression (Dai *et al.*, 1995, Yang *et al.*, 1994). The treatment of immunocompetent mice with first generation adenoviruses in conjunction with immunosuppressive drugs lengthens the period of transgene expression considerably (Dai *et al.*, 1995).

Although deletion of the E1 region was intended to block the expression of viral genes, it was demonstrated that both early and late viral genes were still expressed (Yang *et al.*, 1994) as the E2a gene can be activated by cellular E2F

proteins (Reichel *et al.*, 1988). Expression of late genes is one of the main reasons for immune induction against these vectors.

Several groups have shown that immune responses against transgenes are also a limitation in the administration of these vectors. Immune responses against certain transgenes, for example β-galactosidase, have resulted in increased toxicity and reduced persistence of transgenes (Juillard *et al.*, 1995, Michou *et al.*, 1997). In contrast, the administration of vectors expressing transgenes that were recognised as native by the host immune system resulted in longer periods of transgene expression (Michou *et al.*, 1997, Yang *et al.*, 1996).

It became apparent from this research that E1 and E3 deletions alone were insufficient for the complete prevention of viral gene expression and therefore evasion of the immune system.

1.4.3.2 Second Generation Vectors

It was generally accepted that the immunogenicity of first generation vectors was due to cellular complementation and expression of adenovirus late genes. To overcome this, further deletions in essential early regions were proposed. The majority of strategies deleted the E2a (DNA polymerase) and E4 regions. Deletions in these regions have the additional benefit of increasing the packaging capacity.

E2a-deleted second generation vectors have been investigated in both tissue culture and in vivo (Zhou *et al.*, 1996, Andrews *et al.*, 2002). Vector replication is not observed (Lusky *et al.*, 1998) and transgene expression has been demonstrated in a variety of tissues of experimental animals such as mice, rabbits and monkeys (Bristol *et al.*, 2001, O'Neal *et al.*, 1998, Andrews *et al.*, 2002). However, when compared with first generation vectors transgene expression is not extended (Morral *et al.*, 1997, Wen *et al.*, 2000) and immune responses elicited to the vectors are not reduced (Lusky *et al.*, 1998). Furthermore, vector

induced hepatotoxicity is comparable to that induced by first generation vectors (O'Neal *et al.*, 1998).

The situation with E4-deleted second generation vectors is far more complicated. E4 products have multiple roles in the HAd life cycle (section 1.4.2.2). Although low level expression of viral genes has been detected from E4-deleted vectors (Lusky *et al.*, 1998), they generally have lower cytotoxicity in animals (Wang *et al.*, 1997, Christ *et al.*, 2000). However, several groups have reported lower transgene expression from such vectors (Brough *et al.*, 1997, Wang *et al.*, 1997, Christ *et al.*, 2000). Christ et al. examined the specific roles of E4 proteins in determining transgene expression and found that vectors retaining either ORFs 3 and 4 or ORFs 3, 6 and 7 prolonged transgene expression. In addition, retention of ORFs 3 and 4 is associated with reduced hepatotoxicity (Christ *et al.*, 2000). Investigations into the induction of immune responses by E4 defective adenoviruses have produced varied reports (Gao *et al.*, 1996, Wang *et al.*, 1996, Dedieu *et al.*, 1997); however, when Lusky *et al.*, compared an E4-deleted vector with a first generation vector the immune responses were similar (Lusky *et al.*, 1998).

It is clear from work on first and second generation vectors that there are many variables to consider in the delivery of HAd vectors. Transgene expression and immune responses will not only be affected by the nature of HAd backbone but also the route and titre of inoculation, the transgene and even strain of animal used.

1.4.3.3 Third Generation/Gutless Vectors

Third generation adenoviral vectors only possess the packaging sequences, ITR and Ψ, which flank the transgene, and as such require the assistance of helper viruses for their propagation. Helper viruses are usually E1-deleted and the gutless vector is propagated in their presence on E1 expressing 293 cells (Fisher *et al.*, 1996). These vectors are otherwise known as gutless, gutted, or helper-dependent (HD) adenovirus vectors. The elimination of almost the entire viral

genome enables the insertion of up to 37 kbp of heterologous DNA (Morsy *et al.*, 1998, Morsy & Caskey, 1999), allowing the expression of large transgenes or multiple transgenes. The removal of all viral coding regions ensures that no viral replication or transcription can take place and, it was hoped that this would minimise immune responses to the vector, leading to longer term transgene expression. Although transgene expression was extended with such vectors compared to first and second generation vectors (Morral *et al.*, 1999, Roth *et al.*, 2002) initial investigations demonstrated an eventual loss of transgene expression associated with immune responses to the vector (Chen *et al.*, 1997). When this was expanded further and immune responses against transgenes investigated, several groups demonstrated that by eliminating immune induction to the transgene, expression could be maintained (Chen *et al.*, 1997, Maione *et al.*, 2001, Kochaneck *et al.*, 1996).

The successful use of HAds for gene delivery in primates has been demonstrated (Morral *et al.*, 1999). A HAd5 based HD vector expressed the human α-antitrypsin gene (hAAT) transgene for over a year in the livers of 2 out of 3 young baboon models after a single intravenous injection (Morral *et al.*, 1999). Transgene expression from equivalent first generation vectors lasted only 3-5 months, and as no hAAT antibodies were detected, this suggested that transgene loss was due to anti-HAd immune responses.

It is important to note that pre-existing antibodies to capsid proteins are likely to limit the effectiveness of HD adenovirus vectors (Parks *et al.*, 1999). Vectors derived from less common adenovirus serotypes are likely to avoid these pre-existing immune responses in the majority of patients.

1.4.3.4 Chimeric Vectors

Chimeric adenovirus vectors have capsid proteins originating from different adenovirus serotypes. The rationale behind this is that anti-adenovirus neutralising antibodies specifically recognise capsid proteins (see Chapter 5) and therefore immune responses to common adenovirus serotypes might be evaded

by developing vectors that possess capsid proteins from less prevalent adenovirus serotypes. This was demonstrated by Roy *et al,* who in 1998 developed a vector that had a chimeric HAd5/HAd12 hexon with 99.2% identity with the HAd12 hexon. Mice that had been immunised with HAd5 were unable to neutralise the HAd5/HAd12 chimera and the vector successfully transduced the livers of all mice tested. Chimeric adenoviruses have also been produced with a view to targeting adenoviruses; this is discussed further in Chapter 4.

1.4.3.5 Conditionally Replicative Adenovirus Vectors

An alternative field of adenovirus vector research is the development of conditionally replicative adenoviruses (CRADs) (reviewed by Curiel 2000). CRADs are developed to preferentially replicate within and lyse tumour cells. This is achieved by mutating or deleting adenovirus genes whose function can be complemented by the tumour cell but not normal cells. For example, the adenovirus mutant ONYX-015 has a 827 kb deletion in the E1b gene that prevents E1b inactivation of cellular p53. As such, ONYX-015 replication is limited in normal cells due to a p53 response (see section 1.4.2.2) however in tumour cells, which often lack a p53 gene, ONYX-015 is able to undergo lytic replication, destroying infected cells (Bischoff *et al.*, 1996). Human trials have proven promising with this vector, with its administration leading to the significant regression of tumours when administered with and without chemotherapeutic drugs (Nemunaitis *et al.*, 2000, reviewed by Ries & Korn, 2002). However, it was found that replication of these vectors was not strictly restricted to p53 mutated cells and replication was noted in non-tumour cells (Vollmer *et al.*, 1999). Another method of directing cytolysis is to replace the E1a promoter with that of a tumour specific promoter, so that E1a expression will only occur in tumour cells resulting in selective lysis (Rodriguez *et al.*, 1997). This technology could be used in combination with an E1b deleted vector such as ONYX-015. The main limitation with CRADs at present is that they require local administration because of the wide tropism of adenoviruses. The systemic delivery of CRADs will rely on the development of specifically targeted adenovirus vectors.

1.4.3.6 Adenovirus Vaccine Vectors

Recombinant vaccines are attenuated viruses that have genes from other pathogens inserted into their genome. These viruses enter cells and express the chosen antigens, which are then processed and presented to the immune system. Adenoviruses are well characterised and the immune response to them has been studied in depth, which has aided their development for use as vaccine vectors in replication-competent or replication-deficient form. Although these vaccines are effective at eliciting immune protection against heterologous immunogens, immunity is also elicited to the adenovirus (Ye *et al.*, 1991, Buge *et al.*, 1997).

The choice of vaccine vector is dependent on the pathogen being vaccinated against and the immune response required to protect against infection. The method of administration often dictates the type of immune response induced and, where possible, administration via the same route as wild-type antigen presentation is favourable, as it is more likely to induce immunity similar to that against the wild type pathogen. Natural adenovirus infection occurs via the airways. As such, adenovirus based vaccines are likely to offer efficient protection against other pathogens that infect mucosal surfaces. Examples of such viruses are hepatitis B, herpes simplex viruses types 1 and 2 and HIV, all of which have proven difficult to vaccinate against.

As with other forms of gene therapy, the existence of a pre-existing immune response is likely to reduce the efficiency of a recombinant vaccine vector. Many adenoviruses are capable of abortive infection in cells from hosts of heterologous species, and therefore have the potential to express transgenes in these cells (discussed in Chapter 3). The reduced possibility of patients having pre-existing immunity to an adenovirus of a heterologous species opens up the possibility of using a range of adenovirus serotypes. The examples that follow describe vaccination using adenoviruses of heterologous host species as vectors.

Vaccination of monkeys with either a replication incompetent HAd5 vector, plasmid DNA or a modified vaccinia Ankara virus vector, each expressing the simian immunodeficiency (SIV) gag protein, was compared (Shiver *et al.*, 2002). The most effective was found to be based on HAd5. Furthermore, a replication-deficient adenovirus vector induced anti-measles IgG antibodies in 100% of mice, compared with 30% in those treated with the equivalent DNA vaccine (Fooks *et al.*, 2000).

An E1 and E3 deleted HAd5 vector backbone was used to deliver glycoproteins of bovine herpesvirus type 1 (BHV-1) to calves intranasally (Gogev *et al.*, 2002). Administration induced neutralising antibodies that were still evident 3 weeks later and protected these calves from intranasal wild-type BHV-1 challenge.

Buge *et al.*, used an animal model to demonstrate that vaccination against HIV in humans using a heterologous adenoviral backbone is feasible. SIV genes delivered by a HAd5 vaccine vector induced humoral, cellular and mucosal immune responses specific to SIV in rhesus macaques (Buge *et al.*, 1997). These responses provided a degree of protection against vaginal challenge with SIV. Although full protection was not conferred, in some of the subjects the infection was transient and where the infection was persistent, viral burden was decreased compared to infected control macaques.

Large deletions from the adenovirus genome can be made that render the virus replication-deficient and also permit the expression of large transgenes. HAds have an upper packaging limit of 105% (Bett *et al.*, 1993) and large deletions allow the presentation of epitopes from multiple pathogens, producing multivalent vaccines. A vaccine that expressed 3 different hepatitis B antigens from different loci of a HAd7 vector induced significant antibody responses to each of the 3 antigens in inoculated dogs. (Ye *et al.*, 1991). Multiple deletions within the vector genome reduces the risk of reversion of vector virus.

Often vaccination that results in a rapid immune response is particularly desirable. For example, Moraes *et al.*, recently demonstrated that a single dose of a replication defective HAd 5 vaccine vector expressing foot-and-mouth

disease virus (FMDV) capsid proteins was sufficient to protect against challenge by wild-type FMDV as early as 7 days after vaccination in pigs (Moraes *et al.*, 2002).

Intranasal vaccination proved more effective than intraperitoneal administration with a HAd 5 based vector carrying the glycoprotein B gene of HSV-1 (HSV1-gB) (Gallichan *et al.*, 1993). After intranasal administration, HSV1-gB specific antibodies were produced on mucosal tissues and specific CTLs were found on the spleen and lymph nodes; intranasal challenge was protected against. However, intraperitoneal administration induced less antibodies in the nasal mucosa and none in the lung mucosa, no CTLs were found in the lymph nodes.

The above examples demonstrate that adenoviruses are suitable as vaccine vectors for many diseases, by a number of different administration routes. The broad range of adenovirus serotypes will allow their use in several host species.

1.4.4 Animal Adenovirus Vectors

Most other adenoviruses in vector development belong to the genus Mastadenovirus, with the exception of ovine adenoviruses (OAV), which are Atadenoviruses (Universal virus database of the International Committee for Taxonomy of Viruses). In addition some avian adenoviruses (Aviadenovirus) are being developed.

Several animal adenoviruses have been found to infect human cell-lines abortively in culture, examples of these are bovine adenovirus type 3 (BAV-3), canine adenovirus types 1 and 2 (CAV-1 and CAV-2), ovine adenovirus (OAV) and porcine adenovirus type 3 (PAV-3) (Gehle & Smith 1969, Thoelen *et al.*, 2001, Klonjkowski *et al.*, 1997, Xu *et al.*, 1997, Reddy *et al.*, 1999). CAV-1, CAV-2, BAV-3 and PAV-3 have been shown to have colinear genetic organisations to HAds and the functions of many animal adenovirus genes are comparable with those of HAds (Morrison *et al.*, 1997, Klonjkowski *et al.*, 1997, Reddy *et al.*, 1998b, Reddy *et al.*, 1998a). Moffatt *et al.* immunised mice with HAd5, BAV-3 or PAV-3, then challenged them with a different adenovirus recombinant. The mice developed virus specific antibodies that could prevent transgene expression when challenged with the same vector, however there was no cross-neutralisation between human and non-human adenoviruses (Moffatt *et al.*, 2000). These results provide support for the development of animal adenoviruses as gene therapy vectors in humans. Animal adenoviruses are discussed further in section 3.1.

1.4.4.1 Bovine Adenoviruses

There are 10 known bovine adenovirus (BAV) serotypes, which are divided into 2 subgroups classified by cell culture characteristics and the presence or absence of complement-fixing antigens (Bartha 1969). Of these, BAV-3 has been studied in the most depth. It is similar in diameter (75nm) (Niiyama *et al.*, 1975) and genetic organisation to the HAds (Reddy *et al.*, 1998b). Many of the BAV-3 genes have similar functions to the equivalent genes in HAds. The E1a region is essential for activating transcription of other BAV-3 early genes (Zhou *et al.*, 2001). As with HAds, the BAV-3 E3 region is non-essential for virus growth in culture. At 1.5 kbp, it is approximately half the size of the E3 regions of HAd2 and HAd5 (Evans *et al.*, 1998, Mittal *et al.*, 1995). One of the four E3 ORFs shows significant homology and has functions similar to the 14.7 kDa protein of HAd5 (Mittal *et al.*, 1993, Zakhartchouk *et al.*, 2001). However, the E4 region of BAV-3 differs from HAds significantly. It has only 5 ORFs, each of which can be deleted individually from the BAV-3 genome with no loss of viability and therefore each separate E4 ORF is nonessential (Baxi *et al.*, 2001). In addition, ORFs 1-3 and 3-5 are nonessential in complex. However, Baxi *et al.* were unable to isolate a BAV-3 vector with the whole E4 region deleted and suggested that the deletion of the E4 region may have been destabilising for adjacent regions of the genome. In contrast to other adenovirus fibres, the BAV-3 fibre was found to be both bent and significantly longer. It has been suggested that with the added length of this fibre, bending is necessary for secondary penton interactions and therefore internalisation of the virus (Ruigrok *et al.*, 1994).

BAV-3 is capable of abortive infection in cultured human cells (Mittal *et al.*, 1995). A BAV-3 vector with a firefly luciferase gene expressed from E3 region, which had a 696 bp deletion, was produced. Although vector replication in Madin-Darby bovine kidney (MDBK) cells was 10-fold less than with wild type BAV-3, luciferase expression was detected. In addition, the BAV-3 vector was able to express the luciferase gene in human 293 cells for at least 6 days. With this potential realised, the production of replication defective BAV-3 vectors may result in a useful alternative to the HAds for human gene therapy.

1.4.4.2 Murine Adenoviruses

There are 2 murine adenoviruses (MAV) serotypes (Hamelin *et al.*, 1988). Murine adenovirus type 1 (MAV-1) was discovered in 1960 by Hartley and Rowe and is the most extensively studied of the two serotypes. It naturally infects heart, kidney and brain cells of mice and has low sequence homology with HAds 2 and 5 (Meissner *et al.*, 1997). This adenovirus appears to be a potential candidate for human gene therapy as it has been shown by RT-PCR that MAV-1 is able to enter mouse, monkey and human epithelial cells and express E1 genes (Nguyen *et al.*, 1999). Like the HAd E3 region, the MAV-1 E3 region functions in the modulation of the host immune system and is dispensable for viral growth in culture (Beard & Spindler 1996).

As well as providing another possible human gene therapy vector, research with MAV-1 may be able to provide an animal model for gene therapy since it can be studied with relative ease in its natural host, which would parallel the use of HAds in humans to a certain extent.

1.4.4.3 Ovine Adenovirus

There have been 7 OAVs described to date (Both, 1999). With a genome size of approximately 29 kbp these are considerably smaller than the HAds (Vrati *et al.*, 1996), and have significant structural and genomic differences to other adenoviruses. The shaft region of the fibre is only 120 residues in length in comparison to approximately 200 residues of other adenoviruses (Khatri *et al.*, 1997) and the E1a/E1b, E3 and E4 regions show little homology to those of other adenovirus. A 4.3 kbp insert in OAV can be tolerated without any deletion in the viral genome, and it was found that a 2 kbp deletion could be made, which does not affect virus viability and increases the maximum potential insert capacity to approximately 6.3 kbp (Xu *et al.*, 1997). These differences may offer OAVs advantages over other adenoviruses for human gene therapy since the immune response to them may be expected to be significantly different. There were no antibodies against OAV detected in human sera collected from 15 healthy individuals, all of which had significant anti-HAd titre. Cross-administration

studies with HAd5 and OAV vectors showed that antibodies to HAd5 did not block OAV vector transduction and vice versa (Hofmann *et al.*, 1999).

Several OAV vectors have been investigated in human cells *in vitro* and in mice *in vivo*. One OAV vector was shown to transduce a broad range of human cell types (lung and foreskin fibroblasts, liver, prostate, heart, colon and retinal) and also bovine and rabbit cells *in vitro* (Khatri *et al.*, 1997). *In vivo,* expression from replication competent OAVs was found to be comparable with replication-deficient HAd vectors (Hofmann *et al.*, 1999, Voeks *et al.*, 2002). However, whereas expression from a HAd vector was found predominantly in the livers of mice, OAV vector expression was found in mice spleens, kidneys, hearts and livers at similar levels. Retargeting of an OAV to non-permissive human 293 cells has been demonstrated by swapping the fibre knob domain with that of HAd5 (Xu & Both, 1998). In addition, the infectivity of this vector in other human cell-lines was enhanced.

Despite being quite distinct from HAds, OAV shows great promise in the development of a human gene therapy vector. Further investigation of the genome is required in order to identify other genes that can be deleted, either because they are non-essential for transcription or can be provided *in trans*, allowing the insertion of larger transgenes.

1.4.4.4 Porcine Adenoviruses

Five porcine adenoviruses (PAV) have been isolated which generally do not cause disease in the pig population (reviewed in Tuboly & Nagy, 2000). Of these PAV-3 has been studied most for gene therapy purposes. It has a genome of approximately 34 kbp, similar in size to HAd serotypes, and the E3 region is non-essential in culture. A recombinant PAV-3, with E1a and partial E3 deletions, expressing a GFP gene, was propagated in a porcine cell-line transformed with HAd5 E1 proteins (Reddy *et al.*, 1999). The resultant recombinant virus was able to transduce canine, ovine, bovine and human cells (Reddy *et al.*, 1999).

The E3 region of another porcine adenovirus type 5 (PAV-5), which is more closely related, genetically, to certain BAVs than other PAVs, was also found to be non-essential for virus replication, which would allow an additional vector capacity of at least 2.9 kbp (Tuboly & Nagy, 2000, Nagy *et al.*, 2001, Tuboly & Nagy, 2001).

As the widespread exposure of humans to PAVs is unlikely, they may provide yet another alternative to HAds in the development of gene therapy vectors for humans.

1.4.4.5 Avian Adenoviruses

An Aviadenovirus developed with a view to application in human gene therapy is chicken embryo lethal orphan virus (CELO), otherwise known as fowl adenovirus type 1, which was first identified in 1957 (Yates *et al.*, 1957). It has the largest known adenovirus genome of approximately 44 kbp in length (Chiocca *et al.*, 1996). The virus is structurally similar to the mammalian adenoviruses except that it has two fibres of different lengths at each capsid vertex (Hess *et al.*, 1995). The short fibre, fibre 2, is essential for normal viral infection (Tan *et al.*, 2001). Mutation of the fibre gene 1, which encodes the long fibre, allows the production of infectious virus, although the virus loses the ability to transduce human cells – suggesting that CELO fibre 2 binds a receptor on chicken cells that is not present on human cells. However, the replication of this recombinant CELO in chicken embryos was impaired demonstrating that both fibre genes are necessary for efficient viral infection. CELO was also found to be able to use human CAR as a receptor; a CELO vector transduced chinese hamster ovary (CHO) cells that express CAR with 100-fold higher efficiency compared with naturally CAR-deficient CHO cells (Tan *et al.*, 2001).

CELO is significantly different from the Mastadenoviruses in that it has no regions that correspond to early regions E1, E3 or E4 of HAd5 (Chiocca *et al.*, 1996). Michou *et al.* identified a set of ORFs of CELO that could be deleted and complemented *in trans*, and also another region that was completely dispensable for viral replication (Michou *et al.*, 1999). This group found that CELO vectors

expressing a luciferase or (enhanced green fluorescent protein (EGFP) gene under the influence of CMV promoter were able to express transgenes in mammalian cell-lines, including human cells, to levels comparable with HAd5 vectors expressing the same transgenes from the same promoter.

The large genome of CELO suggests that it may be able to accommodate larger transgenes. Humans are unlikely to have a pre-existing immune response to CELO. CELO can therefore be added to the group of non-human adenoviruses with potential for human gene therapy. However, further investigations into the molecular biology of this virus and the human immune response against it are required.

1.5 CANINE ADENOVIRUSES TYPES 1 AND 2

CAV-1 was first isolated in 1954 by Cabasso *et al.*, from a dog with acute hepatitis and was originally known as infectious canine hepatitis virus (ICH virus). In 1962, Ditchfield *et al.* isolated a virus similar to CAV-1 from dogs suffering from laryngotracheitis that was found to be antigenically distinguishable from CAV-1. This virus was originally known as canine laryngotracheitis virus and was later renamed canine adenovirus type 2 (CAV-2). These viruses both have genomes of approximately 30 kbp, which is smaller than HAds, but their structures are typical of adenoviruses. They are both approximately 80nm in diameter and are made up of 252 capsomeres. Haemagglutination inhibition tests and restriction digest analysis of viral DNA confirmed the distinct genomic differences between the two canine adenoviruses (Daria *et al.*, 1985, Ditchfield *et al.*, 1962, Hamelin 1984, MacCartney *et al.*, 1988).

1.5.1 Pathogenesis of CAV-1 and CAV-2

CAV-1 has a wide tissue tropism, replicates in endothelial cells of the vascular system (Greene, 1998) and can cause chronic diseases of the nervous system, respiratory tract, gastrointestinal tract, liver, kidneys and eyes (Koptopoulos & Cornwell 1981). CAV-2 is normally restricted to replication in respiratory epithelial cells, though CAV-2 variants have been isolated from the intestine of a 7-week-old puppy that had died from haemorrhagic diarrhoea and from the faeces of kennelled dogs with diarrhoeal disease (Hamelin *et al.*, 1986, MacCartney *et al.*, 1988).

Like some HAds, CAV-1 is able to transform cells in culture, which, when injected into newborn hamsters, cause tumours (Kinjo *et al.*, 1968). CAV-2 also contains genes that transform cells and are tumourigenic in newborn rats (Yamashita *et al.*, 1985, Tsukiyama *et al.*, 1988). However, there is no evidence of canine adenovirus associated tumour formation in the natural host.

1.5.2 Genomic comparisons between CAV-1 and CAV-2

There are notable biological differences between CAV-1 and CAV-1. Early DNA hybridisation experiments demonstrated that there is less that 70% overall homology between their genomes (Jouvenne & Hamelin, 1986).

The E3 regions of CAV-1 and CAV-2 are approximately 1300 nucleotides and 1800 nucleotides, respectively, (Linne 1992) compared with the 4300 nucleotides of HAd3 E3 region and 3300 nucleotides of HAd2. Most HAds have 10 E3 ORFs (reviewed by Shenk 2001) whereas both canine adenoviruses have only two ORFs in this region. The E3 regions of CAV-1 and CAV-2 both encode 13.3 kDa polypeptides, which have 78% amino acid identity. However, the major ORF of region E3 of CAV-1 encodes a 22kDa and the corresponding region of CAV-2 encodes a 40.7 kDa polypeptide. The amino- and carboxy- termini of these polypeptides are similar but the CAV-2 genome has 500 nucleotides that are not present in CAV-1. This variance between hosts and serotypes of adenoviruses in the E3 region may contribute to the biological differences in host and cell tropism, and pathogenesis of adenoviruses.

The differences in CAV-1 and CAV-2 tropism may be attributable, in part, to differences in their fibre genes. They have approximately 80% amino acid sequence identity although the CAV-2 has been found to be about 10nm longer than that of CAV-1 (Rasmussen *et al.*, 1995, Yamamoto & Marusyk, 1968, Marusyk *et al.*, 1970).

1.5.3 Canine Adenoviruses type 2 as a Gene Therapy Vector

A research group in France is currently developing CAV-2 as a gene therapy vector for use in humans and most of their work has been published concurrently with the investigation presented here. The genetic organisation of CAV-2 early and late genes, and the growth kinetics of CAV-2 are comparable to those of HAd5 in culture (Chillon & Kremer, 2001). Although CAV-2 is able to use CAR to enter cells, it can also transduce cells lacking CAR, albeit at a low efficiency (Soudais *et al.*, 2000). It also lacks the RGD motif that, in HAds, interacts with integrins for internalisation of the virus. CAV-2 attachment and internalisation is clearly different from HAds implying that the tropism of this virus might differ significantly from HAds (Soudais *et al.*, 2000). Nevertheless, CAV-2 DNA replication and gene expression has been demonstrated in human cells (293, A549 and HeLa) to varying degrees, although the formation of infectious particles was not detected (Rasmussen *et al.*, 1999). A CAV-2 vector expressing GFP has also been found to preferentially transduce neurons both *in vitro* in rodents and *ex vivo* in human brain tissue (Soudais *et al.*, 2001). E1 deleted CAV-2 vectors have been produced which express the lacZ transgene, under the control of an RSV promoter, in human, mouse, rat and monkey cell-lines *in vitro* and also in a variety of cells in 2 day old chick embryos *in vivo* (Kremer *et al.*, 2000, Klonjkowski *et al.*, 1997).

Neutralisation studies from a lung cancer clinical trial showed that sera from patients containing HAd5 neutralising antibodies had little effect on CAV-2 infectivity (Klonjkowski *et al.*, 1997). Although sera from 3 out of 7 of these patients contained CAV-2 neutralising antibodies, another study showed that 49/50 samples from healthy individuals did not contain detectable levels of CAV-2 neutralising antibodies (Kremer *et al.*, 2000).

1.5.4 *Canine Adenoviruses type 1 as a Gene Therapy Vector*

CAV-1 possesses the positive attributes that are true of HAds for gene therapy. These include a broad range of host cells (Koptopoulos & Cornwell, 1981), a large insert capacity (between +6 and +9%, compared with +5% of HAds) (Morrison *et al.*, 1997, Bett *et al.*, 1993) and relative ease with which the viral genome can be manipulated (Morrison *et al.*, 2002).

Like many adenoviruses, CAV-1 binds human CAR (unpublished data Morrison *et al.* 2002) and may thus have a common primary receptor with the HAds, however, little is known regarding the secondary routes by which CAV-1 can enter cells. In nature CAV-1 has a wider range of host tissues than CAV-2 and as such may be able to express recombinant genes in tissues of the human body that CAV-2 is unable to transduce. It is hoped that with the natural ability of CAV-1 to infect a broad range of cell types, combined with targeting technology (see Chapter 4), that the use of CAV-1 based vectors in humans would show efficient and specific transduction of a wide range of tissue types.

Although there have been no reports of CAV-1 infection in humans, in 1969 Gehle and Smith demonstrated that a CAV-1 strain could infect and replicate in human cells *in vitro,* albeit at low levels, which indicated that CAV-1 had potential for development as a human gene therapy vector. As CAV-1 and CAV-2 cross-neutralise, it would be expected that CAV-1 would have the same neutralisation profile in human sera as CAV-1. In 1970, a study in the Bexar County, Texas, USA, reported that a high proportion of normal adults had antibodies that neutralised CAV-1 (Smith *et al* 1970). It is not known if these antibodies were specific to CAV-1 or were cross-reactive antibodies elicited in response to prior infection by human adenoviruses. These results differ greatly to the CAV-2 study by Kremer *et al.* in France, 2000, however, there are geographical differences, the Smith study is over 30 years old and the methodology is different. Theoretically, it is possible that anti-CAV-1 immune responses could be elicited in the UK population through exposure to human adenoviruses [Smith *et al.,* found that anti-HAd8 neutralising antibodies cross react with CAV-1 (Smith *et al.,* 1970)], CAV-1 or CAV-2. However, there is no

reservoir for either CAV-1 or CAV-2 in the domestic canine community as vaccination is common practice in the UK; vaccination against the canine adenoviruses is mediated by administration of live-attenuated CAV-2 (Appel *et al.*, 1975, Tribe & Wolff, 1973). Nevertheless, canine adenoviruses are found in the wild, with the fox as an example of a host and the cross-neutralisation of CAV-1 by other HAds may be relevant (Davidson *et al.*, 1992, Truyen *et al.*, 1998).

The full sequence of CAV-1 is available and the genome has a similar genetic organisation to that of published HAds (Figure 1.3) (Morrison *et al.*, 1997). In addition, recombinant CAV-1 vectors have been developed that are deleted singly or multiply in the E1, E3, and E4 regions , and E1 and E4 complementing cell-lines have been produced for the propagation of E1 and E4 deleted vectors (Morrison *et al.*, 2002, unpublished data Morrison *et al.*). Towards the end of the PhD project presented herein, a first generation, E3 deleted, vector with a GFP reporter gene also became available.

1.5.5 Objectives of this project

The aim of this project was to investigate the potential application of CAV-1 as a vaccine or gene therapy vector in non-canine species. Specifically the following questions were posed –

1) could wild type CAV-1 transduce, and express transgenes, within feline and human cells?

2) could a system be developed whereby CAV-1 fibre modified viruses could be generated (that could later be exploited to improve vector delivery to target tissues)?

3) would pre-existing antibodies in feline and human recipients limit efficacy of vectors?

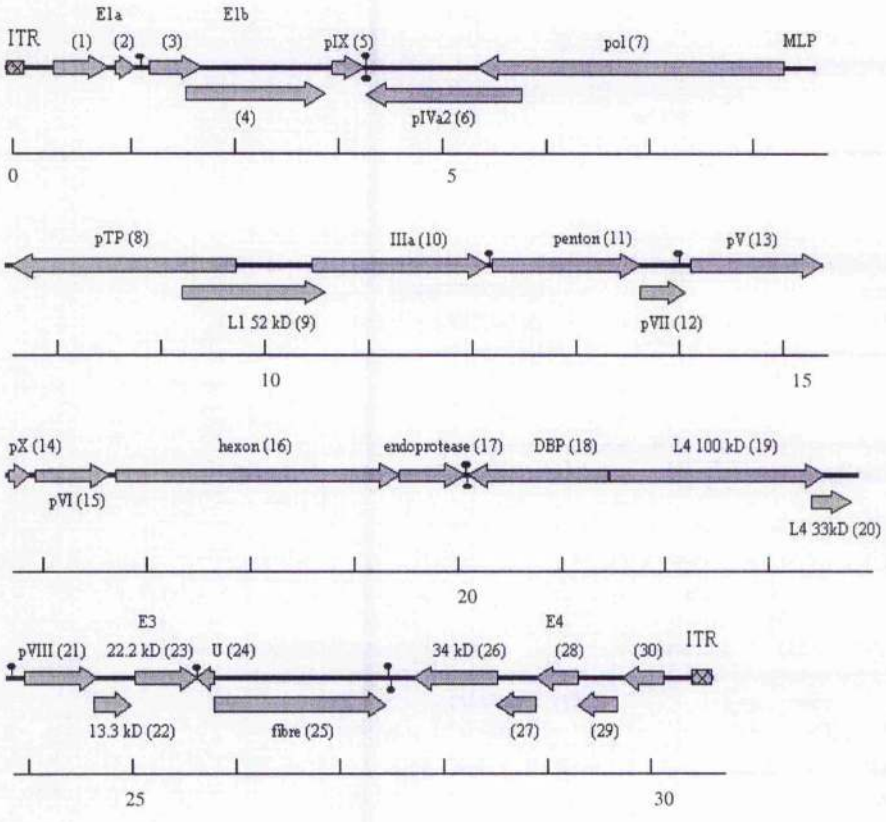


Figure 1.3 Schematic of CAV-1 genome. Open reading frames are indicated by arrows and numbered (in brackets), polyA sites are shown as ↟ for upper strand or ↡ for lower strand. The location of the major late promoter (MLP) is indicated. A scale (marked in 1 kb increments) is given underneath (Morrison *et al.*, 1997).

CHAPTER 2

Materials & Methods

2.1 MATERIALS

2.1.1 General

2.1.1.1 Major Equipment

- ABI Prism 3100 genetic analyser (Applied Biosystems, UK).
- ABI Prism 770 sequence detector system (Applied Biosystems, UK).
- Automated Sequencing Apparatus and appliances: Licor 4000 (Licor Inc., USA).
- Benchtop orbital shaker (New Brunswick Scientific, USA).
- C25 incubator shaker (New Brunswick Scientific, USA).
- Centrifuges
  - Microcentrifuge 5415 (Eppendorf GMBH, Germany).
  - Benchtop centrifuge GS-6R (Beckman Instruments Inc., USA).
  - Ultracentrifuge J2-21 (Beckman Instruments Inc., USA).
- CO_2 incubator for tissue culture (Leec Ltd., UK).
- Gel Documentation System (Ultra Violet Products Inc., USA).
- High Performance Ultraviolet Transilluminator (Ultra Violet Products Inc., USA).
- Pipetteman (P20, P100, P200, P1000) (Gilson Medical Electronics, France).
- Spectrophotometer: WPA UV1101 Biotech Photometer (Jencons-Pls, UK).
- Thermal Cyclers: GeneAmp 9600 (The Perkin Elmer Corporation, USA), PCR Express (Hybaid Ltd., Middlesex, UK).
- Vacuum Dessicator (Jencons-Pls, UK).
- Water baths (Grant Instruments Ltd., UK).

2.1.1.2 *Consumables*

- 0.2 ml PCR tubes (The Perkin Elmer Corporation, USA).
- 15 ml and 50 ml falcon tubes (Becton Dickinson labware, UK).
- 2 ml Cryogenic vials (Corning Incorporated, USA).
- 200 µl and 100µl pipette tips (Sarsteds, Germany).
- Cell scrapers (Greiner Labortechnik Ltd, UK).
- Disposable sterile scalpels (Swann-Morton, UK).
- Falcon tubes: 15 and 50 ml (Corning Incorporated, USA).
- Filter pipette tips (Costar, Corning Inc., USA; Greiner Labortechnik Ltd, UK).
- Flat ended gel loading tips (Sorenson Bioscience Ltd., Denmark).
- PCR tubes (The Perkin Elmer Corporation, USA).
- Bijoux and universals (Greiner Labortechnik Ltd, UK).
- Screw top and flip top 1.5ml microfuge tube (Treff AG, Switzerland)
- Single use syringe filter, 0.2 and 0.45 µm (Sartorius, Germany).
- Syringes of 1, 2, 5, 10, 20 and 50 ml (Becton Dickinson Labware, UK).
- Tissue culture disposables: flasks (TRP, Switzerland), multiwell plates and pipettes (Corning Incorporated, USA).

2.1.1.3 *Complete kits*

- ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems, UK).
- Access RT-PCR System (Promega, UK) for reverse transcription and PCR amplification of specific target RNA from total RNA.
- DNeasy® Tissue Kit (Qiagen, UK) for purification of DNA from cultured cells.
- Purescript® RNA isolation kit (Flowgen, UK) for the isolation of RNA from cultured cells.
- LipofectAMINE Reagent (Invitrogen Life Technologies, UK).

- Generation® Capture Column Kit (Flowgen, UK) for the isolation of DNA from cultured cells.
- Qiagen Plasmid Mega Kit supplied by Qiagen (Qiagen, UK) was used to isolate up to 2.5mg of high-purity plasmid DNA from 500 ml bacterial culture.
- Qiaprep Miniprep Kit (Qiagen, UK) was used to isolate up to 20µg of high-purity plasmid DNA from 3 ml bacterial culture.
- QIAquick Gel Extraction Kit (Qiagen, UK) for purification of DNA and extraction of DNA from agarose gel.
- TOPO TA Cloning Kit (Invitrogen Life Technologies, UK) for the cloning of PCR products with A overhangs generated by *Taq* polymerase.

2.1.1.4 DNA plasmid vectors

- pCI-neo Mammalian Expression Vector (Promega, USA).
- pCR®2.1-TOPO TA cloning vector (Invitrogen Life Technologies, UK) as part of the TOPO TA cloning kit, supplied as linearised DNA with 3' T overhangs.

2.1.1.5 Enzymes

- Calf Intestinal Alkaline Phosphatase (CIAP) (Invitrogen Life Technologies, UK).
- DNaseI (Invitrogen Life Technologies, UK).
- Restriction enzymes and appropriate buffers (New England Biolabs, UK, or Invitrogen Life Technologies, UK).
- RNaseA (Invitrogen Life Technologies, UK)
- T4 DNA Ligase and buffer (New England Biolabs, UK, or Invitrogen Life Technologies, UK).
- *Taq* DNA polymerase (Qiagen, UK).

2.1.1.6 Molecular size standard

1kb DNA Ladder (size range 100 bp to 12 kb) (Invitrogen Life Technologies , UK).

2.1.1.7 Reagents and solutions

The following list represents commonly used solutions. Other reagents are listed in the appropriate methods section of each chapter.

- Water for the preparation of media and general solutions was purified using a Millipore RO10 system (Millipore, UK). Ultrapure water (milli-Q H_2O) for work using recombinant DNA and protein was purified by a Millipore Q50 water purification system (Millipore, UK).
- All general chemicals used were of analytical (Analar) or ultra pure grade, supplied by Sigma Chemical Company, UK, or BDH Ltd., UK, unless otherwise specified.
- 1 M Tris-HCl: 121 g Tris base, 800 ml dH_2O adjusted to desired pH with concentrated HCl and made up to 1 litre.
- 10 x DNA loading buffer: 50% (v/v) glycerol, 0.5% (w/v) bromophenol blue, 0.5% (w/v) xylene cyanol, 100 mM EDTA (pH 8) in milli-Q H_2O.
- 10 x TBE: 0.9 M Tris-HCl, 0.9 M Boric acid, 25 mM EDTA (pH 8.3). Dilute to 1 x with milli-Q H_2O.
- Ethidium bromide: 10 mg/ml stock in milli-Q H_2O, working solution at 3 mg/ml with milli-Q H_2O; stored at room temperature.
- Phosphate buffered saline (PBS): 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.3).
- STET: 8% (w/v) sucrose, 50 mM Tris-HCl (pH 8), 50 mM EDTA pH8, 5% triton X100 made up in dH_2O, filtered (0.22 mm).

2.1.1.8 Oligonucleotides

All oligonucleotides were synthesised and supplied freeze dried by the manufacturer MWG-Biotech and were then suspended in milli-Q H_2O for use. Sequencing primers for Licor model 4000 were modified by IRD 800 labelling. Sequencing primers for ABI Prism 7700 system were unmodified. Specific primers are detailed in the appropriate chapters.

2.1.2 Bacteria

2.1.2.1 Bacterial strains

- BJ5183 electrocompetent *E.coli* cells were a kind gift from M. Mehtali (Transgene S.A., Strasbourg).
- TOP10 cells comprising One Shot chemically competent *E. coli* (Invitrogen Life Technologies, UK).
- MAX Efficiency STBL2 competent cells (Invitrogen Life Technologies, UK).

2.1.2.2 Bacterial media and supplements

- Ampicillin: 100 mg/ml in dH_2O, filtered through a 0.22 mm filter, aliquoted and stored at -20°C. Used at a final concentration of 100 µg/ml.
- Chloramphenicol: 34 mg/ml in ethanol. Stored at -20°C. Used at a final concentration of 100 µg/ml.
- Luria-Bertani (LB) medium: 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride in dH_2O, autoclaved and stored at room temperature.
- LB-agar: LB medium containing 1.5% (w/v) agar.
- SOC Medium: 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose.

2.1.3 Cell culture

2.1.3.1 Cell-lines

- 293 cells (American Tissue Culture Collection, Manassas, USA) are primary human embryonal kidney transformed by human adenovirus type 5 (Ad 5) DNA.
- A549 cells (American Tissue Culture Collection, Manassas, USA) were derived from human lung carcinomatous tissue.
- CrFK cells were isolated from feline kidney cortex and FEA cells are feline embryonic fibroblast cells. Both cell-lines were kind donations from Mathew Golder (Feline Virus Unit, University of Glasgow).
- Canine cell-lines A72, D17 and CML10 were supplied by Lizzie Gault (Division of Small Animal Clinical Studies, Faculty of Veterinary Medicine, University of Glasgow). A72, D17 and CML10 cells are skin fibroma, osteosarcoma and melanoma cells, respectively.
- MDCK cells (kindly provided by Anne Weir of the Feline Virus Unit, University of Glasgow) are normal canine kidney cells.

2.1.3.2 Media

All media and supplements were purchased from Invitrogen Life Technologies, UK, unless otherwise stated.

- Dulbeccos's Modified Eagle's Medium (DMEM) with 1000mg/L D-glucose, sodium pyruvate and pyridoxine
- Foetal Calf Serum (FCS), virus screened and mycoplasma screened.
- Heat-Inactivated Foetal Calf Serum (HI-FCS), virus screened and mycoplasma screened-glutamine, supplied as a 200mM (100 x) stock solution.
- Minimum Essential Medium (MEM), containing Earle's Salts and L-glutamine.

- MEM Non Essential Amino Acids (NEAA). Supplied as a 100x solution containing L-alanine (890 mg/L), L-asparagine (1320 mg/L), L-glutamic acid (1470 mg/L), glycine (750 mg/L), L-proline (1150 mg/L) and L-serine (1050 mg/L)
- Penicillin/streptomycin (Pen/Strep), supplied as a 100 x stock solution of 10,000 units penicillin and 10 g streptomycin per ml.
- 1 x Trypsin-EDTA solution: 0.05% Trypsin (w/v), 0.53 mM EDTA·4Na.

2.1.4 Viruses, Viral DNA, Cosmids and Plasmids

- CAV-1 isolate RI261 was used throughout. RI261 was obtained from the lungs of a naturally infected dog, and as such was more likely to represent wild-type CAV-1 than strains that had been passaged extensively in tissue culture (Morrison *et al,* 1997).
- Human Adenovirus type 5 was a kind gift from Dr Andrew Baker (Department of Medicine and Therapeutics, University of Glasgow).
- Canine Adenovirus type 2 DNA was donated by Anne Weir (Feline Virus Unit, Department of Veterinary Pathology, University of Glasgow).
- Human Adenovirus type 5 DNA was supplied by Laura Blackwood (Division of Small Animal Clinical Studies, Faculty of Veterinary Medicine, University of Glasgow).
- The CAV-1 vector ΔE3-GFP-CAV-1, which has a 844 bp section of the E3 region replaced by a GFP reporter gene under the control of the CMV immediate early promotor (see section 3.2.5), plasmids pSwapmeCm^R, KXCm^R and cosmid FLR-CAV were donated by Dr Mark Morrison (Vaccine Research Group, Department of Veterinary Pathology, University of Glasgow).

2.2 METHODS

2.2.1 General Molecular biology

2.2.1.1 Quantitation of DNA

The concentration of DNA in a solution was determined by spectrophotometry with a WPA UV1101 Biotech spectrophotometer (Jencons-Pls, UK). The spectrophotometer was initially calibrated using dH_2O as a blank. DNA was diluted in dH_2O at a ratio of 1:100 and transferred to a quartz cuvette. The optical density readings were obtained at 260 nm and 280 nm. The ratio between readings at 260 nm and 280 nm (A_{260}:A_{280}) provided an estimate of the sample purity; a ratio approximately 1.8 indicated that preparations contained essentially no protein contamination. As 1 OD_{260} is equivalent to 50 µg/µl DNA, the concentration of nucleic acid in samples was deduced by 5 x OD_{260}.

2.2.1.2 Restriction Enzyme Digests

Restriction digests were performed using the appropriate enzymes and reaction buffers according to the manufacturer's instructions. Typically, plasmid DNA was incubated with 2 units of restriction enzyme/µg DNA in a buffered solution at the temperature recommended by the manufacturer. Restriction digestion of small quantities of plasmid or cosmid DNA (0.2-1 µg) was carried out in a 20µl reaction volume for 1-3 hours. Restriction digestion of larger quantities of DNA (1-5 µg) for gel extraction (section 2.2.1.6) was carried out in a volume of 50-100 µl for 3 hours, or overnight.

2.2.1.3 Dephosphorylation of Vector DNA

Vector DNA was dephosphorylated at its termini to prevent re-ligation. After vector DNA had been linearised by digestion, the reaction mixture was adjusted

by adding milli-Q H_2O, dephosphorylation buffer (Invitrogen Life Technologies, UK) to make a 1 x solution, and 5 units of CIAP. The reaction mixture was incubated at 37°C for an hour. Enzyme activity was terminated by heating to 72°C for 10 minutes.

2.2.1.4 Blunting of Restriction Endonuclease Termini

T4 DNA Polymerase was used to fill termini created by restriction enzyme digestion of DNA as follows. For each reaction, 0.5-2.5 µg of DNA was mixed with 20 µl 5 x T4 DNA polymerase buffer, 20 µl 0.5 mM dNTP mixture, 1 µl 50 mM dithiothreitol (DTT) and 10 units of T4 DNA polymerase, and milli-Q H_2O added to bring the volume to 100 µl. The reaction was incubated at 11°C for 15 minutes, followed by incubation of ice.

2.2.1.5 Agarose Gel Electrophoresis

A perspex horizontal gel cast apparatus was used. Agarose gel mix to 0.7% (w/v) agarose, unless otherwise stated, was dissolved in 1 x TBE buffer by heating the solution in a glass conical flask in a microwave. 5 µl of ethidium bromide working solution was added per 100 ml of gel to allow visualisation of bands under UV after running. The gel was poured when the agarose was hand hot and a comb with the required number and size of teeth was placed immediately into the gel to form the sample wells. Once the gel had set it was submerged in 1 x TBE buffer. The samples containing 1 x loading buffer were loaded in each well alongside 1Kb DNA ladder and electrophoresed at a constant voltage of 10 V/cm gel length. DNA fragments were visualised by illumination with short wave (312 nm) UV light and photographed using the Gel Documentation System (Ultra Violet Products Inc., USA).

2.2.1.6 *Isolation and Purification of DNA Restriction Fragment from Agarose Gel*

Restriction enzyme digested DNA (section 2.2.1.2) was visualised by agarose gel electrophoresis as described in section 2.2.1.5. Desired fragments were cut out of the gel with a clean scalpel blade and individual gel slices placed in microfuge tubes. Extraction of DNA fragments was achieved using the QIAquick gel extraction kit following the manufacturer's instructions. DNA was eluted in 30 µl of dH_2O.

2.2.1.7 *Ligation of DNA Fragments*

DNA was analysed by agarose gel electrophoresis in order to estimate quantity (section 2.2.1.5). DNA fragments were ligated to dephosphorylated vectors (100 ng) at a ratio of 3:1. The vector and inserted DNA were incubated together in a reaction containing 1 x ligase buffer and 1 unit of T4 ligase at 16°C overnight.

2.2.1.8 *Preparation of LB plates*

LB plates were prepared by melting 200 ml of LB agar in a microwave at the lowest temperature setting for 8 minutes. The melted agar was cooled until hand hot, 100 µg/ml ampicillin was added (unless otherwise stated) and approximately 20 ml of agar was poured into each 10 cm^2 petri dish.

2.2.1.9 *Transformation of Bacterial Hosts*

Transformation of TOP10 and Max Efficiency STBL2 bacteria was performed using commercial kits according to the manufacturer's instructions. Electrocompetent BJ5183 cells were prepared and transformed as follows.

Preparation of electrocompetent BJ5183 cells

A BJ5183 glycerol was streaked out on antibiotic free LB agar plates and incubated at 37°C overnight. 20 ml of antibiotic free L broth was inoculated with a single BJ5183 colony and shaken at 200 rpm for approximately 5 hours, until the $OD_{600} = 0.5 - 0.7$. The bacteria were then chilled on ice for 20 minutes followed by centrifugation for 20 minutes at 3 K at 2°C in a benchtop centrifuge. The supernatant was removed and the pellet resuspended in 20 ml of ice cold dH_2O and centrifuged for 20 minutes at 3K, 2°C. The procedure was repeated and the pellet resuspended in an equal volume of ice cold dH_2O and stored on ice until electroporation.

Electroporation of electrocompetent BJ5183 cells

50µl aliquots of electrocompetent cells were added to cold electrocuvettes on ice. DNA samples for cotransformation were added to the electrocuvettes immediately prior to electroporation. The electrocuvette was placed in a prechilled cuvette holder and pulsed using a pulse controller set to 200 ohms, gene pulser at 25 µFD and 2.5 V. 1 ml of SOC medium was added to the cells, which were then shaken at 37°C for 1 hour to allow expression of antibiotic resistance genes.

2.2.1.10 Small Scale Preparation of Plasmid DNA (Miniprep)

A single bacterial colony or 50 µl from a 2.5 ml overnight bacterial culture was used to inoculate 2.5ml of L-Broth (section 2.1.2.2) containing antibiotic (100 µg/ml ampicillin unless otherwise stated) and grown overnight at 37°C with shaking. 1.5ml of bacterial culture was pelleted by centrifugation in a microcentrifuge (14000 rpm) for 15 seconds. Supernatant was removed and plasmid DNA was prepared from the pellet using either Qiagen's QIAprep Spin plasmid miniprep kit according to the manufacturer's instructions or the Stet/Lysosyme minipreparation method as follows.

Stet/Lysosyme Miniprep

After centrifugation the supernatant was removed and the bacterial pellet resuspended in 100µl STET containing 3mg/ml of lysosyme (Sigma Chemical Company, UK). The sample was then boiled for 45 seconds and centrifuged at 14 K for 15 minutes. The pellet containing cell debris was picked out with a toothpick and discarded. 100µl of isopropanol was added and the sample incubated at -20°C for 30 minutes to precipitate DNA, followed by centrifugation at 14 K for 6 minutes. The supernatant was removed and the pellet air dried. DNA was resuspended in 20µl or 30µl of milli-Q H_2O.

2.2.1.11 Large Scale Preparation of Plasmid DNA (Maxiprep)

A single bacterial colony or 1 ml of a 2.5ml overnight bacterial culture was used to inoculate 400 ml of L-broth containing the appropriate antibiotic (100 µg/ml ampicillin unless otherwise stated) in a 2 litre conical flask. The culture was incubated overnight at 37°C with shaking. Bacterial cells were pelleted by centrifugation (7 K, 15 min, 4°C). DNA was prepared from the pellet using the Qiagen Megaprep kit according to the manufacturer's instructions.

2.2.1.12 Glycerol Stocks

Bacterial cultures were preserved for further use by making glycerols from overnight cultures. 800 µl of an overnight culture was mixed with 200 µl of 80% glycerol in a 1.5 ml microcentrifuge tube and stored at -70°C. To recover the bacteria from glycerol stocks, the glycerols were thawed, streaked out onto an LB-agar plate with appropriate antibiotics using a sterilised platinum wire and incubated overnight at 37°C.

2.2.2 Polymerase Chain Reaction (PCR)

2.2.2.1 PCR Primer Design

Primers used were between 18 and 25 nucleotides in length, with a G/C concentration of 50-60% where possible. Melting temperatures were determined for the binding sequences of each primer using the formula:

$$T_m = 4\ (G + C) + 2(A + T)$$

Where T_m = melting temperature in °C, and G, C, A and T represent the number of guanine, cytosine, adenine and thymine residues in the primer, respectively (Itakura *et al* 1984). Wherever possible, primer pairs with similar melting temperatures were used to ensure similar annealing temperatures for both primers.

2.2.2.2 Taq PCR

Qiagen master mixes were prepared in bulk in a designated PCR clean area. PCR master mixes contained 200 µM each dNTP, 2.5 units of *Taq* polymerase in a total volume of 25 µl containing 2 x Qiagen PCR buffer, in 0.2ml sterile reaction tubes. Approximately 30 ng of DNA template, 100 pmol of each primer and PCR grade H_2O were added to a total volume of 50 µl. PCR amplification was carried out using a Perkin Elmer 9600 Thermal Cycler. Unless otherwise stated, an initial denaturation step at 94°C for 3 minutes was followed by 30 cycles of denaturation, annealing and extension as follows. The samples were denatured by heating at 95°C for 45 seconds, followed by annealing at approximately 5°C below the melting temperature (T_m) of the primers for 45 seconds. The extension step was carried out at 72°C for approximately 1 minute per Kb of DNA. 5µl of each PCR reaction was analysed by agarose gel electrophoresis (section 2.2.1.5).

2.2.2.3 *RT-PCR*

RNA was incubated with 1 unit of DNaseI in a solution buffered with React I at 37°C for 30 minutes. The reaction was terminated by incubation at 65°C for 10 minutes. RNA was then reverse transcribed, and the resultant cDNA amplified in a single tube using Promega's "Access" kit according to the manufacturer's instructions. For each reaction of 50µl total volume, 5µl of RNA was mixed with 10µl AMV/*Tfl* 5x Reaction Buffer, 1µl dNTP Mixture (10mM each of dATP, dCTP, dGTP and dTTP), 2µl 25mM $MgSO_4$, 1µl AMV Reverse Transcriptase (5U/µl), 1µl *Tfl* DNA Polymerase (5U/µl), 10µl primer mix (50pmol of each per reaction) (see Table 3.2), 20µl PCR grade H_2O. Reverse transcriptase free controls were carried out on selected samples to ensure there was no DNA contamination. Cycling conditions were as follows: 45 minutes at 48°C for reverse transcription; followed by 5 minutes at 94°C to denature the reverse transcriptase and 45 cycles of 30 seconds at 94°C (denaturation), 30 seconds at 52°C (annealing) and 1 minute at 68°C (extension), followed by a final extension step of 72°C for 10 minutes.

2µl of 10x loading buffer was added to 20µl aliquots of the RT-PCR reactions, which were analysed by electrophoresis of 2% agarose TBE gels stained with 0.5µg/ml ethidium bromide.

2.2.2.4 *TaqMan*

The development of TaqMan PCR has enabled quantitative PCR, which was in the past laborious and time consuming, to be carried out effortlessly and quickly. TaqMan is made possible by the use of a hybridisation probe that is labelled with fluorogenic dyes. This probe is designed to specifically anneal between 5' and 3' primers on the target DNA strand (see Figure 2.1). At the 5' end the probe is labelled with a reporter dye (e.g. 6-carboxy fluorescein [FAM]) and at the 3' end it is labelled with a quencher dye (e.g. 6-carboxy-tetramethyl-rhodamine [TAMRA]), which fluoresce at different wavelengths. Whilst the probe is intact

the quencher dye absorbs the fluorescent emission of the reporter dye. However, during target amplification the exonuclease activity of *Taq* polymerase degrades the hybridised probe from the 5' end, releasing the reporter dye from the vicinity of the quencher dye (Heid *et al.*, 1996). This results in an increase of reporter fluorescence, which is measured, along with the fluorescence of the quencher, by an ABI prism sequence detector in real-time. From these measurements the software calculates a value termed ΔRn, which is the difference between the reporter fluorescence in the sample and that in the no template control. The ΔRn reflects the amount of probe that has been degraded, allowing fluorescence from the reporter dye. The amount of fluorescence generated during each amplification cycle is directly proportional to the degree of amplification of the target sequence. The ΔRn value is plotted on the y-axis of an 'amplification plot' against the cycle number on the x-axis (see Figure 2.2).

A threshold value is assigned to the plot either by the user or by the computer at a level where there is a significant increase in reporter fluorescence above baseline. The cycle at which the amplification plot for a given sample intersects the threshold is defined as the threshold cycle (C_T), which is indicative of the initial target quantity, with C_T values decreasing linearly with increasing target quantity.

The requirements for three hybridisation events results in a sensitive and precise technique. In addition, the buffer used for most TaqMan contains uracil-N-glycosylase (UNG). This prevents the amplification of dUTP-containing PCR products as it hydrolyses uracil containing amplicons. The use of dUTP instead of dTTP in the PCR reaction means that only the amplification of template DNA is detected during TaqMan PCR.

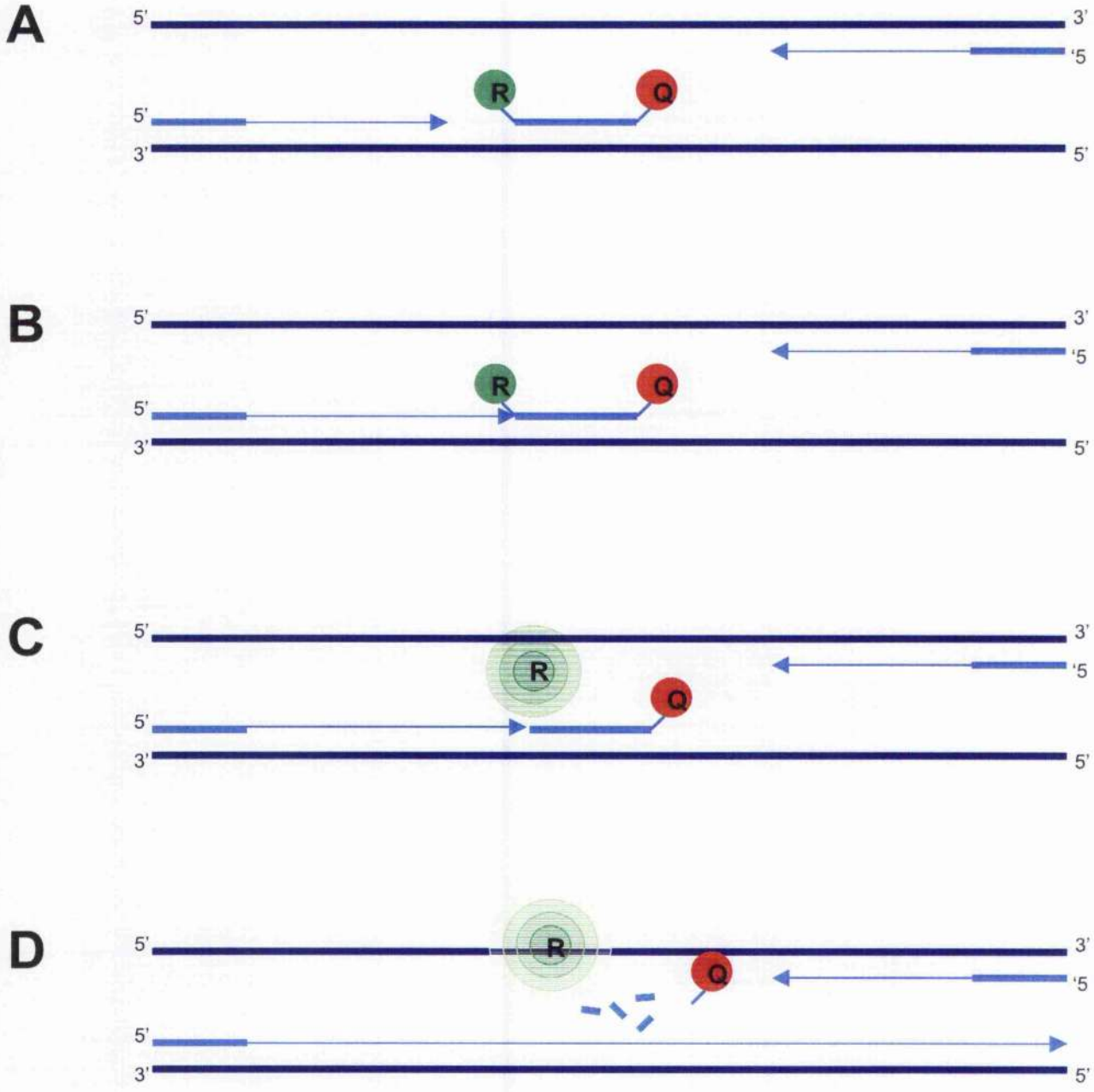


Figure 2.1 TaqMan PCR

A Forward and reverse primers, and fluorogenic probe anneal to the template DNA strand. Primers are extended by *Taq* polymerase.
B The DNA produced by elongation of the primer displaces the fluorogenic probe at the 5' end.
C and **D** The exonuclease activity of *Taq* polymerase cleaves the reporter from the probe and releases it from the quencher generating a fluorescent signal which is detected by the ABI Prism sequence detection system.

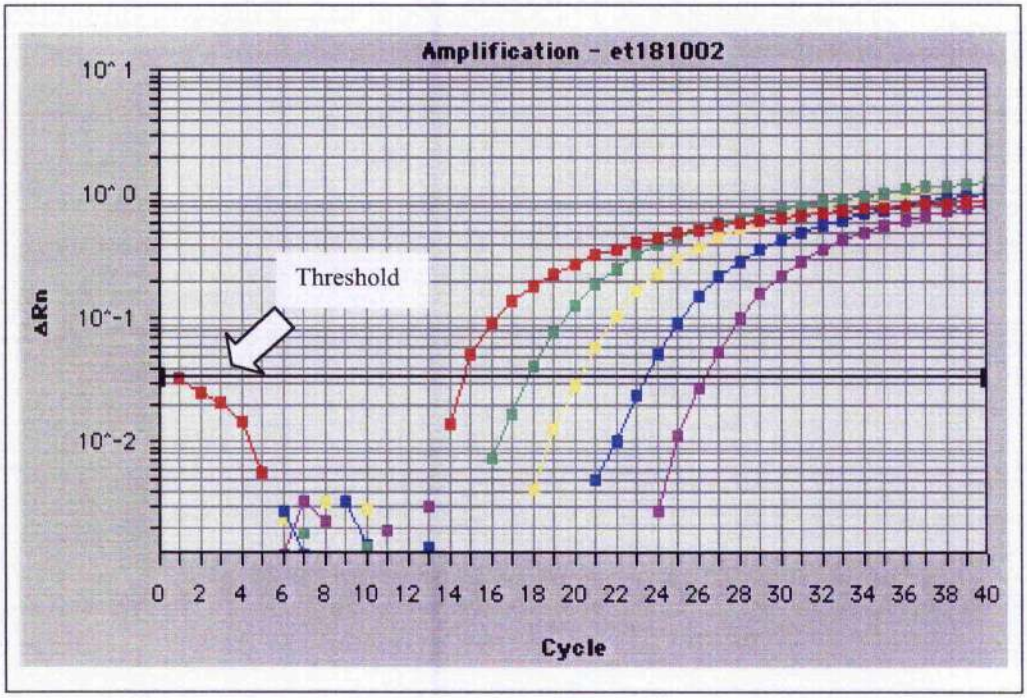


Figure 2.2 TaqMan Amplification Plot

An example of an amplification plot as produced by Applied Biosystems Sequence Detection Software. The ΔRn value is plotted against the cycle number. The cycle that the amplification plot crosses the threshold is referred to as the threshold cycle (C_T).

TaqMan Analysis of DNA

Sample DNA was assayed in triplicate for both E1a and rDNA by an ABI Prism 7700 Sequence Detection system (PE Applied Biosystems). E1a was used as target for viral DNA. Cellular rDNA levels were analysed to provide an endogenous control, the levels of rDNA present in each sample provided a reference with which the relative amount of viral DNA detected could be quantitated. A master mix was prepared which contained dNTPs with dUTP (200μM dCTP, dGTP and dATP), and 400 μM dUTP, 1x PE buffer, 5.5 μM $MgCl_2$, Amplitaq (1.5U/50μl), 300nM of each forward and reverse primers (Table 2.1) and 200nM TaqMan probe (reporter dye- FAM; quencher dye-

TAMRA) (Table 2.1) (rDNA primer and probe sequences from Dr S Dunham, Retrovirus Research Laboratory, University of Glasgow), and PCR grade water to a final volume of 45µl. 5µl of DNA was added to each reaction. The cycling conditions were as follows: initial 5 minute 95°C denaturing step followed by 45 cycles of 15 seconds at 95°C and 1 minute at 60°C (combined annealing/extension step). Fluorescence was measured at the end of each cycle. All PCR data was captured using Sequence Detector Software (SDS version 1.6, PE Applied Biosystems). The threshold value must be set at the same value for separate TaqMan experiments to be comparable. The threshold was set at 0.43 for E1a samples and 0.14 for rDNA samples. The Sequence Detector Software calculated the threshold cycle (C_T) value for each sample, this value was used for calculations that were later performed in Microsoft Excel.

Gene Represented		Nucleotide Sequence	Location on CAV-1	Amplicon Length (bp)
CAV-1 E1a	Primer 1 Primer 2 Probe	5' GAG GCT GAC AGT GAA TC 3' 5' CCG GCG GAG TGT TCA AAG T 3' 5' AAG CCA ACA ACA TCA GTC TCC GTG GAA ATT GT 3'	649-665 715-697 695-667	70
Cellular rDNA	Primer 1 Primer 2 Probe	5' CCA TCG AAC GTC TGC CCT 3' 5' TCA CCC GTG GTC ACC ATG 3' 5' CGA TGG TGG TCG CCG TGC CTA 3'	N/A	67

Table 2.1 Primers and probes for Real-Time PCR

Standards

DNA isolated from wild-type CAV-1 infected MDCK cells, consisting of a mixed population of viral and cellular DNA, was used for standards in TaqMan. These were serially diluted and stored at -20°C in 50µl aliquots. As the actual amount of cellular and viral DNA was unknown in these standards, relative standard curves were produced. Relative standards allow quantification and comparison of individual samples using arbitrary units. 5µl of standards

containing 0.64pg, 3.2pg, 16pg, 80pg, 400pg, 2000pg or 10000pg of total DNA underwent TaqMan analysis in duplicate using both E1a and rDNA probe and primer sets. Standard curves were produced for both cellular and viral DNA for each TaqMan experiment. This allowed the quantitation of CAV-1 DNA relative to cellular rDNA in each sample. The C_T values of the standards were plotted against log DNA values on graphs to give standard curves for both E1a and rDNA.

Excel Calculations

The log DNA value of each sample was obtained by extrapolation of the C_T values from the standard curve. At each time point the average E1a and average rDNA values, and the standard deviations of the averages were calculated from the triplicates. The average E1a value was normalised (to account for differences in cell number) by dividing with the average rDNA value. As both values were means with associated standard deviations, the coefficient of variance was calculated to determine the standard deviation of the normalised mean:

$$cv = \sqrt{(cv_1^2 + cv_2^2)}$$

$$\text{where the cv} = \frac{\text{standard deviation}}{\text{mean value}}$$

and standard deviation of the normalised mean = (cv)(mean).

The –fold increase in E1a was calculated for each time point by dividing each normalised E1a value by that of the 0 hour time point.

The results for each time point from different experiments were averaged and the standard error of the mean (SEM) calculated as follows:

$$\text{SEM} = \frac{\text{Standard Deviation of mean}}{\sqrt{(\text{number of samples})}}$$

Where the Standard Deviation = (cv)(mean)
with cv = to the coefficient of variance and the mean = normalised E1a value.

Analysis of variance (ANOVA) tests the hypothesis that means from two or more samples are equal. A confidence level is set for the ANOVA test and if the p-value of the test is less than the confidence level specified the hypothesis being tested is rejected. For each TaqMan time course ANOVAs were performed between the replicates of samples, with a confidence level of 0.05, to test whether the means of the samples could be considered equal at each time point. All had p-values greater than 0.05.

The pooled results were plotted on a logarithmic scale graph with error bars showing the SEM.

2.2.3 Sequencing

2.2.3.1 LiCor Sequencing

LiCor sequencing reactions were prepared using the ThermoSequenase Fluorescent Labelled Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Pharmacia, UK). Cycle sequencing is based on the chain termination method described by Sanger *et al* 1977.

A reaction mix of 500 ng DNA, 1 pmol of IRD800 labelled primer and 2 µl of reaction mix containing 45 mM of dGTP, dATP, dTTP and dCTP, reaction buffer and thermostable DNA polymerase was prepared in a 0.2 ml reaction tube and dH_2O was added to a volume of 8 µl. Cycle sequencing was as follows: 5 min 95°C denaturing step, 30 cycles of 30 second denaturing step (95°C), 30 second annealing step (5°C below the T_m of the primer) and 30 second extension step (72°C). 4µl of loading buffer provided with the kit was added to each reaction.

66 cm glass plates were thoroughly cleaned and polished with isopropanol. 330 µl of 0.05% (v/v) bind saline in 100% ethanol was mixed with 10 µl of 10% (v/v) acetic acid and applied to the top and bottom of the plates, allowed to dry and then buffed with paper towel. The glass plates were then assembled in the casting stand with spacers. The gel was prepared using 7.2 ml 10 x TBE (216 g Tris, 1110 g Boric acid, 80 ml 10.5 M EDTA pH 8, made up to 2 litres with milli-Q H_2O), 25.2 g urea and 4.8ml Long Ranger™ solution (National Diagnostics, USA) and H_2O to a final volume of 60ml. A volume of 400 µl of 10% ammonium persulphate (APS) was added and 3ml was removed and placed in a bijou. 40µl of 10% (w/v)APS and 4µl N'-tetramethyl-ethylenediamine (TEMED) were added to the 3ml of solution and applied to the channels at the bottom of glass plates. Once this plug had polymerised 40µl of TEMED was added to the remaining solution and poured into the electrophoresis gel plates using a 50 ml syringe. A comb was placed in the top of the gel and the gel was allowed to polymerise for approximately 2 hours. The gel was placed in a LiCor 4000 system with 1 x TBE added to the buffer tanks. The comb was removed, the wells flushed with buffer and a pre-electrophoresis step performed which focussed the microscope, adjusted the gain controls and warmed the gel. 1.5 µl of each sample was loaded into separate wells. The gel was electrophoresed and sequence data was recorded. The data was analysed using the GCG package (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin, USA).

2.2.3.2 ABI Prism Sequencing

Cycle sequencing reactions were prepared in 0.2 ml PCR tubes using a ready made BigDye Terminator RR master mix (Applied Biosystems, UK). This consisted of 3.2 pmol of the appropriate primers, dNTPs, dye terminators, magnesium chloride, buffer, Ampli*Taq* DNA polymerase and FS, which is a variant of *Thermus aquaticus* DNA polymerase. In general, around 500 ng of high-quality double stranded DNA template was included in the cycling reaction. The reactions were made up to a total volume of 20µl with dH_2O.

The cycle sequencing reaction was carried out under the following conditions: 25 cycles of the following: rapid thermal ramp to 96°C, 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes.

Reactions were either purified by ethanol precipitation or by using the Performa DTR gel filtration cartridge kit (Edge Biosystems, USA) - see below. DNA pellets were resuspended in 20 µl Hi Di Formamide (Applied Biosystems, UK). The samples were loaded onto a 96 well plate and electrophoresed on an ABI prism 3100 Genetic Analyser. The chromatogram was studied by eye and sequences were analysed using the GCG package (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin, USA).

Ethanol precipitation

Cycle sequencing reactions were diluted with 80 µl of dH_2O. 1 µl of 3 M sodium acetate and 200 µl of 100 % ethanol and the mixture was incubated at -20°C for 30 minutes to precipitate DNA. DNA was pelleted by centrifugation at 12 K for 10 minutes in a microcentrifuge. The supernatant was removed and the pellet was washed with 150 µl 70% ethanol and centrifugation at 12 K for 5 minutes. The supernatant was removed and the pellet was air dried.

Performa DTR gel filtration cartridge kit

A fresh spin column was centrifuged at 750 g for 2 minutes to remove interstitial fluid. The column was removed from the wash tube and placed in a new collection tube and the cycle sequencing reaction was applied to the centre of the binding column and centrifuged for 2 minutes at 750 g. The binding column was then removed and the DNA pellet dried for 15 minutes using a vacuum pump.

2.2.4 Cell Culture and Transfection

All cell culture was performed using strict aseptic techniques inside a BioMAT laminar flow hood (Medical Air Technology Ltd., UK).

2.2.4.1 Maintenance of Cells

All cell-lines were maintained in plastic tissue culture flasks and incubated at 37°C in a wet atmosphere containing 5% (v/v) CO_2. 5 ml of medium was used in 25 cm^2 flasks, 15 ml of medium was used in 75 cm^2 flasks and 30 ml of medium was used in 175 cm^2 flasks.

MDCK cells were maintained in MEM, 5% (v/v) FCS, 1% (v/v) L-glutamine, 1% (v/v) NEAA and 1% (v/v) Pen/Strep.

293 cells were maintained in MEM, 10% (v/v) HI-FCS, 1% (v/v) L-glutamine, 1% (v/v) NEAA and 1% (v/v) Pen/Strep.

A549, A72, CML10, CrFK, D17 and FEA cells were maintained in DMEM, 10% (v/v) HI-FCS, 1% (v/v) L-glutamine and 1% (v/v) Pen/Strep.

2.2.4.2 Seeding of Cell-lines

Cells were subcultured twice a week at a density determined by the rate of cell growth. The subculture routine was performed by removing the culture medium, washing the cells once with 2 ml (25cm^2 flasks), 3 ml (75cm^2 flasks) or 5 ml (175cm^2 flasks) of PBS, followed by the addition of 1ml (25cm^2 flasks), 2ml (75cm^2 flasks) or 4ml (175cm^2 flasks) of trypsin and incubation at 37°C (20 min for MDCK, 5 min for A549, A72, CML10, CrFK, D17 and FEA cells). Trypsin is neutralised by serum, therefore, 2 ml (25cm^2 flasks), 4 ml (75cm^2 flasks) or 6 ml (175cm^2 flasks) of the appropriate growth medium was added to the trypsinised cells. The cells were then split according to the growth rate of each cell-line. Cells were resuspended in 5ml of medium in 25cm^2, 15ml of medium

in 75cm^2 and 30ml of medium in 175cm^2 flasks, and incubated as described above.

2.2.4.3 Counting cells

Cells were counted by trypan exclusion of dead cells. 50 µl of cell suspension was diluted with 50 µl trypan blue (Invitrogen Life Technologies, UK) and 10µl was loaded onto a haemocytometer with cover slip. Cells in 5 of the 9 open squares were counted under the 10 x objective of an inverted microscope. The sum of the counted cells was divided by 5 (the number of open squares counted), multiplied by 2 (dilution factor) and multiplied by 10^4 to determine the number of cells per ml.

2.2.4.4 Storage and Revival of Cells

To freeze cell stocks for storage, confluent cell cultures were trypsinised and pelleted by centrifugation at 1000 rpm for 5 minutes. The cells were then resuspended at a concentration of approximately 10^6 cells/ml in FCS containing 10% (v/v) DMSO. The DMSO in the medium acts as a cryoprotectant. Suspensions were divided into 1 ml aliquots in 1-2 ml cryogenic vials and placed at -70°C overnight. The following day the tubes were placed in liquid nitrogen.

Frozen stocks were recovered by removing the vials from liquid nitrogen and placed into water at 37°C. Once thawed, the cells were added to 15 ml of growth medium in 75 cm^2 flasks and maintained as described above

2.2.4.5 Transfection of Cells

Cells were subcultured at 5 x 10^5 cells in 25cm^2 tissue culture flasks. The following day transfections were carried out using LipfectAMINE Reagent (Invitrogen Life Technologies, UK) as follows. The required amount of DNA was added to 250µl OptiMEM (mixture A) and 25µl of lipofectamine was added to 250µl OptiMEM (mixture B). Mixture A and mixture B were combined and

incubated at room temperature for 30 minutes to allow interaction of DNA and lipofectamine. The mixture was diluted with 2ml of OptiMEM and added to MDCK cells that had been washed twice with PBS. Cells were incubated at 37°C for 4-6 hours, OptiMEM removed and cells washed once with 5% medium before incubating overnight in 5ml of fresh medium at 37°C.

2.2.4.6 $TCID_{50}$ method

Whole log dilutions were made by diluting 200µl of samples, or previous dilution, with 1.8ml of the appropriate medium. Half log dilutions were made by diluting 1ml of whole log dilutions with 2.2ml of the appropriate medium. 100 µl of each virus dilution was added to multiple wells of a 96-well plate containing 1.5 x 10^4 cells. Plates were scored for c.p.e. every day over a period of 7-10 days. $TCID_{50}$ values were calculated using the Karber formula:

$$\log TCID_{50} = D - \Delta(S - 0.5)$$

where $TCID_{50}$ = tissue culture dose where 50% of cells die, D = log of lowest dilution showing 100% c.p.e., Δ = difference between successive log dilutions, S = sum of the proportional mortalities (Karber 1931).

2.2.4.7 Adenovirus Neutralisation Assay

Complement in serum samples was inactivated by incubation at 56°C for 30 minutes. 4-fold dilutions of the serum samples were made by adding 30 µl to 90 µl of the appropriate medium (1:4 dilution). Growth medium containing 2 % serum (v/v) was used throughout. 25 µl of this dilution was added to each of 4 wells of a 96 well plate, each containing 75 µl of growth medium (1:16 dilution). 25 µl of each 1:16 dilution was diluted by adding it to 75 µl of growth medium (1:64 dilution). This step was repeated 4 times to make 1:256, 1:1024, 1:4096 and 1:16384 dilutions. 100 $TCID_{50}$ units of the appropriate virus were added to each well in 50 µl of medium. Serum/virus mixtures were incubated at 37°C for 1 hour, followed by further incubation at room temperature for 1 hour to allow

virus antibody interactions to form. 50 µl of growth medium containing 1 x 10^4 of the appropriate cells was added to each well. Plates were incubated at 37°C for 6 days. The plates were then read for cytopathic effect and neutralising antibody titres were determined using Table 2.2 as follows. The number of wells showing c.p.e. in the first dilution demonstrating c.p.e. determines the neutralising antibody titre of the appropriate sample. For example, a sample which had no cytotoxicity in the 1/16 and 1/64 dilutions and one well of c.p.e. in the 1/256 dilution would have a neutralising antibody titre of 362 (the appropriate cell on the table is highlighted in grey).

No. of wells showing c.p.e./dilution	1/16 dilution column	1/64 dilution column	1/256 dilution column	1/1024 dilution column	1/4096 dilution column	1/16384 dilution column
4	0	32	128	512	2048	8192
3	11	45	181	724	2896	11584
2	16	64	256	1024	4096	16384
1	23	91	362	1448	5792	23168

Table 2.2 Adenovirus Neutralisation Assay Table

CHAPTER 3

Canine Adenovirus type I (CAV-1):
Potential as a Gene Therapy and Vaccine Vector in Heterologous Species

3.1 INTRODUCTION

Immunological responses pose a challenge to the development of safe and efficacious adenovirus–based vaccine and gene therapy regimes. The existence of a pre-existing immune response is likely to reduce the efficiency of adenovirus based vectors in either therapeutic gene therapy or vaccination. This is a particular problem for the use of vectors based on ubiquitous serotypes HAd2 and HAd5 in humans as a high proportion of the population have pre-existing immunity (see Chapter 5). In addition, development of immunity to administered Ad vectors can compromise efficacy of subsequent administrations of vector necessary for sustained transgene expression. Adenovirus vectors of less common human serotypes or from heterologous species are less likely to encounter pre-existing immune responses to either natural infections or previous vector administration and therefore offer an alternative to common HAds for vector development.. There is therefore a need to develop vectors from less prevalent adenovirus serotype, or from Ads of different host species. Before an adenovirus can be developed as a vector in a host other than its natural host it is necessary to investigate the life cycle of the wild type virus in the target cell type. This research has been carried out for a range of animal adenoviruses in human cells, as discussed below.

3.1.1 Animal Adenovirus Infection of Human Cells

Animal adenoviruses CAV-2, BAV-3 and PAV-3 are similar in size to HAds 2 and 5, have similar genetic organisations (Campbell, 1996, Babiuk *et al.*, 2002, Reddy *et al.*, 1998a) and the functions of many of their genes are comparable (see section 1.4.4). All of these viruses are capable of expressing their genes in human cells (Rasmussen *et al.*, 1999, Reddy *et al.*, 1999).

Infection of human A549 and HeLa cells by wild-type CAV-2 is semi-productive and viral proteins are expressed (Rasmussen *et al.,* 1999). Rasmussen *et al.,* detected an increase in viral particles and observed cytotoxicity during CAV-2 infection of these cells. Although the infection was only semi-productive, in that limited numbers of infectious virus particles were produced, CAV-2 transduced 91% of the HeLa cells. As with the common C group HAds, deletion of the E1 region rendered this virus replication defective and such a first generation vector was efficient in the transduction of human cells to levels comparable with a HAd5 vector (Kremer *et al.,* 2000). Soudais *et al.,* also demonstrated that a GFP expressing CAV-2 vector was able to transduce neurons in human brain tissue biopsies (Soudais *et al.,* 2001).

Wild-type BAV-3 infection of human cells was found to be abortive (Rasmussen *et al.,* 1999). E1a and late gene expression were detected by RT-PCR, however no viral protein synthesis was evident and no cytotoxicity was observed in any of the 3 human cell-lines tested when infected at a m.o.i. of 10 p.f.u./cell. A BAV-3 vector partially deleted in the E3 region was the first non-human adenovirus vector reported (Mittal *et al.,* 1995). It was capable of expressing a firefly luciferase transgene in non-permissive human 293 cells as early as 12 hours after infection and for up to 6 days. The peak expression of the luciferase gene was only 10% less than in its native Madin Darby bovine kidney cells (MDBK). Rasmussen *et al.,* also demonstrated that an E1-deleted BAV-3 was able to transduce 6 human cell-lines investigated and express a lacZ transgene (Rasmussen *et al.,* 1999).

A first generation replication defective PAV-3 vector (E1a and E3 deleted) was able to express a GFP transgene gene in human cells (Reddy *et al.,* 1999).

MAV-1, CELO and OAV have lower homology to HAds 2 and 5 (Spindler, 1990, Chiocca *et al.,* 1996, Both, 1999) but these too are able to transduce human cells. MAV-1 infected human endothelial cells and gene expression from the E1a region was detected by RT-PCR (Nguyen *et al.,* 1999). Wild-type CELO was found to be replication defective in human cells, but was capable of expressing a GFP transgene in human hepatoma, lung epithelial and dermal fibroblast cells

(Michou *et al.*, 1999). Similarly, OAV infection of human cells is non-productive, however, DNA replication, both early and late viral transcription and protein synthesis were demonstrated (Kümin *et al.*, 2002). A replication-competent OAV vector was also able to express transgenes in several human cell-lines (Khatri *et al.*, 1997).

3.1.2 Adenovirus infection in domestic cats

Adenovirus infections are prevalent in humans and, before the development of an effective vaccine, were also widespread in dogs (see section 1.5.4). However, there have been few reports of adenoviral infection in the cat. In 1993 Kennedy and Mullaney reported disseminated adenovirus infection in the intestinal contents of a domestic cat that presented with a swollen liver and kidneys, and high volumes of fluid in the abdominal cavity. Electron microscopy revealed the presence of 'roughly icosahedral' particles of approximately 65 nm in diameter, which formed crystalline arrays and this group concluded that this was an adenovirus. However no other biological techniques were used to confirm the identity of this virus. Another cat, which presented with transient liver failure, was seropositive for purified HAd1 hexon antigen over 18 months (Lakatos *et al.,* 1999). Pharyngeal and rectal swab samples were examined for adenoviral hexon sequences by PCR. The rectal sample was positive and pharyngeal sample was negative. 12 months later both rectal and pharyngeal samples were found to be positive. The PCR primers used were modified versions of those used for the general detection of HAds (Allard *et al.,* 1990) that amplify a portion of the hexon region of most mammalian adenoviruses (Kiss *et al.,* 1996). All 3 PCR amplicons were sequenced, found to be identical, and to have 95% similarity to both the HAd2 and HAd5 hexon sequences. This indicates a persistent adenovirus infection which resulted in the shedding of virus from this animal, however, it us unclear what the source of this adenovirus was. This domestic cat was a single pet kept in isolation. The close homology to HAd sequences suggest that it may be possible that the adenovirus was originally acquired from an infected human and had mutated for survival in a heterologous host or that it was an alternative HAd serotype.

PCR of DNA from infected Hungarian cats, with primers for human adenovirus hexon, yielded bands at the expected size. After serial passages c.p.e. was observed in HeLa cells inoculated with rectal swabs taken from these cats (Ongrádi 1999). Immunofluorescent labelling of the isolated virus confirmed the

presence of adenovirus hexons in the cytoplasm of infected cells. Icosahedral capsids of approximately 88nm in diameter were observed under electron microscopy, although these appeared to be fibreless.

Lakatos *et al.*, investigated field serum samples obtained from 622 cats in Scotland, Hungary, the Netherlands and the USA (Lakatos *et al.*, 2000). Overall, 17.4% of these samples were seropositive for adenovirus when tested by indirect ELISA carried out with purified HAd1 hexon antigens (see Table 3.1). Experiments were carried out using HAd1 hexon antigens on the theoretical basis of the hexon containing a common determinant of immunity to adenoviruses. The lowest percentage was 9.8% in Scotland and the highest was 26% in the USA.

Site	Number of samples	Number and percentage (%) of positive samples
Hungary	294	43 (14.6)
Scotland	102	10 (9.8)
The Netherlands	64	13 (20.3)
USA	162	42 (26.0)
Total	622	108 (17.4)

Table 3.1 Prevalence of anti-HAd1 hexon antibodies in the serum of cats from Hungary, Scotland, The Netherlands and the USA (Lakatos *et al.*, 2000).

Whether these adenoviral infections are from a novel adenovirus of cats or have HAd origins is unknown as all of these results were carried out using detection methods for HAds. The infrequent isolation of adenoviruses from cats and the low seropositivity makes the use of adenoviruses as a vaccine vector in this

species a possibility as there is less chance of pre-existing immunity preventing effective vaccination (see section 1.4.3.6 for an overview of vaccination using adenovirus vectors).

3.1.3 Objective

CAV-1 possesses many positive biological attributes with respect to vector development. These include its wide tropism (Koptopoulos & Cornwell, 1981), a large insert capacity (between 106 and 109%, compared with 105% of HAds) (Morrison *et al.*, 1997, Bett *et al.*, 1993) and relative ease with which the viral gene can be manipulated (Morrison *et al.*, 2002).

With a view to developing CAV-1 as a gene therapy vector for humans and a vaccine vector for cats, it was important to investigate the lifecycle of CAV-1 in both human and feline cells in detail. CAV-1 infection of 2 human cell-lines, A549 and 293 (lung carcinoma and adenovirus transformed embryonic cells, respectively) and 2 feline cell-lines, CrFK and FEA, (kidney and fibroblast cells, respectively) was investigated by isolation of infectious virus, determination of levels of viral DNA, detection of transcription of viral genes and expression of a transgene from a replication competent vector.

3.2 MATERIALS AND METHODS

3.2.1 *De Novo* Production of CAV-1 in Human and Feline Cells

3.2.1.1 De Novo Production of CAV-1 in Human Cells

MDCK, A549 and 293 cells were subcultured at 5 x 10^5 cells, in 5 ml of medium, per 25cm^2 flask and incubated at 37°C overnight. Individual flasks were prepared for each time-point. The cells were infected the following day with CAV-1 at a m.o.i. of 10 p.f.u./cell in 2 ml of medium at 37°C for 1 hour. The medium containing virus was removed and 1 ml aliquots were stored at –70°C. The cells were washed twice with PBS and 5 ml of fresh medium was added to each flask. This was considered the 0 hour time point. At each time point until, and including, 48 hours the medium was removed and 1 ml aliquots were retained. The cells were scraped into 1 ml of fresh medium and stored at -70°C. The flasks assigned for harvest on day 5 were subcultured into a 75cm^2 flask on day 2 to maintain the cells at subconfluency. The culture medium was also transferred to this flask and supplemented with 10 ml of fresh medium. At 5 days the medium was removed and 1 ml aliquots were retained, the cells were scraped into 2 ml fresh medium and 1 ml aliquots were stored.

Infectious virus was isolated and titred using the $TCID_{50}$ method detailed in section 2.2.4.6.

3.2.1.2 De Novo Production of CAV-1 in Feline Cells

MDCK, CrFK and FEA cells were subcultured and infected as described in section 3.2.2.1. At each time point up until, and including, 48 hours the medium was removed and the cells were scraped into 1 ml of fresh medium and stored at

-70°C. The flasks assigned for harvest on day 5 were subcultured into a 75 cm^2 flask on day 2; the surrounding medium was also transferred to this flask and supplemented with 10 ml of fresh medium. At day 5 the cells were scraped into 2 ml of fresh medium and 1 ml aliquots were stored at -70°C.

Virus was isolated from cell samples and titred using the $TCID_{50}$ method detailed in section 2.2.4.6.

3.2.2 CAV-1 DNA Replication in Human and Feline Cells

3.2.2.1 DNA replication over a 14 day time course in human cells infected with CAV-1 at a m.o.i. of 10 p.f.u./cell

MDCK, A549 and 293 cells were subcultured at 2 x 10^5 cells, in 2 ml of medium, per well of a six-well tissue culture plate. These were incubated at 37°C overnight. The following day each well was infected with CAV-1 at a m.o.i. of 10 p.f.u./cell in 0.5 ml of the appropriate medium and incubated at 37°C for 1 hour. The medium containing virus was then removed, the cells were washed twice with PBS and 2 ml of the appropriate medium was added to each well. This was taken as the 0 hour time point. For MDCK cells a separate 6-well plate was used for each time point (0, 6, 12, 24, 48, 72 and 96 hours), MDCK cells exhibited 100% c.p.e. by 48 hours. Separate 6-well plates were used for each time point up until 4 days and 8 days for the A549 and 293 cells, respectively. By these time points the cells were confluent and 2 wells were pooled and subcultured into 75cm^2 flasks with 15 ml of the appropriate medium for each triplicate. At subsequent time-points, 20% of the cell population was retained for DNA isolation with 20% of the remainder returned to the tissue-culture flask. This maintained the cell population at subconfluency. Cells in 6-well plates were washed twice with PBS and trypsinised with 0.5 ml of trypsin, 2 wells were pooled for each sample, at the appropriate time points. DNA was harvested using Gentra's Generation® capture column kit according to the

manufacturer's protocol (Flowgen) (section 2.1.1.3). TaqMan analysis was carried out on samples as described in section 2.2.2.4.

3.2.2.2 DNA replication over a 4 day time course in human cells infected with CAV-1 at a m.o.i. of 0.1 p.f.u./cell

MDCK, A549 and 293 cells were subcultured at 2 x 10^5 cells, in 2 ml of medium, per well of a six-well tissue culture plate. These were incubated at 37°C overnight. The following day each well was infected with CAV-1 at m.o.i. of 0.1 p.f.u./cell in 0.5 ml of the appropriate medium and incubated at 37°C for 1 hour. The medium containing virus was then removed, the cells were washed twice with PBS and 2 ml of the appropriate medium was added to each well, this was considered to be the 0 hour time point. At each time point the cells were washed twice with PBS and trypsinised with 0.5 ml of trypsin. For each sample 2 wells were pooled and DNA was harvested using Gentra's Generation® capture column kit according to the manufacturer's protocol (Flowgen) (section 2.1.1.3) and stored at -20°C. The time points were: 0, 6, 12, 24, 48, 72 and 96 hours. TaqMan analysis was carried out as described in section 2.2.2.4.

3.2.2.3 DNA replication over a 15 day time course in feline cells infected with CAV-1 at a m.o.i. of 10 p.f.u./cell

CrFK and FEA cells were subcultured at 2 x 10^5 cells, in 2 ml of medium, per well of a six-well tissue culture plate and incubated at 37°C overnight. The following day each well was infected with CAV-1 at a m.o.i. of 10 p.f.u./cell in 0.5 ml of medium for 1 hour. The medium containing virus was then removed, the cells were washed twice with PBS and 2 ml of the appropriate medium was added to each well. This was taken as the 0 hour time point. By 72 hours the cells were confluent and cells from 2 wells were pooled and transferred to 75 cm^2 tissue culture flasks. The cells of 75 cm^2 flasks were transferred to 850 cm^2 roller bottles at the 10 day time point. DNA was harvested from 2 pooled wells in triplicate at the following time points: 0, 8, 24, 48 and 72 hours and from the

appropriate vessels at 4, 7, 10 and 15 days. Cells in 6-well plates were washed twice with PBS and trypsinised with 0.5 ml of trypsin. Cells in 75 cm^2 flasks were washed twice with PBS and trypsinised with 1.5 ml of trypsin. Cells in roller bottles were washed twice with PBS and trypsinised with 20 ml of trypsin. DNA was harvested using Gentra's "Generation" DNA isolation kit according to the manufacturer's protocol (section 2.1.1.3). TaqMan analysis was carried out on samples as described in section 2.2.2.4.

3.2.3 Transcription of CAV-1 DNA in Human and Feline Cells

MDCK, A549, 293, CrFK and FEA cells were subcultured at 5 x 10^5 cells per $25cm^2$ flask and incubated overnight at 37°C. Individual flasks were prepared for each time point. The following day the cells were infected with CAV-1 at a m.o.i. of 10 p.f.u./cell in 2 ml of medium and incubated at 37°C for 1 hour. The cells were washed twice with PBS and 5 ml of the appropriate medium was added to the flasks. Cells were harvested from flasks at the following time points: 0, 4, 8, 24, 48 and 72 hours (except for the MDCK cells which had shown 100% c.p.e. by 48 hours and therefore no 72 hour time point was taken). RNA was harvested from cells Gentra's Purescript® RNA isolation kit according to the manufacturer's protocol (section 2.1.1.3). RNA was DNase treated, reverse transcribed, and the resultant cDNA amplified in a single tube using the primers detailed in Table 3.2 and Promega's "Access" kit as described in section 2.2.2.3. Reverse transcriptase free PCR was carried out on the 48 hours samples to check for DNA contamination.

Gene Represented	Primers	Amplicon Length (bp)
E1a	5' TGT GAG TAC TGG ATG GTG G 3' 5' CCT GGT AAT AGG CAC AAC GC 3'	369
E4 - 34kDa homologue	5' TGC CCT GCT CTA TCG ACA TG 3' 5' GGA GGC TGC AAG ATT TAC AC 3'	450
Polymerase	5' AGG AGT GTT GCA AGA ACC AG 3' 5' CAC ATA CTC TCT GGC AAG AC 3'	417
Fibre	5' TGG AAA TGC AGT GTC AGT GC 3' 5' ATC AGG TCC AGT CCA TAG AG 3'	357

Table 3.2 Primers for RT-PCR of CAV-1 E1a, E4 34 kDa homologue, polymerase and fibre transcripts.

2µl of 10x loading buffer was added to 20µl aliquots of the RT-PCR reactions, which were analysed by electrophoresis of 2% (w/v) agarose TBE gels stained with 0.5µg/ ml ethidium bromide.

3.2.4 CAV-1 Maintenance in A549 Cells Over a 28 Day Time Course

A549 cells were subcultured at 1.5 x 10^6 cells per 75cm^2 flask and incubated overnight at 37°C. The following day the cells were infected with CAV-1 at a m.o.i. of 10 p.f.u./cell in 5 ml of medium for 1 hour at 37°C, after which the medium containing virus was removed and 1 ml aliquots were retained. The cells were washed twice with PBS and 15 ml of the growth medium was added to each flask. Cells were maintained at subconfluency for 28 days by routine subculture at 0, 1, 2, 4, 6, 8, 10, 12, 14, 17, 21, 24 and 28 days. At 0, 1, 2, 4, 6, 8, 10, 12, 14, 21 and 28 days p.i. the culture medium was retained and 1 ml samples containing 1/6 of the cells were retained for isolation of cell-associated CAV-1, DNA and RNA.

3.2.4.1 De Novo Production of Infectious CAV-1 in A549 Cells

Cell-associated virus was isolated from cell samples by subjecting cell pellets to three freeze thaw cycles, then both these and the samples of medium were centrifuged at 14K in a benchtop centrifuge for 10 minutes to pellet the cell debris. The supernatant was removed and infectious virus was titred using the $TCID_{50}$ method detailed in section 2.2.4.6.

3.2.4.2 CAV-1 DNA Replication in A549 Cells

DNA was isolated from the cells samples using Gentra's "Generation" DNA isolation kit according to the manufacturer's protocol (section 2.1.1.3). TaqMan analysis was carried out on the DNA samples, as described in section 2.2.2.4.

3.2.4.3 CAV-1 DNA Transcription in A549 Cells

RNA was isolated from the cell samples using Gentra's Purescript® RNA isolation kit according to manufacturer's protocol (section 2.1.1.3). RNA was DNase treated, reverse transcribed, and the resultant cDNA amplified in a single tube using the primers in Table 3.2 and Promega's "Access" kit as described in section 2.2.2.3. Reverse transcriptase free PCR was carried out on the 48 hours samples to check for DNA contamination.

3.2.5 Infection of Human and Feline Cells with a CAV-1 Vector carrying a GFP Transgene

ΔE3-GFP-CAV-1 was constructed as follows. GFP driven by the CMV immediate early promoter was cloned as an Ase I and Mlu I fragment of pEGFP-C2 (Clonetech) into the Eco RV site of pSwapme (Morrison *et al.*, unpublished

data). It was subsequently cloned as a Pme I fragment into the Pme I site of cosmid ΔE3CAV-1. The resultant cosmid was digested with Fse I to release the virus genome. Transfection into MDCK cells led to the recovery of infectious ΔE3-GFP-CAV-1 (Morrison *et al.*, 2002, and unpublished data). MDCK, A549, 293, CrFK and FEA cells were subcultured at 5 x 10^5 cells, in 5 ml of medium in 25 cm^2 tissue culture flasks and incubated at 37°C overnight. The following day the cells were infected at a m.o.i. of 0.1 or 10 p.f.u./cell with ΔE3-GFP-CAV-1. No manipulation of the cells or medium in these flasks took place at later time points. The cells were visualised at 8, 24, 48 and 120 hours using an Axiovert 25 inverted microscope (Zeiss, Germany) and photographed using a Digital CCD Camera (Hamamatsu Photonics K.K., Japan) and Simple PCI Version 5 (Compic Inc., USA) software for the Apple MacIntosh. At each time point the infected cells were compared with uninfected controls.

3.3 RESULTS

In each of the experiments CAV-1 infected MDCK cells were used as positive controls.

3.3.1 CAV-1 Replication in Human and Feline Cells

To investigate whether CAV-1 infection of human and feline cells resulted in *de novo* virus production, canine and human cell-lines were infected at a high m.o.i. with CAV-1 and investigated over a 5 day time course. Virus was isolated from the cells and also from the culture medium surrounding the human cells. The virus titres were determined by the $TCID_{50}$ method. Figures 3.1 and 3.2 show the total log $TCID_{50}$ values at each time point.

3.3.1.1 Productive CAV-1 Infection of Human Cells

At 0 hours the cell-associated and extracellular levels of CAV-1 were similar in each cell-line (Figure 3.1).

The levels of cell-associated MDCK cells initially dropped (6 hour time point), however by 24 hours there was approximately 2 logs more CAV-1 than input and this increased by another 2 logs by 48 hours, at which point 100% c.p.e. was evident and MDCK harvests were discontinued. The amount of CAV-1 in the medium surrounding the MDCK cells had increased slightly by 6 hours and continued to do so. By 48 hours post infection there was over 3 logs more CAV-1 in the extracellular medium than there was at 0 hours.

Cell-associated CAV-1 in A549 cells also decreased at 6 hours, however, in contrast to CAV-1 infected MDCK cells, the levels had dropped even further by 24 hours. Nevertheless, by 48 hours cell-associated CAV-1 had increased to levels higher than input in A549 cells and this increased further by 5 days post infection. At this point there was approximately 1 log more infectious CAV-1

than at 0 hours. Although there was an initial increase in extracellular CAV-1 at 6 hours post infection, the levels fell to below input at 24 hours. The levels then increased gradually and by 5 days p.i. the titre of extracellular virus was also approximately 1 log higher than at the beginning of the time course. This increase in extracellular virus in A549 cells indicates that new virus has been produced, however, this is over 1000-fold less than that produced in infected canine cells.

There was an overall decrease in the amount of both cell-associated and extracellular virus isolated from infected 293 cells over the 5 day time course. A slight increase in extracellular CAV-1 was detected 24 hours p.i., however, by 5 days p.i. there were undetectable amounts of cell-associated virus and the amount of extracellular virus had decreased by 3 logs.

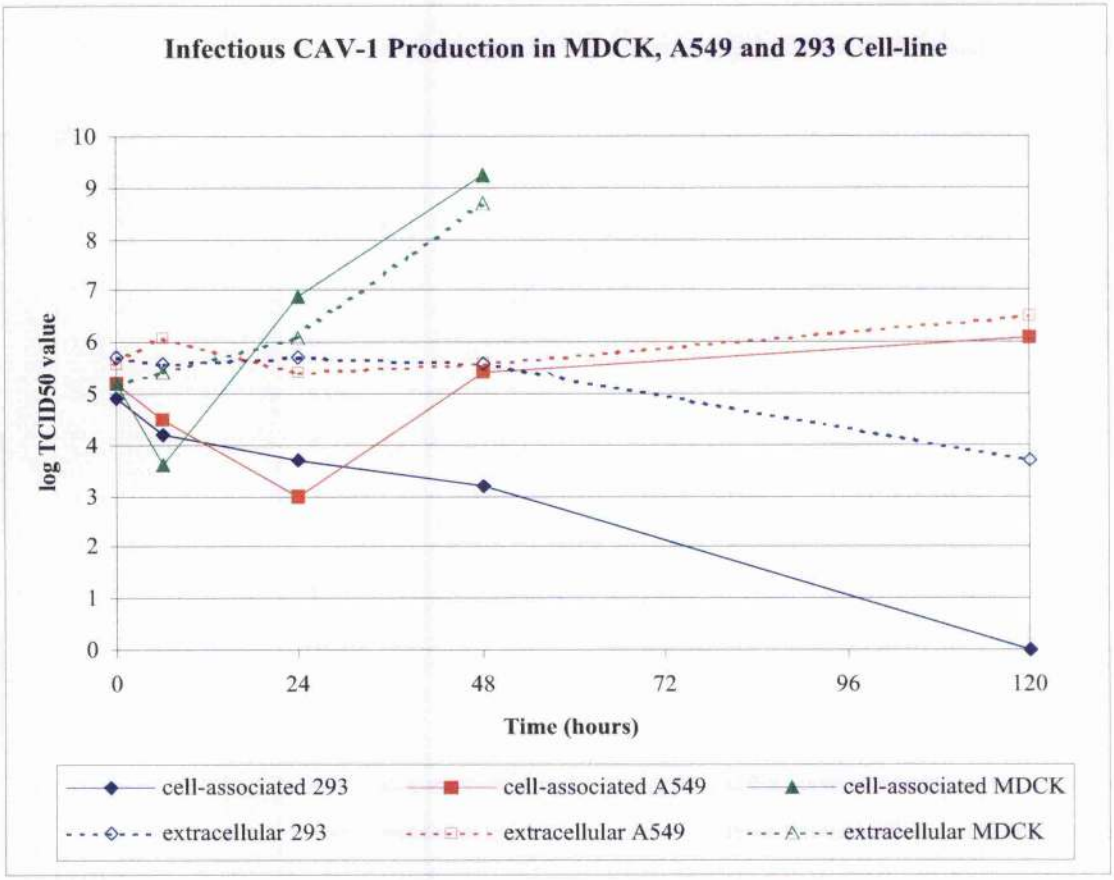


Figure 3.1 Infectious CAV-1 production in human cell-lines. MDCK, A549 and 293 cells were infected with CAV-1 at a m.o.i. of 10 p.f.u./cell. Cells and supernatant were harvested at 0, 1, 2 and 5 day time points and virus titre was determined by $TCID_{50}$.

3.3.1.2 *Productive CAV-1 Infection of Feline Cells*

One-step growth curves were carried out with CAV-1 at a m.o.i. of 10 p.f.u./cell in MDCK and feline cells in order to investigate the production of *de novo* virus particles. Cell-associated virus was isolated and the titre was determined by the $TCID_{50}$ method. Log $TCID_{50}$ values are presented, indicating the total amount of virus at each time point (see Figure 3.2).

An overall increase in CAV-1 particles was observed in all three cell-lines. 48 hours p.i. CAV-1 levels had increased more than 100-fold in MDCK cells, 10-fold in CrFK cells and 3-fold in FEA cells. By 5 days p.i. the overall increase in CAV-1 titre in CrFK cells was almost 500-fold greater than input. There was only a 35-fold increase in titre of CAV-1 in FEA cells over the 5 days of the time course. However, it should be noted that the starting titre in these cells was more than 5-fold less than for both MDCK and CrFK cells and that there was an initial 2-fold decrease in the titre of CAV-1 at 24 hours p.i. in this cell-line.

It should also be noted that the growth curved of the MDCK cells is quite distinct from that of Figure 3.1 and of published results (Morrison *et al.*, 2002). The reasons for this are unknown.

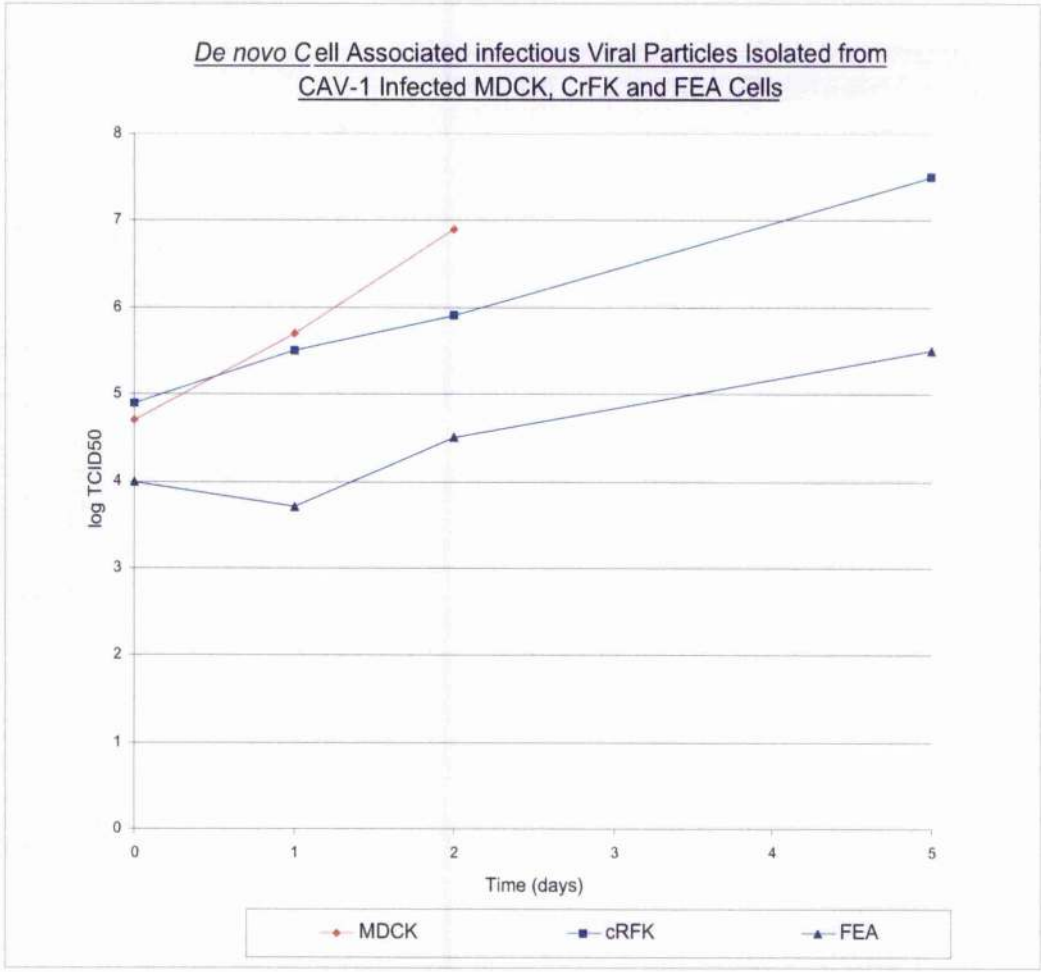


Figure 3.2 Infectious CAV-1 production in feline cell-lines. MDCK, CrFK and FEA cells were infected with CAV-1 at a m.o.i. of 10 p.f.u./cell. Cells were harvested at 0, 1, 2 and 5 day time points and virus titre was determined by $TCID_{50}$.

3.3.2 CAV-1 DNA Replication in Human and Feline Cells

To determine whether viral DNA is replicated in infected human and feline cell-lines, TaqMan PCR was performed on DNA samples isolated from CAV-1 infected cells at different times post infection. The amount of viral DNA was determined relative to the endogenous control, rDNA, and the E1a DNA values at each time point were established in relation to those at day 0. These values were plotted on logarithmic graphs.

3.3.2.1 Infection of Human Cells with CAV-1 at High M.o.i., 14 Day Time Course

All 3 cell-lines showed an initial decrease in CAV-1 DNA 6 hours post infection, which was more pronounced in the human cell-lines (see Figure 3.3). The amount of CAV-1 DNA detected in MDCK cells had increased by approximately 1000-fold by 24 hours. The levels of CAV-1 DNA in human cells remained lower than input until 48 hours p.i. By 48 hours the amount of viral DNA had increased in A549 and 293 cells by 27-fold and 2-fold, respectively. By 14 days viral DNA levels detected in 293 cells fell to lower than that input initially, however, in A549 cells DNA was 6-fold higher than input.

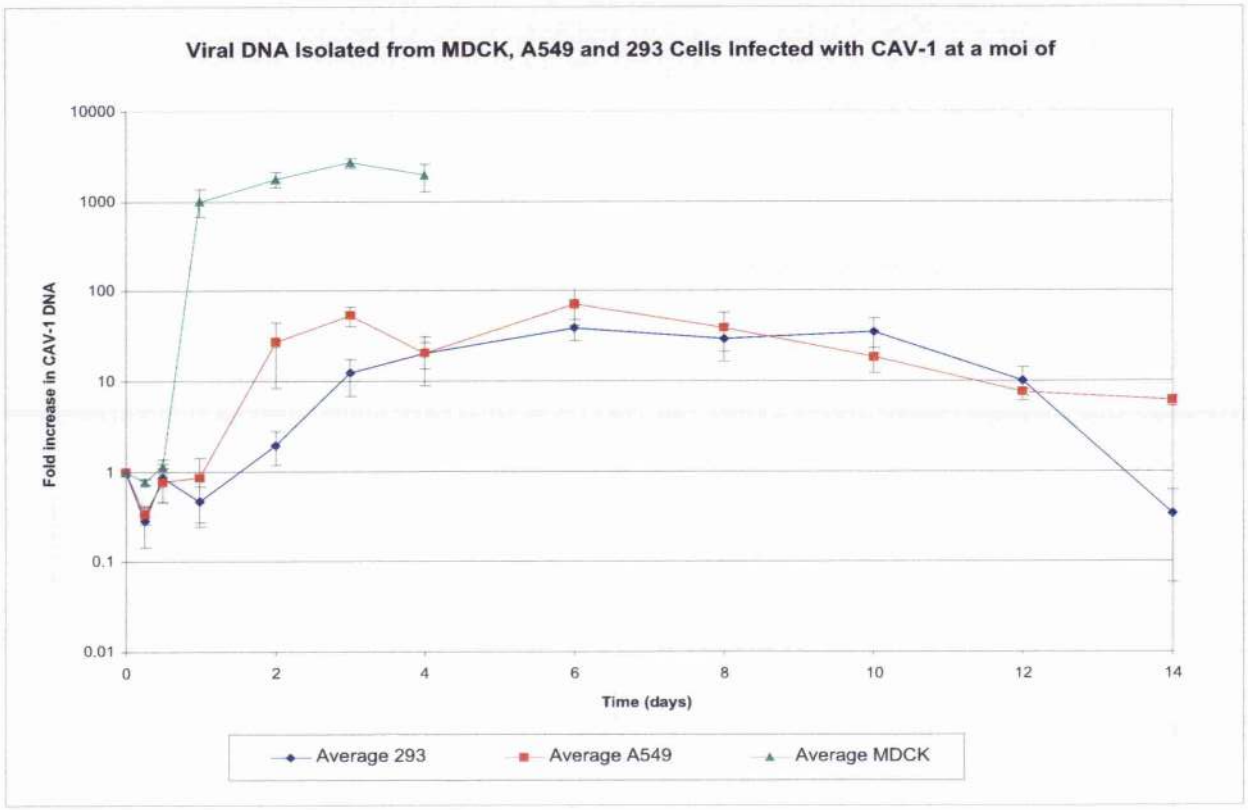


Figure 3.3 DNA replication in human cell lines. MDCK, A549 and 293 cells were infected with CAV-1 at a m.o.i. of 10 p.f.u./cell. DNA was harvested at 0, 6 and 12 hour and 1, 2, 3, 4, 6, 8, 10, 12 and 14 day time points and subjected to TaqMan analysis as described in the text.

3.3.2.2 *Infection of Human Cells at Low M.o.i., 4 Day Time Course*

To investigate whether the high virus to cell ratio increased the levels of viral DNA replication, infection of both cell-lines with CAV-1 was performed at a low m.o.i., 0.1 p.f.u./cell.

There was an initial decrease in CAV-1 DNA levels in each cell-line by 6 hours p.i. A gain in viral DNA was detected by 12 hours in the infected MDCK cells that continued throughout the time course to approximately 24000-fold by 4 days (Figure 3.4). The levels decreased further by 12 hours p.i. in the human cell-lines, however, by 24 and 48 hours p.i. there was a boost in CAV-1 DNA in the A549 cells and 293 cells, respectively. The levels of DNA continued to rise in both cell-lines over the remainder of the time course with an overall increase of approximately 100-fold in the A549 cells and approximately 70-fold in the 293 cells. This is considerably less than that of the MDCK cells, however it demonstrates that DNA replication does take place, even at low m.o.i.

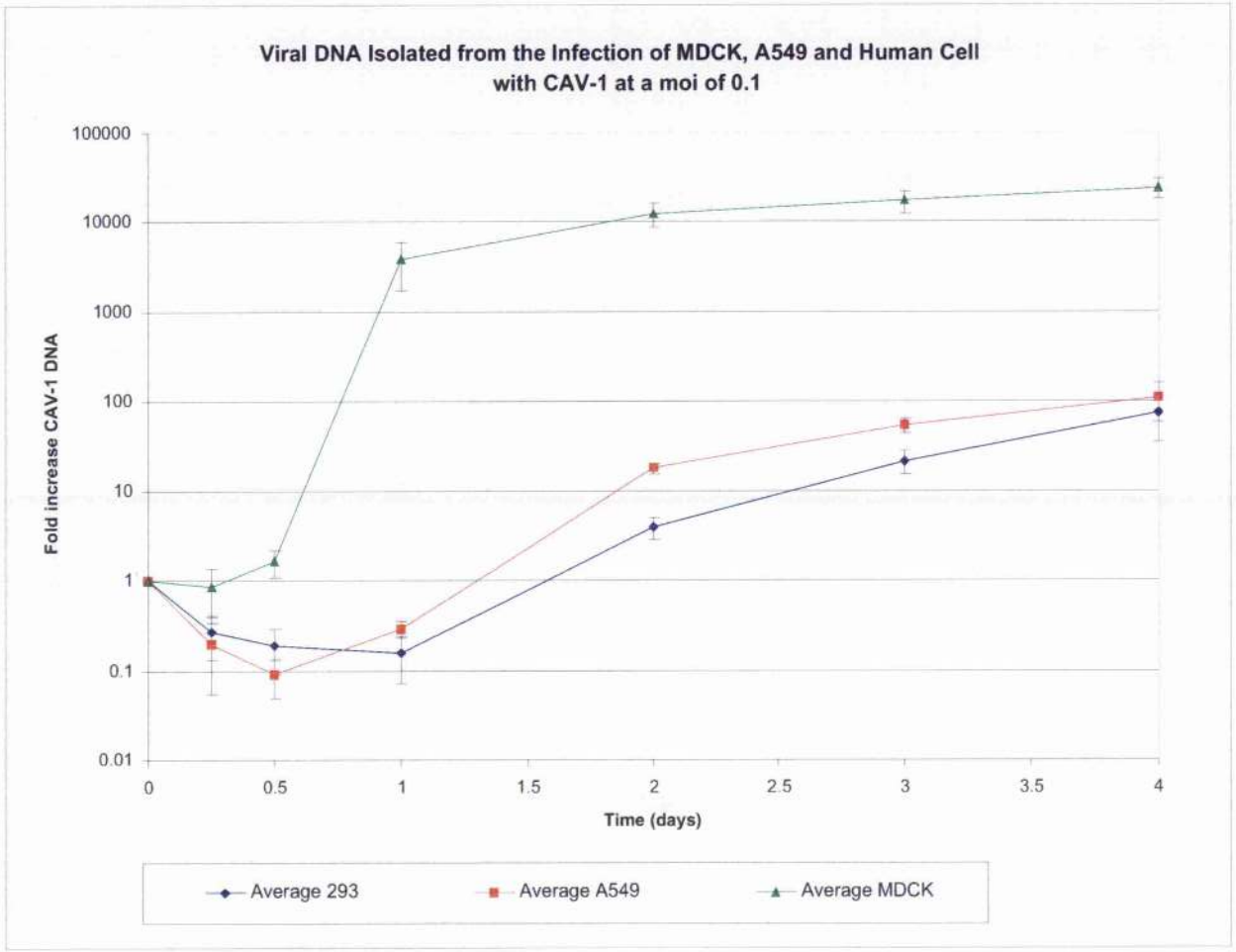


Figure 3.4 DNA replication in human cell lines. MDCK, A549 and 293 cells were infected with CAV-1 at a m.o.i. of 0.1 p.f.u./cell. DNA was harvested at 0, 6 and 12 hour and 1, 2, 3 and 4 day time points and subjected to TaqMan analysis as described in the text

3.3.2.3 *Infection of Feline Cells at High M.o.i., 15 Day Time Course*

There was an initial decrease of approximately 3-fold in the detected amount of CAV-1 DNA in both CrFK and FEA cells by 8 hours p.i. (Figure 3.5). However, 24 hours p.i the amount of CAV-1 DNA had increased by 7- and 471-fold in FEA and CrFK cells, respectively, demonstrating that CAV-1 DNA replication takes place in both cell-lines. Between day 3 and day 10 the levels of CAV-1 DNA in the CrFK cells were consistently more than 1000 fold higher than input (Figure 3.5). However, by day 15 a decrease of approximately 1 log was observed; at this point most of the CrFK cells were rounded and detached from the flask. The increase in CAV-1 DNA detected in FEA cells was slower, nevertheless, by day 10 the amount of E1a detected in the FEA cells had also increased by more than 1000-fold from input.

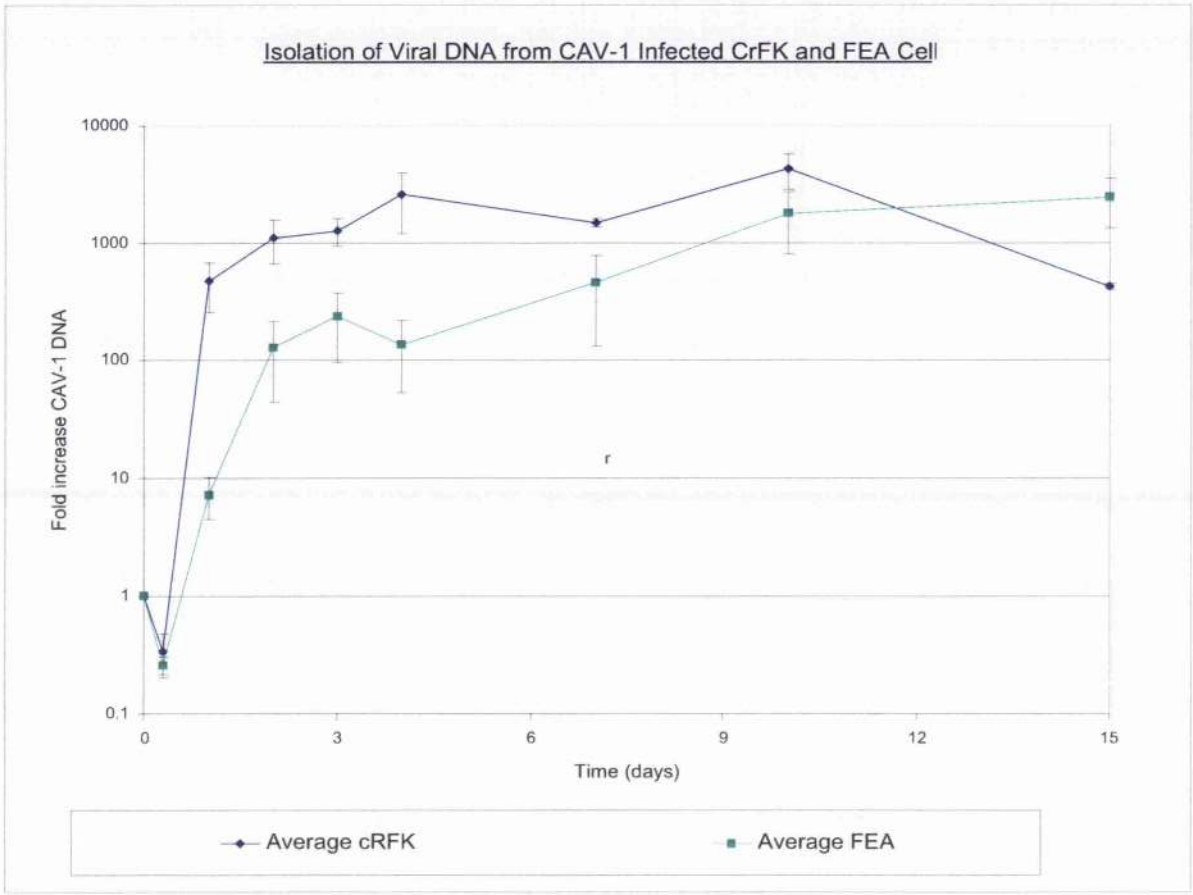


Figure 3.5 DNA replication in feline cell lines. CrFK and FEA cells were infected with CAV-1 at a m.o.i. of 10 p.f.u./cell. DNA was harvested at 0 and 8 hour, 1, 2, 3, 4, 7, 10 and 15 day time points and subjected to TaqMan analysis as described in the text.

3.3.3 CAV-1 DNA Transcription in Human and Feline Cells

Four genes were selected for analysis by RT-PCR based on the following rationale. Each of these genes is found in a different transcription unit, demonstrating transcription from the essential early regions, E1, E2, and E4, and the late region. The E1a gene should be the first to be transcribed and is intimately involved in transcription of other adenoviral genes. The 34 kDa ORF in HAd5 is an essential E4 protein. The polymerase gene must be expressed if the viral DNA is replicating, and therefore acts as an internal control. The late region encodes structural proteins of which the fibre, penton and hexon are the major immunogens.

Figure 3.6 shows the transcripts obtained from MDCK, 293, A549, CrFK and FEA cells infected with CAV-1 at a m.o.i. of 10 p.f.u./cell over a 3 day period. Within 8 hours, E1a and 34 kDa transcripts were detected in the infected MDCK cells, and by 24 hours the polymerase and fibre transcripts were also evident. The presence of all four transcripts was demonstrated at the 48 hour time point. Transcripts were not found in either of the human cell lines until the 24 hour time point, when all four could be detected. The four transcripts were present for the remainder of the time course in A549 cells but there were no transcripts detected by 72 hours in the 293 cells. E1a and fibre transcripts were evident by 24 hours p.i. in samples isolated from infected CrFK cells and the samples taken 48 and 72 hour p.i. contained all 4 transcripts. No transcripts were apparent until 48 hours p.i. in the FEA cell samples. At both this time point and 72 hours p.i. all 4 transcripts could be detected. In all cells the sample taken 48 hours p.i. was analysed for DNA contamination as this proved to be the time point when target was most abundant. These samples were negative indicating the absence of CAV-1 DNA in the RNA samples.

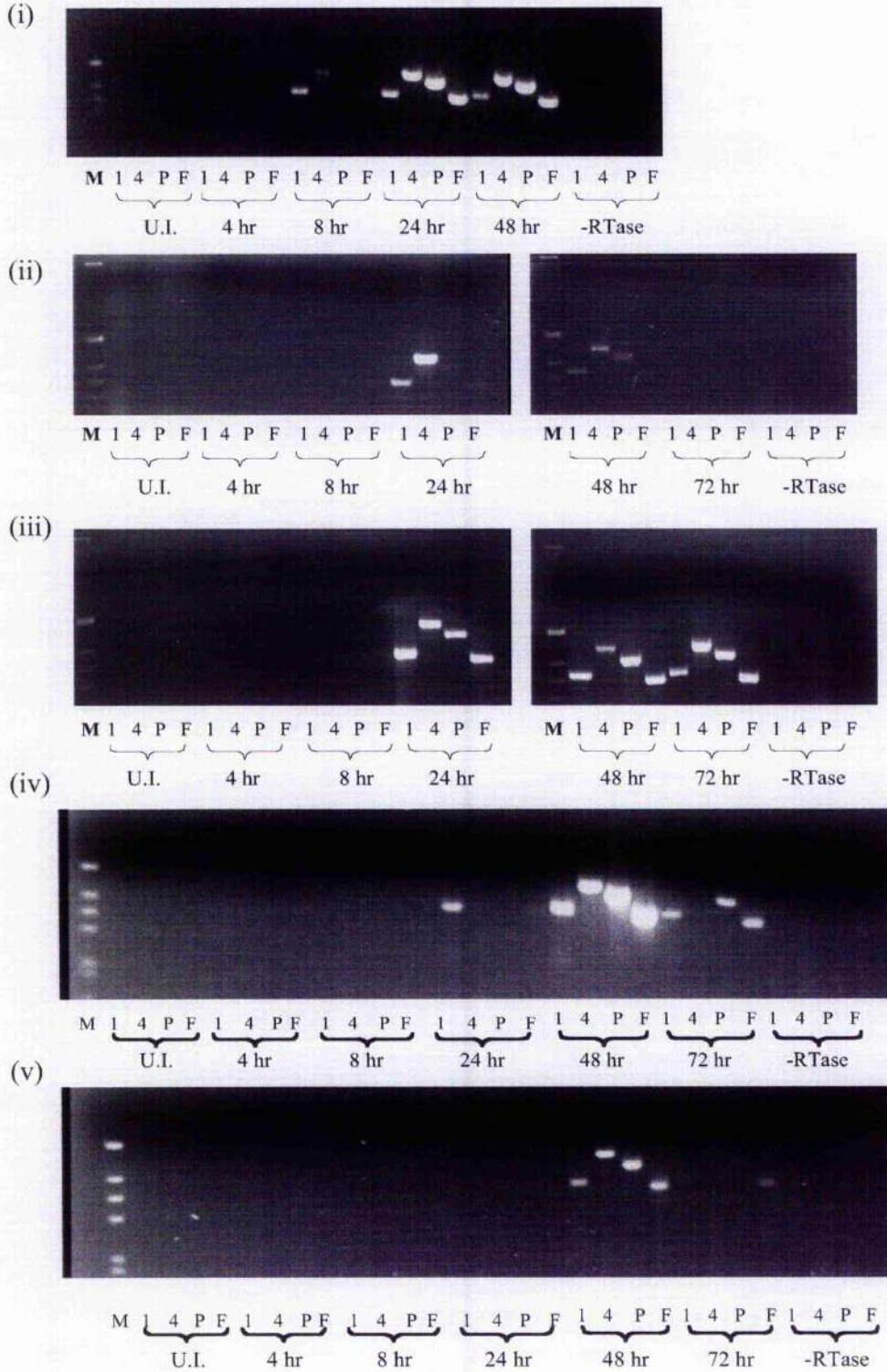


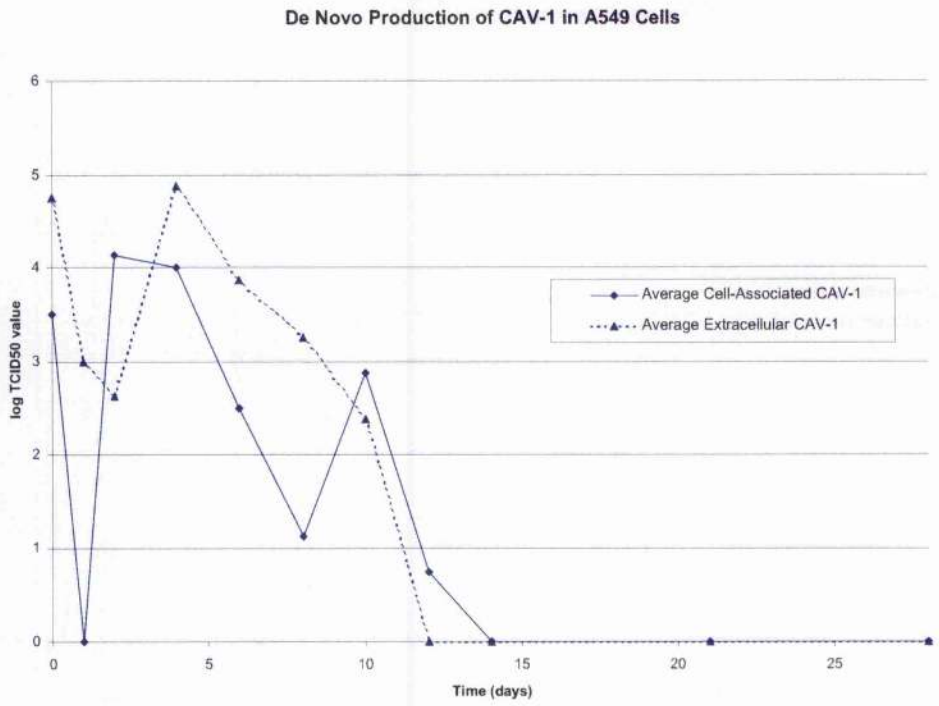
Figure 3.6 RT-PCR of RNA isolated from CAV-1 infected (i) MDCK, (ii) 293, (iii) A549, (iv) CrFK and (v) FEA cells at 0, 4, 8, 24, 48, 72 hour time points. M = 1Kb molecular weight marker, 1 = e1a, 4 = E4 34 kDa homologue, P = DNA polymerase, F = fibre, -RTase = no RTase control for the 48 hours samples.

3.3.4 Maintenance of CAV-1 in Human Cells

A549 cells were infected with CAV-1 at a m.o.i. of 10 in order to investigate maintenance of CAV-1 over an extended time period. Samples were taken over a 28 day period for determination of de novo virus production. DNA was harvested for TaqMan analysis and RNA was isolated and subjected to RT-PCR. Following an initial decrease in cell-associated and extracellular CAV-1 titres, 2 days p.i. cell-associated CAV-1 virus particles were detected at levels approximately 0.6 logs higher than input and by 4 days the amount of extracellular virus detected in the culture medium was also slightly higher than input level (Figure 3.7(i)). Thereafter, the amount of CAV-1 infectious virus particles decreased until, by 14 days, no cell-associated or extracellular CAV-1 could be detected. When compared with Figure 3.1, although both growth curves show a decrease in titres in both the cell-associated and extracellular samples, this is more pronounced in Figure 3.7(i). Nevertheless, cell-associated virus increased by approximately the same amount in both experiments. It is noteworthy that the experimental protocol in this section was carried out quite differently from previous experiments: cells were subcultured more frequently with cell culture medium being discarded and replaced with fresh medium.

TaqMan analysis revealed that CAV-1 levels increased to approximately 38-fold higher than input by 2 days, but declined throughout the remainder of the time course and by 14 days the amount of E1a DNA was 100-fold less than that at the beginning of the time course (Figure 3.7(ii)). In Figure 3.3, where less aggressive subculture took place, the maximum fold increase was approximately 100-fold and the levels of DNA were still higher than input 14 days p.i.

Transcripts were detected at 1 and 2 days but not at any other time point (Figure 3.8). In Figure 3.6 there were transcripts for all 4 CAV-1 genes detected 1, 2 and 3 days p.i. and, although only carried out for 3 days and not quantitative PCR, there is no evidence of the levels of transcripts detected dropping at the 3 day time point. As mentioned above, the experimental conditions were quite distinct from those of Figure 3.6 where cells were untouched over the 3 days of the experiment.



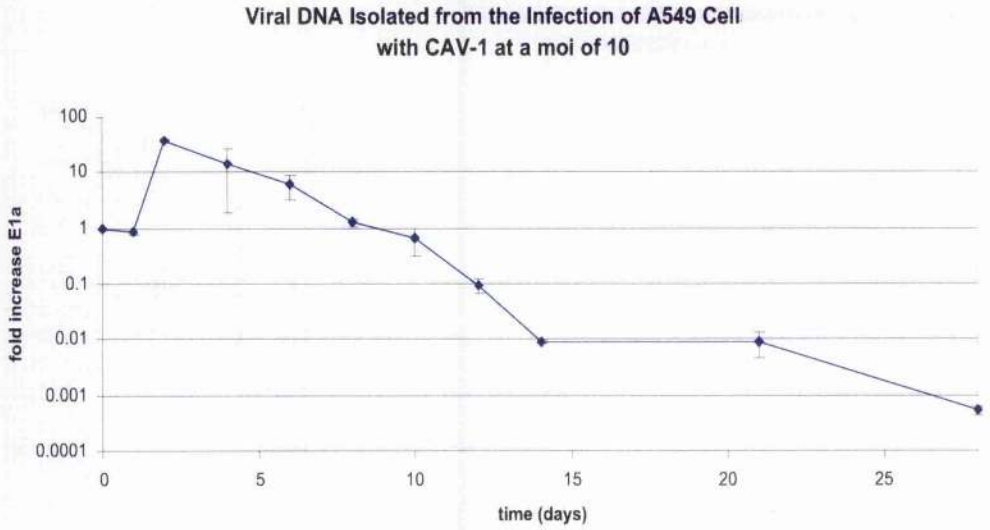


Figure 3.7 Maintenance of CAV-1 infection in A549 cells. A549 cells were infected with CAV-1 at a m.o.i. of 10 p.f.u./cell. Cell and supernatant samples were taken at 0, 1, 2, 4, 6, 8, 10, 12, 14, 21 and 28 day time points. (i) Cell-associated and extracellular virus particles were isolated at the appropriate time points. $TCID_{50}$ values are presented (n=2). (ii) DNA was isolated at the appropriate time points and subjected to TaqMan analysis as described in the text.

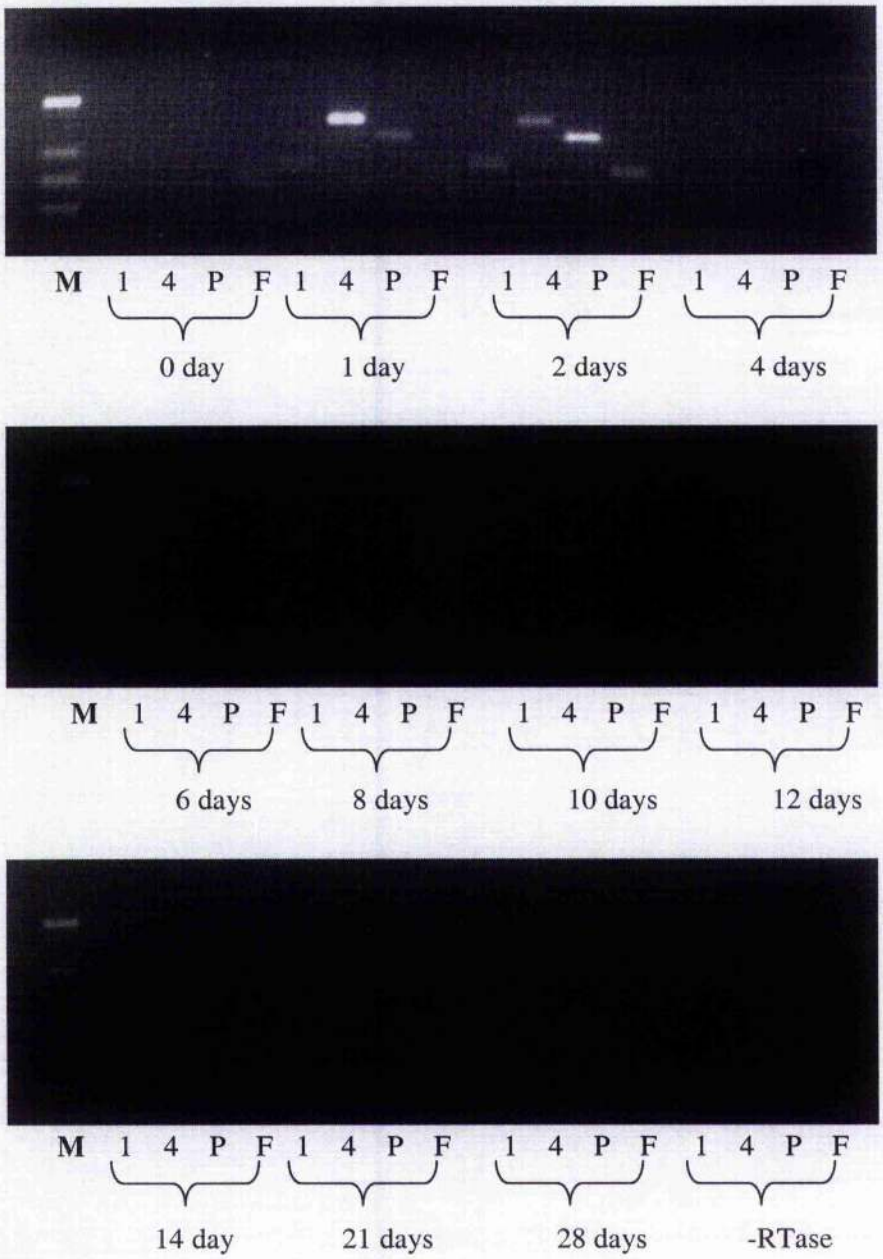


Figure 3.8 RT-PCR of RNA isolated from CAV-1 infected A549 cells over a 28 day time course. M = 1Kb molecular weight marker, 1 = E1a, 4 = E4 34 kDa homologue, P = DNA polymerase, F = fibre. Time points are shown below, -RTase = no RTase control for the 48 hour samples.

3.3.5 Transgene Expression from a CAV-1 Vector in Human and Feline Cells

MDCK, A549, 293, CrFK and FEA cells were infected with ΔE3-GFP-CAV-1 at a m.o.i. of 0.1 or 10 p.f.u./cell. Individual flasks were set up for each time point and no subculture took place. The cells were photographed at 8, 24, 48 and 120 hours post infection using light and fluorescent microscopy (see Figures 3.9 – 3.28), the same field was photographed for both light and fluorescent microscopy.

MDCK cells infected at a m.o.i. of 0.1 p.f.u./cell expressed the GFP transgene by 8 hours p.i., albeit at a very low intensity and in only very few of the cells (Figure 3.9). Nevertheless by 24 hours p.i. (Figure 3.10), GFP was evident in a higher proportion of the cells and the intensity of fluorescence had clearly increased. These features had become even more pronounced by 48 hours p.i. (Figure 3.11). At this time almost all the cells displayed high intensity fluorescence, although under a light microscope there was no evidence of c.p.e.. Productive adenovirus infection is characterised by the formation of 'grape-like' clusters of rounded cells and by 120 hours p.i., c.p.e. had reached 100% and all cells showed high intensity fluorescence (Figure 3.12). By 8 hours p.i. more than half the MDCK cells infected at a m.o.i. of 10 p.f.u./cell showed a degree of fluorescence (Figure 3.9) and at 24 hours p.i. there was almost 100% c.p.e. (Figure 3.10) and all the cells present fluoresced intensely. 100% c.p.e. was evident by 48 hours p.i. (Figure 3.11).

By 8 hours p.i. there was no fluorescence observed in the 293 cells infected at a m.o.i. of 0.1 and those infected at a m.o.i. of 10 showed minimal fluorescence (Figure3.13). There was also no fluorescence evident in the A549 cells infected at a m.o.i. of 0.1, although a few of those infected at a m.o.i. of 10 showed very low levels of fluorescence (Figure3.17). The expression of GFP was similar throughout the time course in A549 and 293 cells at later time points. There was evidence of GFP expression observed in the either human cell-line infected at both a m.o.i. of 0.1 and 10 by 24 hours p.i. (Figures 3.14 and 3.18), although this was sporadic and none of the fluorescing cells appeared to be excessively rounded. By 48 hours p.i. more human cells infected at a m.o.i. of 10 were fluorescing, and of these a proportion were

rounded (Figures 3.15 and 3.19). However, human cells infected at a m.o.i. of 10 showed less GFP expression at 120 hours than at 48 hours p.i. (Figures 3.16 and 3.20). There was no change in fluorescence in the human cells infected at a m.o.i. of 0.1 after the 24 hour time point.

GFP expression was evident 8 hours p.i. in CrFK cells infected at both 0.1 and 10 m.o.i. (Figure 3.21). This expression was very scattered in those infected at a m.o.i. of 0.1 p.f.u./cell., however, at a m.o.i. of 10 p.f.u./cell, a substantial number of cells fluoresced weakly. By 24 hours p.i. there was fluorescence to a high intensity evident in the CrFK cells infected at a m.o.i. of 10 p.f.u./cell and most of these cells were significantly rounded (Figure 3.22). There was also rounding and GFP expression in CrFK cells infected at a m.o.i. of 0.1 p.f.u./cell at the 24 hour time point, however, the fluorescence was weaker and the rounding was less distinct and GFP expression was evident in a smaller proportion of the cells. From 48 hours p.i. onwards, the CrFK cells infected at a m.o.i. of 10 p.f.u./cell showed high levels of GFP expression and c.p.e. (Figure 3.23), however this c.p.e. had not reached completion by the end of the time course. 5 days p.i. CrFK cells infected at a m.o.i. of 0.1 p.f.u./cell showed no evidence of c.p.e. but more than 50% of the cells demonstrated expression of GFP (Figure 3.24).

There was no evidence of transgene expression in FEA cells infected at a m.o.i. of 0.1 p.f.u./cell until 24 hours p.i. (Figure 3.26). At this time-point there were only very few cells fluorescing, however this fluorescence was intense. The number of GFP expressing cells increased slightly by 48 hours p.i. (Figure 3.27) and even more so by 120 hours p.i. (Figure 3.28). The fluorescent cells were rounded, although classical adenoviral c.p.e. was not observed. However, infection of FEA cells at a m.o.i. of 10 p.f.u./cell did result in a significant level of cell death. Although by 120 hours p.i. there was distinct rounding and clumping of many of the cells present there was also a proportion that of cells that did not demonstrate c.p.e..

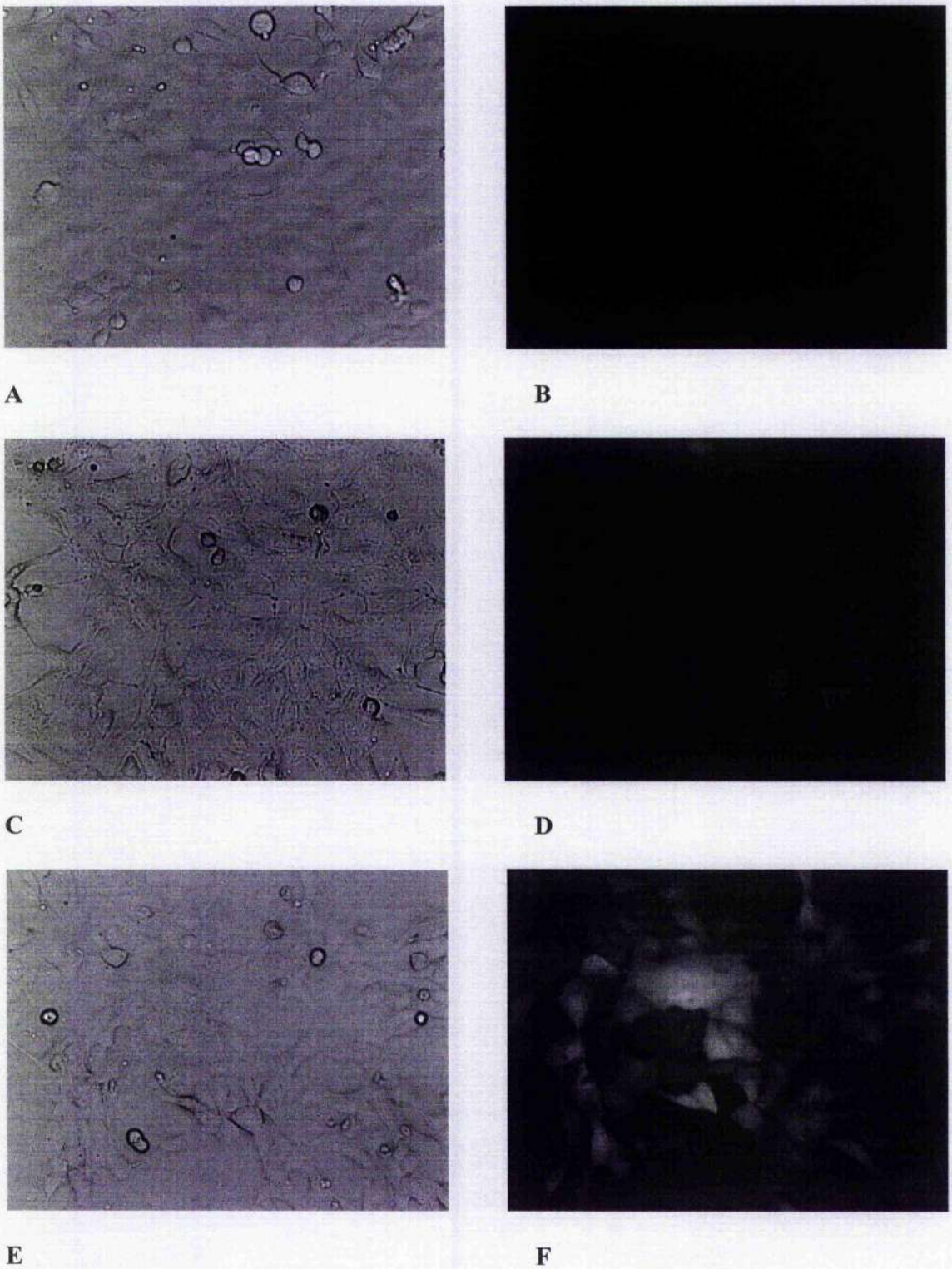


Figure 3.9 GFP expression in MDCK cells 8 hours post infection
MDCK cells were either uninfected (A and B), infected at a moi of 0.1 with ΔE3GFPCAV-1 (C and D) or infected at a moi of 10 with ΔE3GFPCAV-1 (E and F). The same cells were viewed with both a light microscope (A, C and E) and with a microscope fitted with fluorescence (B, D and F) 8 hours p.i. (see section 3.2.5).

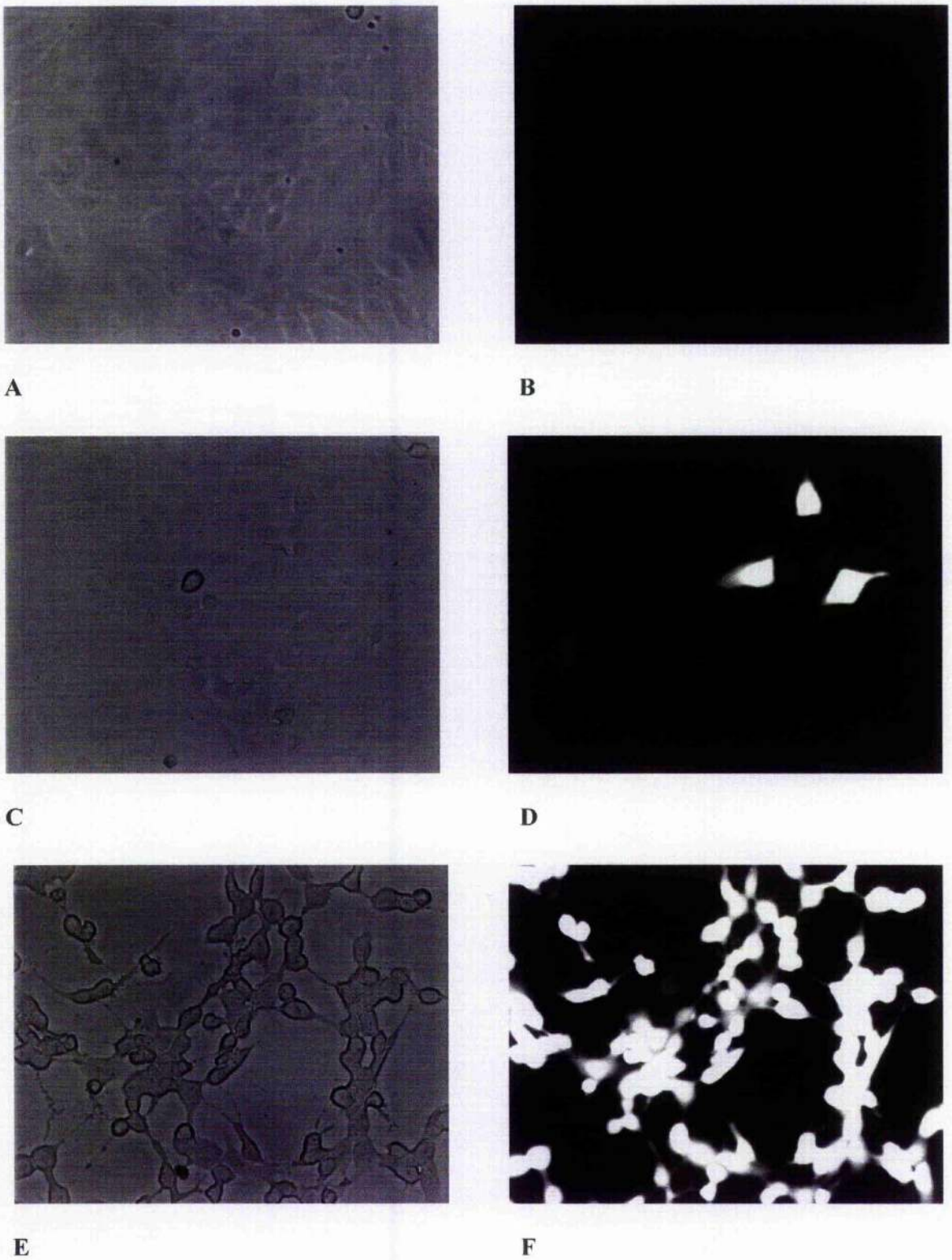


Figure 3.10 GFP Expression in MDCK cells 24 hours post infection
MDCK cells were either uninfected (A and B), infected at a moi of 0.1 with ΔE3GFPCAV-1 (C and D) or infected at a moi of 10 with ΔE3GFPCAV-1 (E and F). The same cells were viewed with both a light microscope (A, C and E) and with a microscope fitted with fluorescence (B, D and F) 24 hours p.i. (see section 3.2.5).

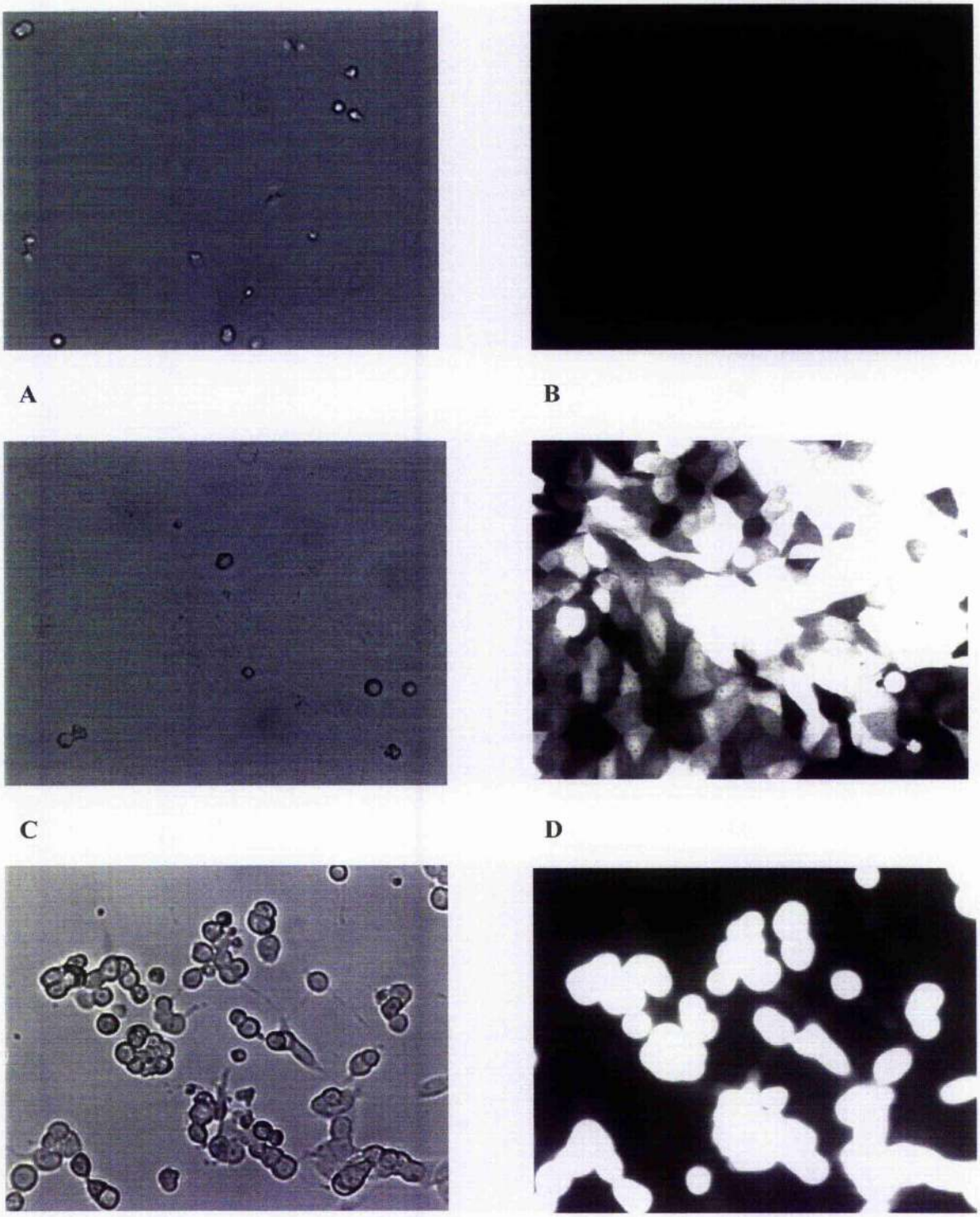


Figure 3.11 GFP expression in MDCK cells 48 hours post infection
MDCK cells were either uninfected (A and B), infected at a moi of 0.1 with ΔE3GFPCAV-1 (C and D) or infected at a moi of 10 with ΔE3GFPCAV-1 (E and F). The same cells were viewed with both a light microscope (A, C and E) and with a microscope fitted with fluorescence (B, D and F) 48 hours p.i. (see section 3.2.5).

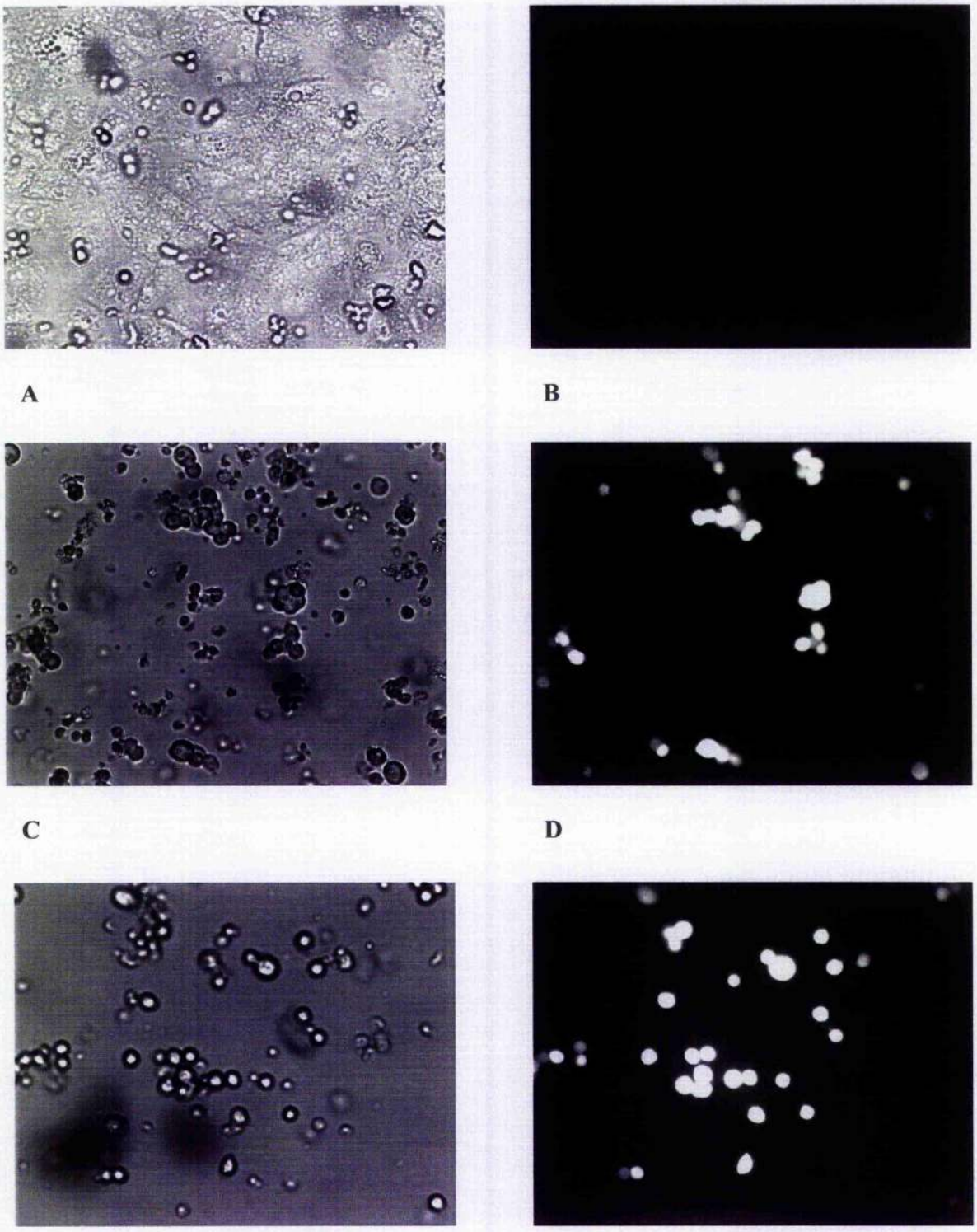


Figure 3.12 GFP expression in MDCK cells 5 days post infection
MDCK cells were either uninfected (A and B), infected at a moi of 0.1 with ΔE3GFPCAV-1 (C and D) or infected at a moi of 10 with ΔE3GFPCAV-1 (E and F). The same cells were viewed with both a light microscope (A, C and E) and with a microscope fitted with fluorescence (B, D and F) 120 hours p.i. (see section 3.2.5).

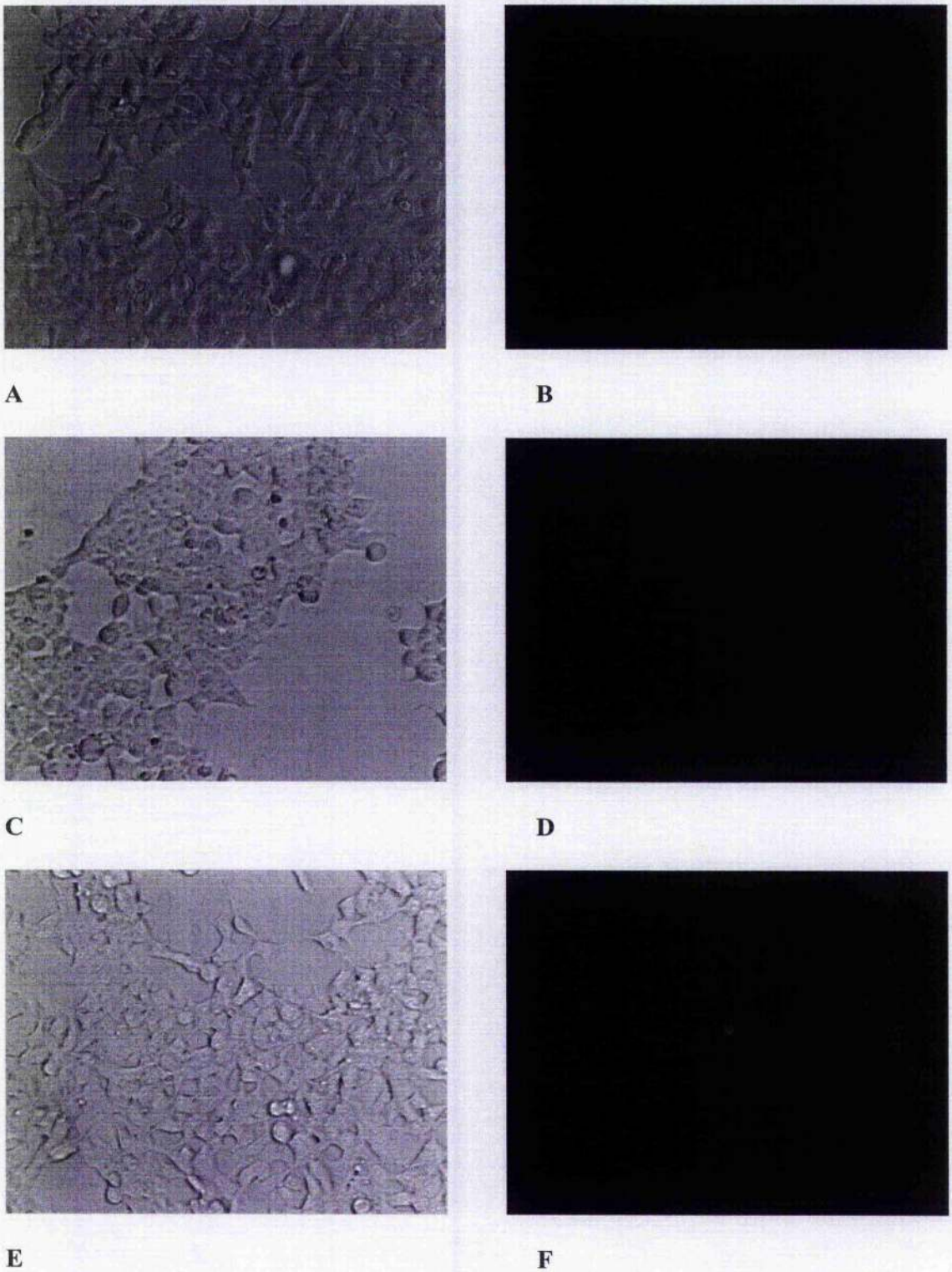


Figure 3.13 GFP expression in 293 cells 8 hours post infection
293 cells were either uninfected (A and B), infected at a moi of 0.1 with ΔE3GFPCAV-1 (C and D) or infected at a moi of 10 with ΔE3GFPCAV-1 (E and F). The same cells were viewed with both a light microscope (A, C and E) and with a microscope fitted with fluorescence (B, D and F) 8 hours p.i. (see section 3.2.5).

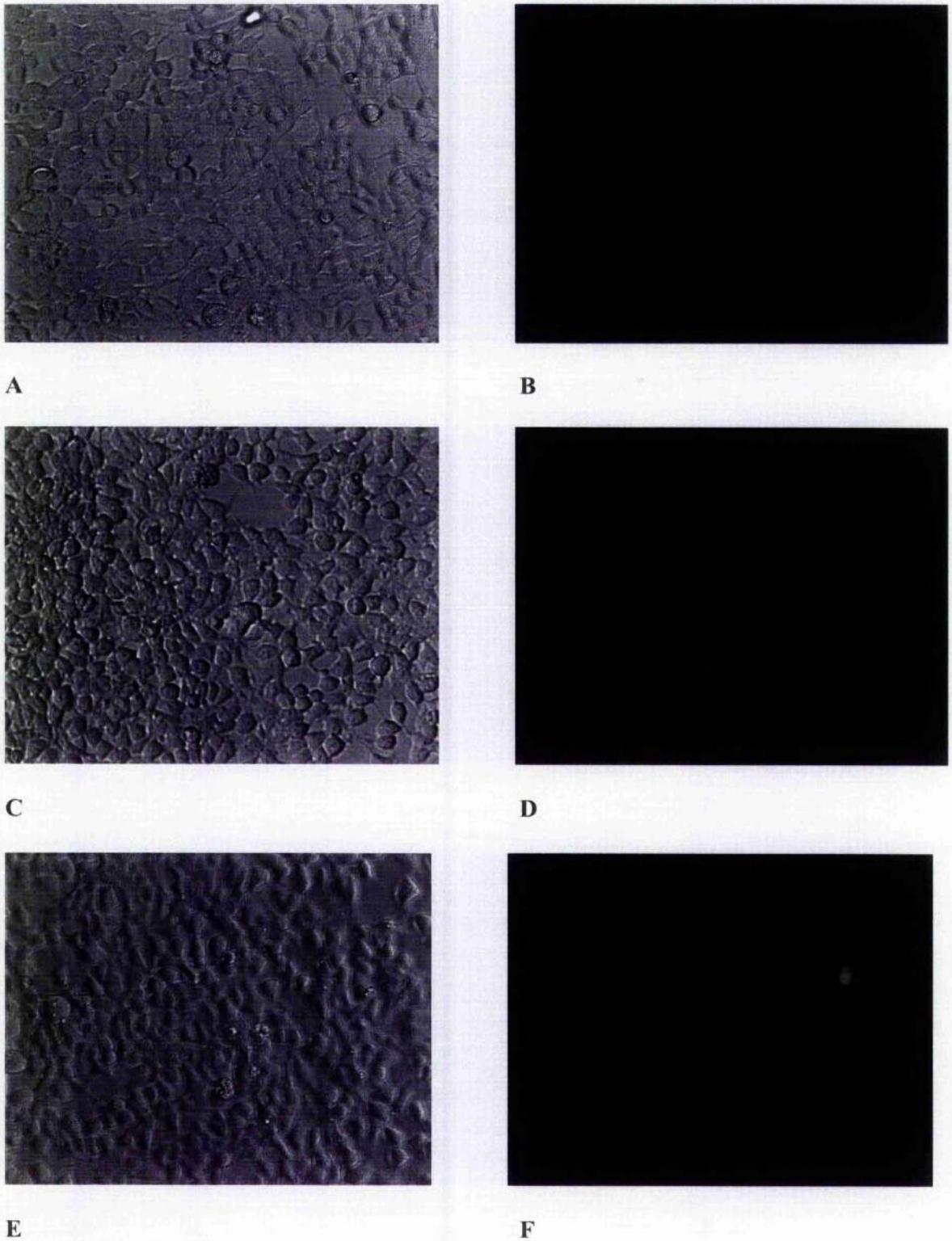


Figure 3.14 GFP expression in 293 cells 24 hours post infection
293 cells were either uninfected (A and B), infected at a moi of 0.1 with ΔE3GFPCAV-1 (C and D) or infected at a moi of 10 with ΔE3GFPCAV-1 (E and F). The same cells were viewed with both a light microscope (A, C and E) and with a microscope fitted with fluorescence (B, D and F) 24 hours p.i. (see section 3.2.5).

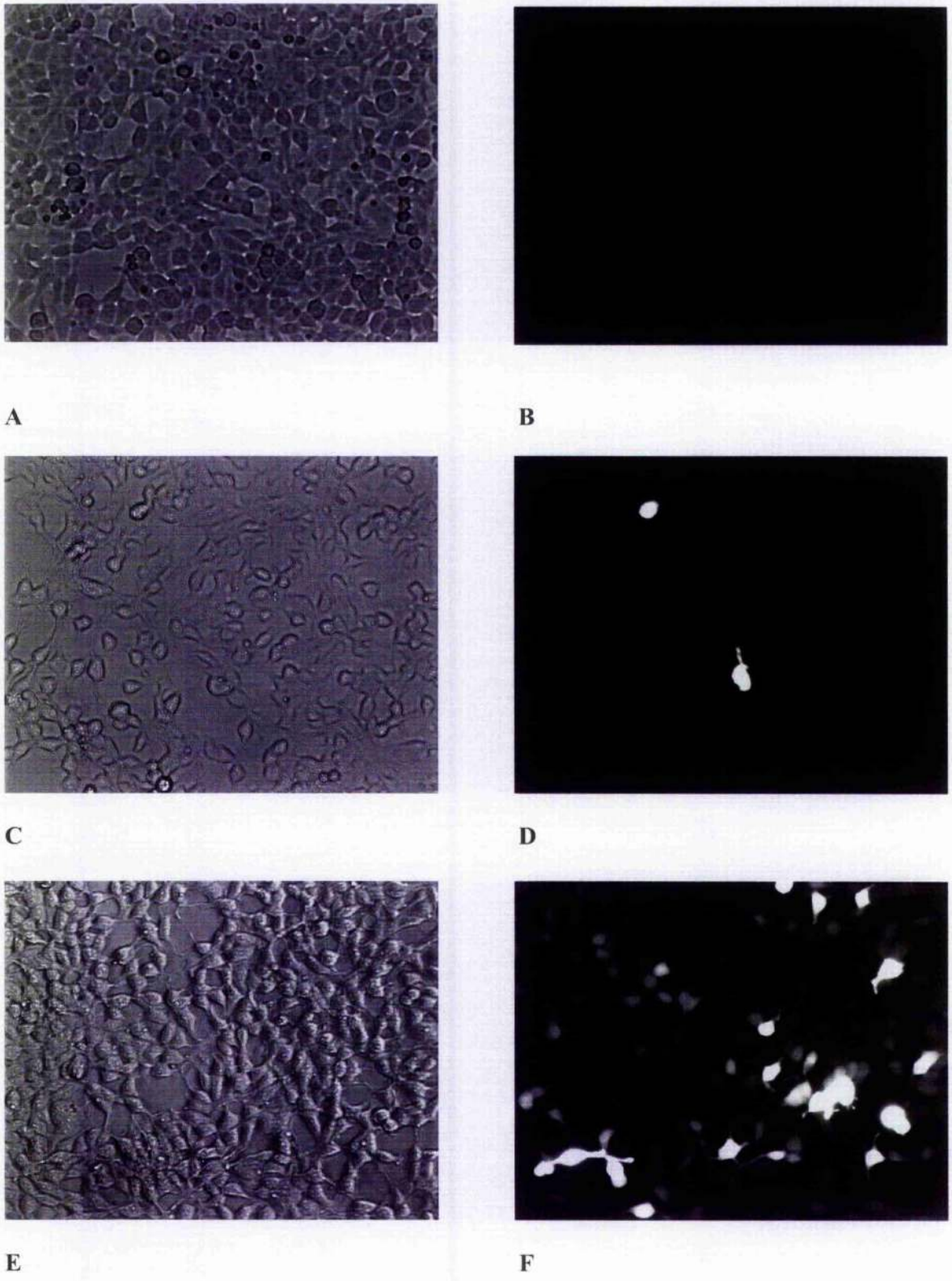


Figure 3.15 GFP expression in 293 cells 48 hours post infection
293 cells were either uninfected (A and B), infected at a moi of 0.1 with ΔE3GFPCAV-1 (C and D) or infected at a moi of 10 with ΔE3GFPCAV-1 (E and F). The same cells were viewed with both a light microscope (A, C and E) and with a microscope fitted with fluorescence (B, D and F) 48 hours p.i. (see section 3.2.5).

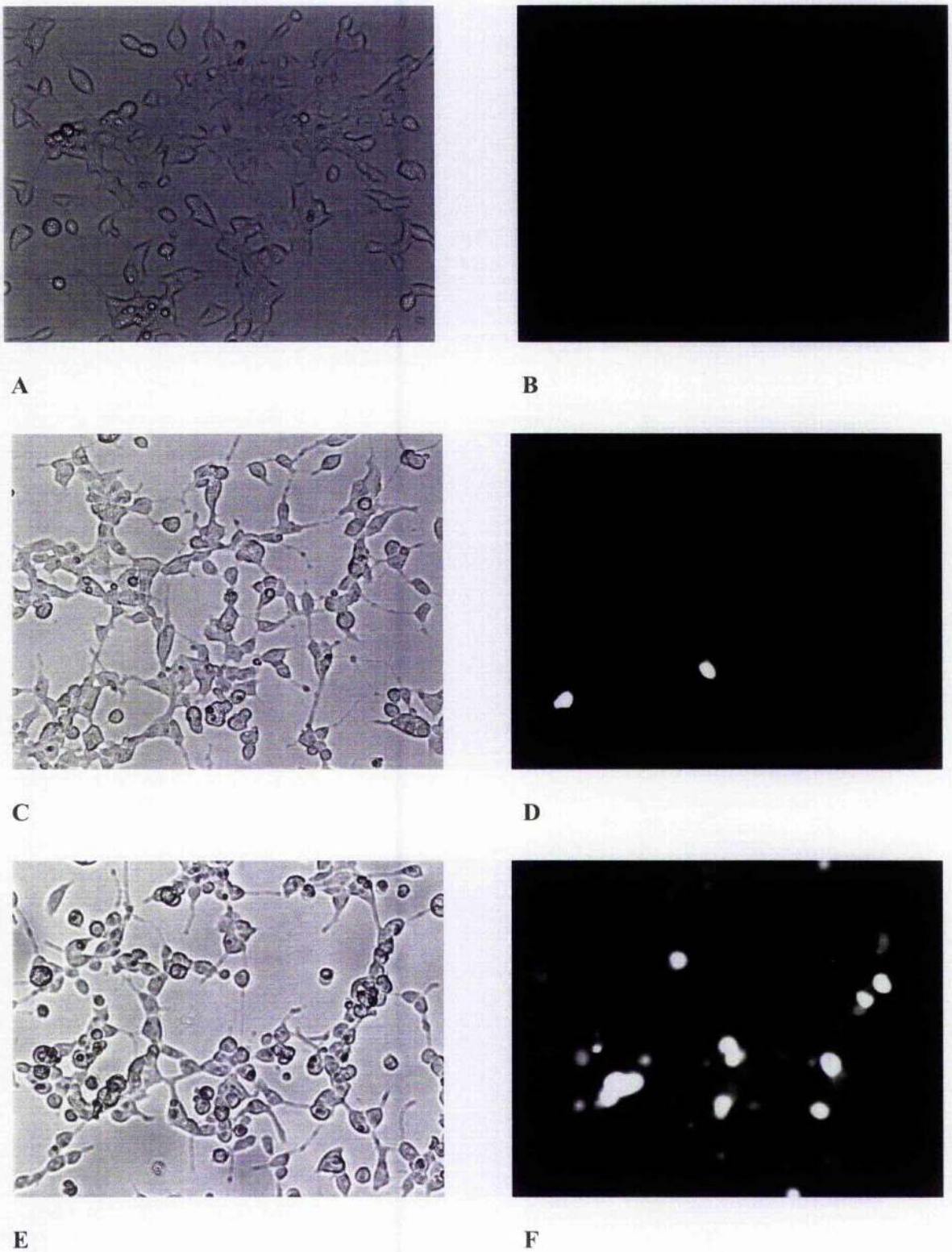


Figure 3.16 GFP expression in 293 cells 5 days post infection
293 cells were either uninfected (A and B), infected at a moi of 0.1 with ΔE3GFPCAV-1 (C and D) or infected at a moi of 10 with ΔE3GFPCAV-1 (E and F). The same cells were viewed with both a light microscope (A, C and E) and with a microscope fitted with fluorescence (B, D and F) 120 hours p.i. (see section 3.2.5).

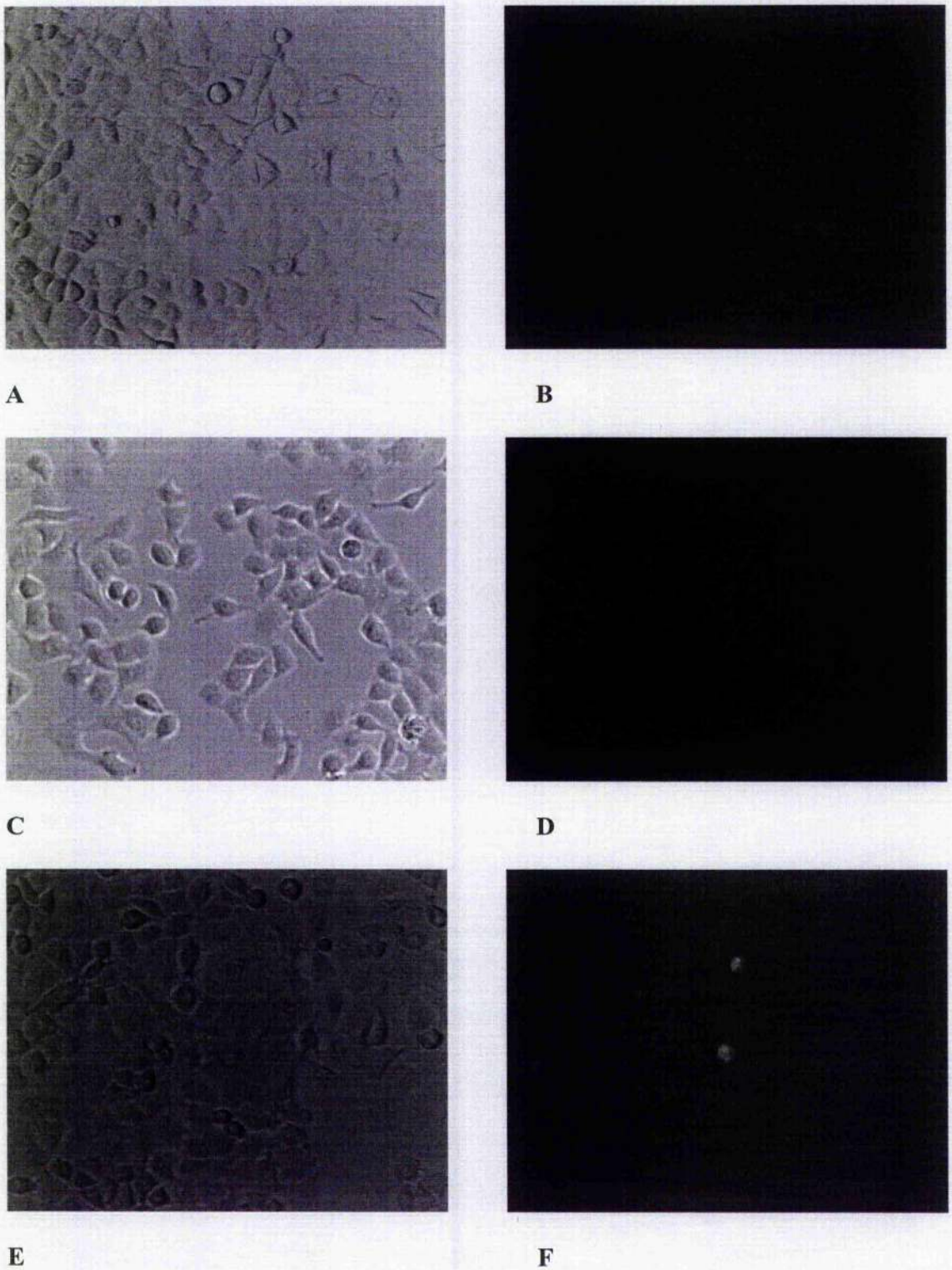


Figure 3.17 GFP expression in A549 cells 8 hours post infection
A549 cells were either uninfected (A and B), infected at a moi of 0.1 with ΔE3GFPCAV-1 (C and D) or infected at a moi of 10 with ΔE3GFPCAV-1 (E and F). The same cells were viewed with both a light microscope (A, C and E) and with a microscope fitted with fluorescence (B, D and F) 8 hours p.i. (see section 3.2.5).

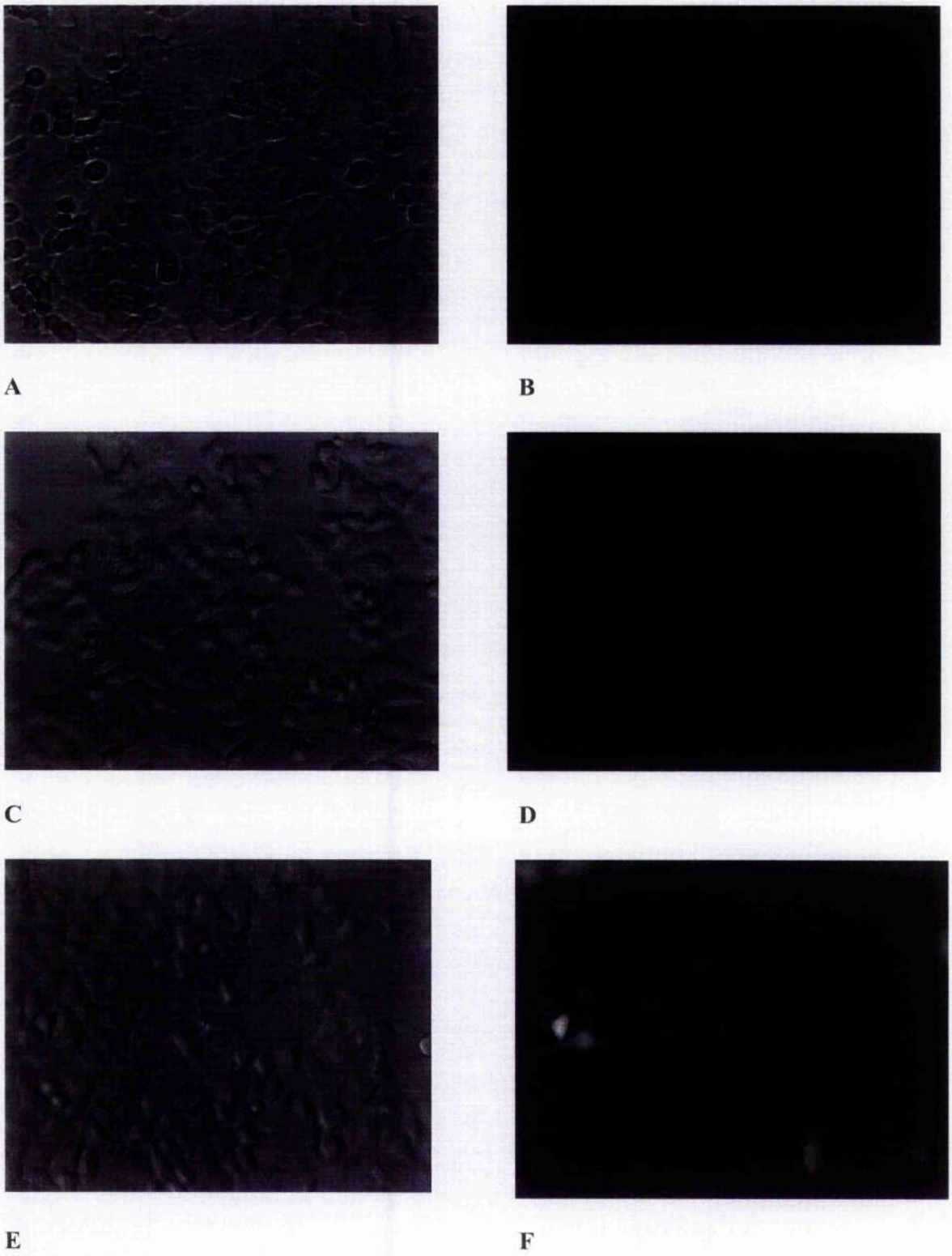


Figure 3.18 GFP expression in A549 cells 24 hours post infection
A549 cells were either uninfected (A and B), infected at a moi of 0.1 with ΔE3GFPCAV-1 (C and D) or infected at a moi of 10 with ΔE3GFPCAV-1 (E and F). The same cells were viewed with both a light microscope (A, C and E) and with a microscope fitted with fluorescence (B, D and F) 24 hours p.i. (see section 3.2.5).

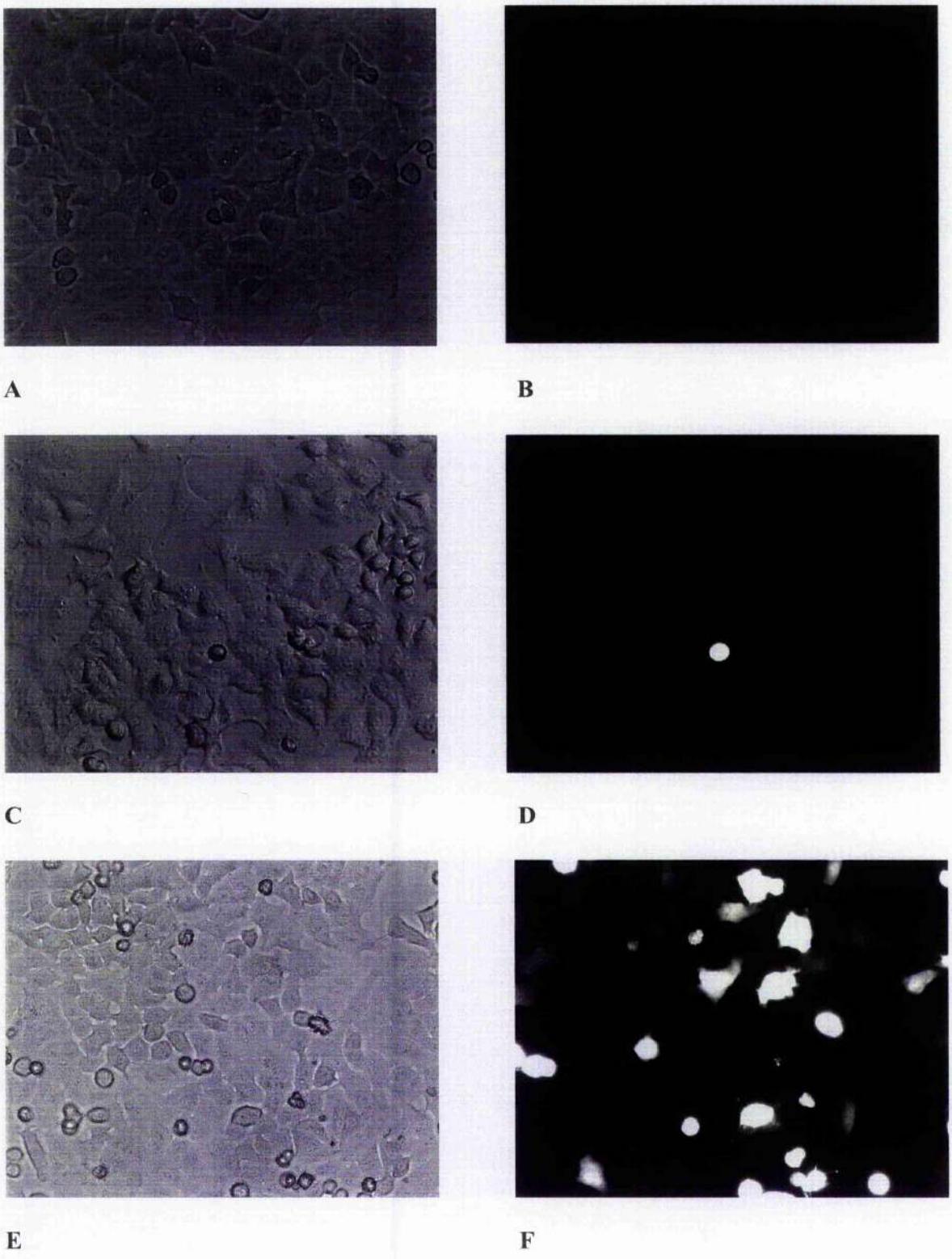


Figure 3.19 GFP expression in A549 cells 48 hours post infection
A549 cells were either uninfected (A and B), infected at a moi of 0.1 with ΔE3GFPCAV-1 (C and D) or infected at a moi of 10 with ΔE3GFPCAV-1 (E and F). The same cells were viewed with both a light microscope (A, C and E) and with a microscope fitted with fluorescence (B, D and F) 48 hours p.i. (see section 3.2.5).

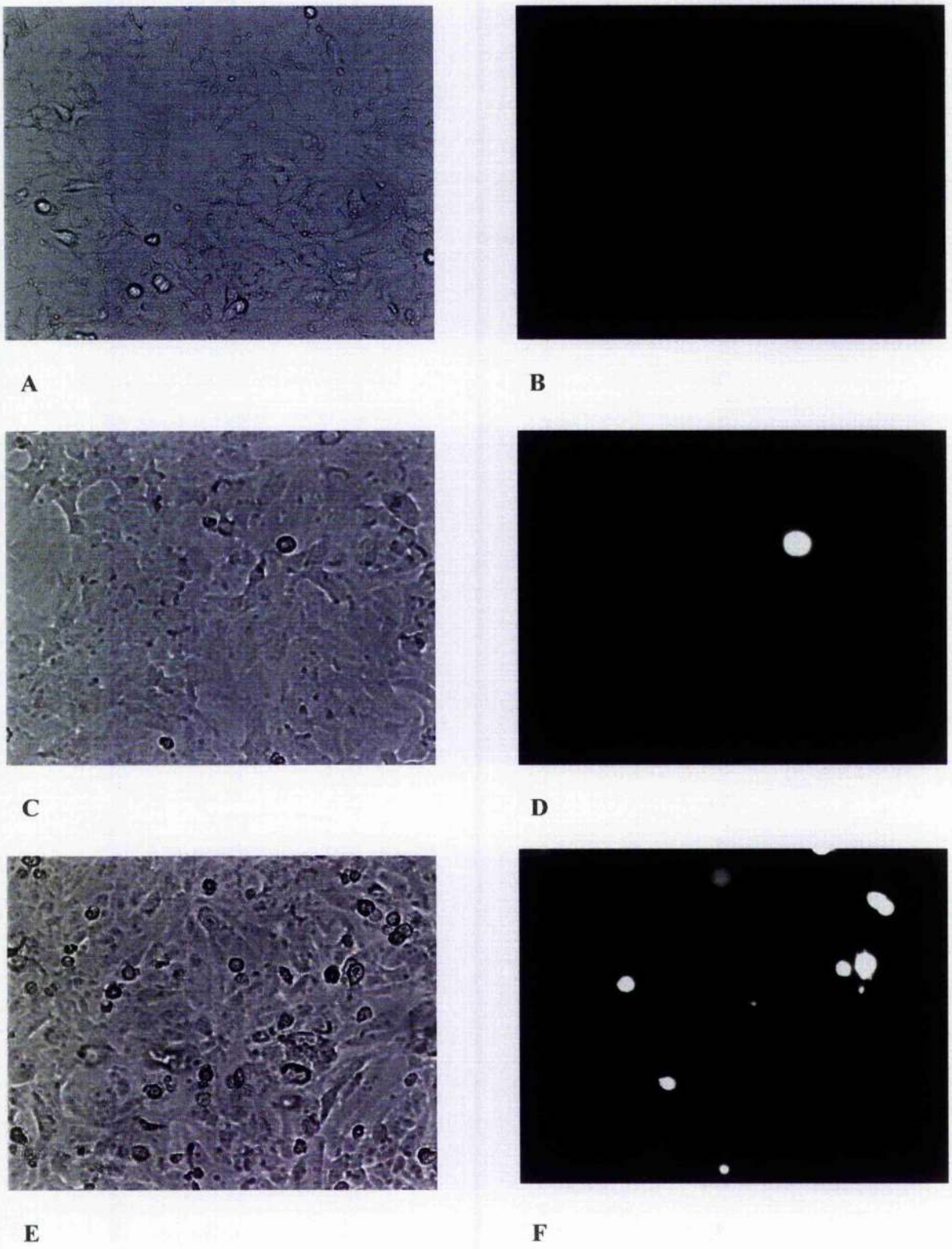


Figure 3.20 GFP expression in A549 cells 5 days post infection
A549 cells were either uninfected (A and B), infected at a moi of 0.1 with ΔE3GFPCAV-1 (C and D) or infected at a moi of 10 with ΔE3GFPCAV-1 (E and F). The same cells were viewed with both a light microscope (A, C and E) and with a microscope fitted with fluorescence (B, D and F) 120 hours p.i. (see section 3.2.5).

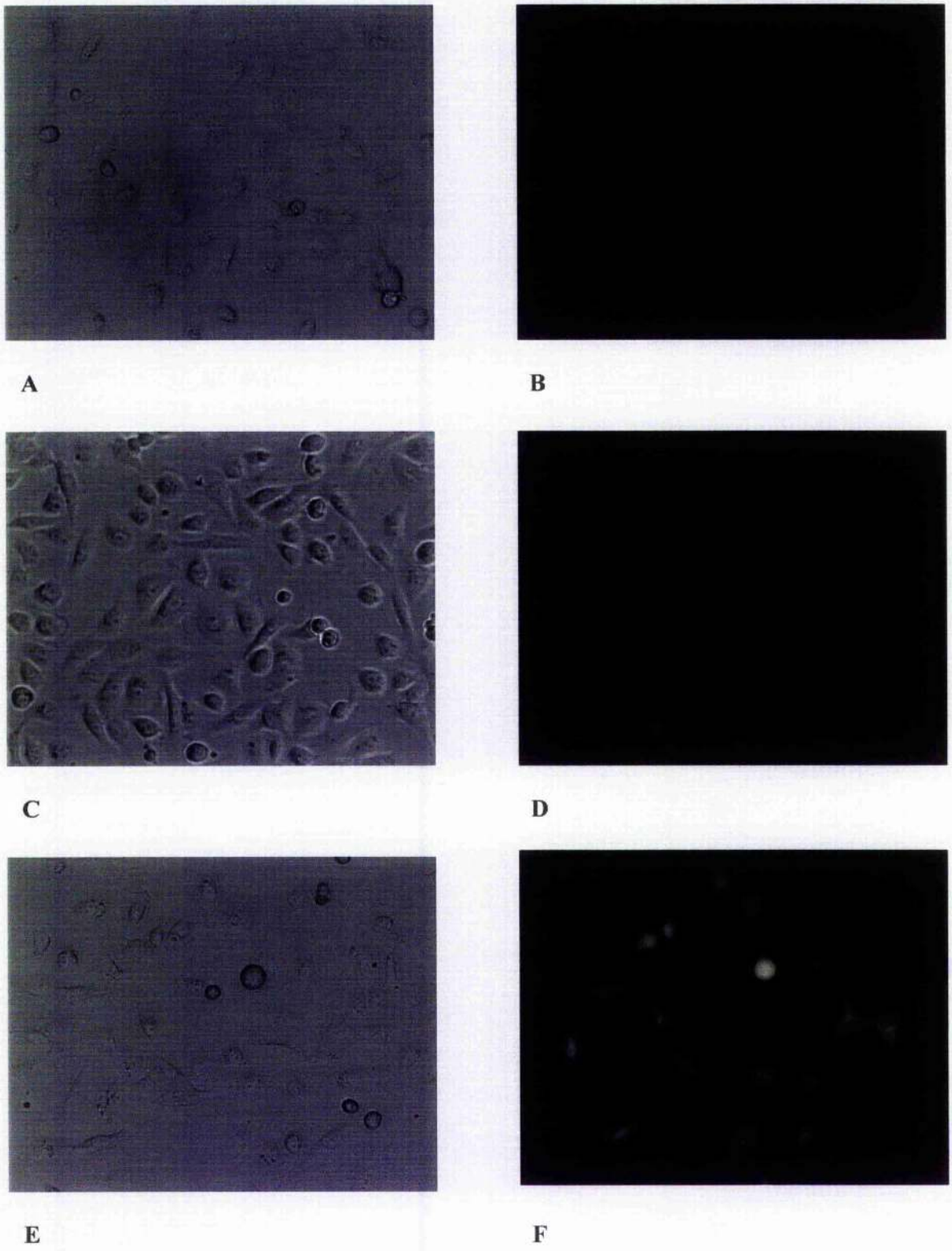
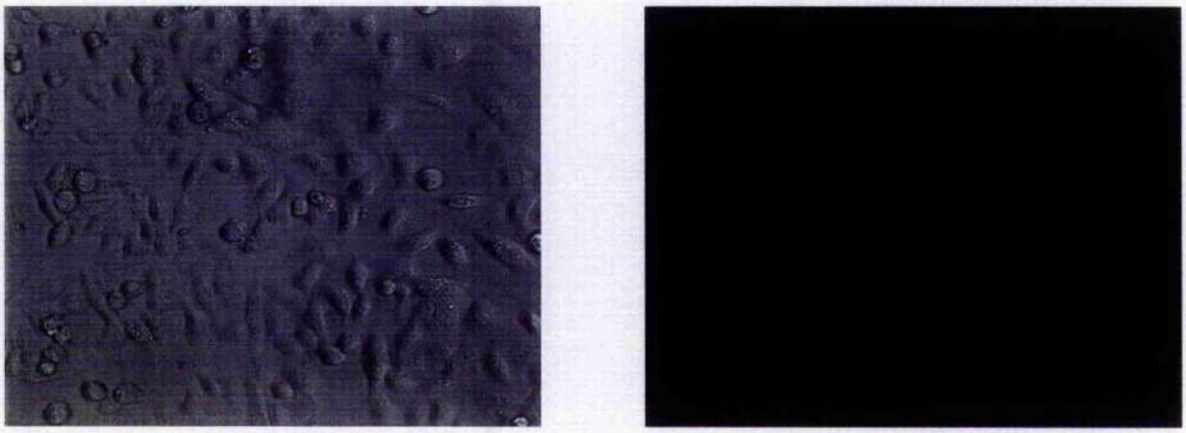


Figure 3.21 GFP expression in CrFK cells 8 hours post infection
CrFK cells were either uninfected (A and B), infected at a moi of 0.1 with ΔE3GFPCAV-1 (C and D) or infected at a moi of 10 with ΔE3GFPCAV-1 (E and F). The same cells were viewed with both a light microscope (A, C and E) and with a microscope fitted with fluorescence (B, D and F) 8 hours p.i. (see section 3.2.5).



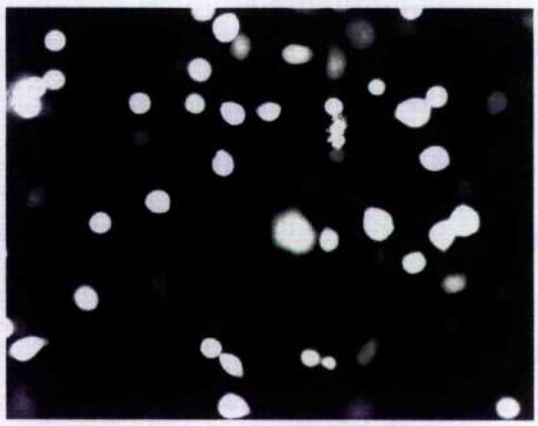
A

B

C

D

E



F

Figure 3.22 Transgene Expression in CrFK cells 24 hours post infection. CrFK cells were either uninfected (A and B), infected at a moi of 0.1 with ΔE3GFPCAV-1 (C and D) or infected at a moi of 10 with ΔE3GFPCAV-1 (E and F). The same cells were viewed with both a light microscope (A, C and E) and with a microscope fitted with fluorescence (B, D and F) 24 hours p.i. (see section 3.2.5).

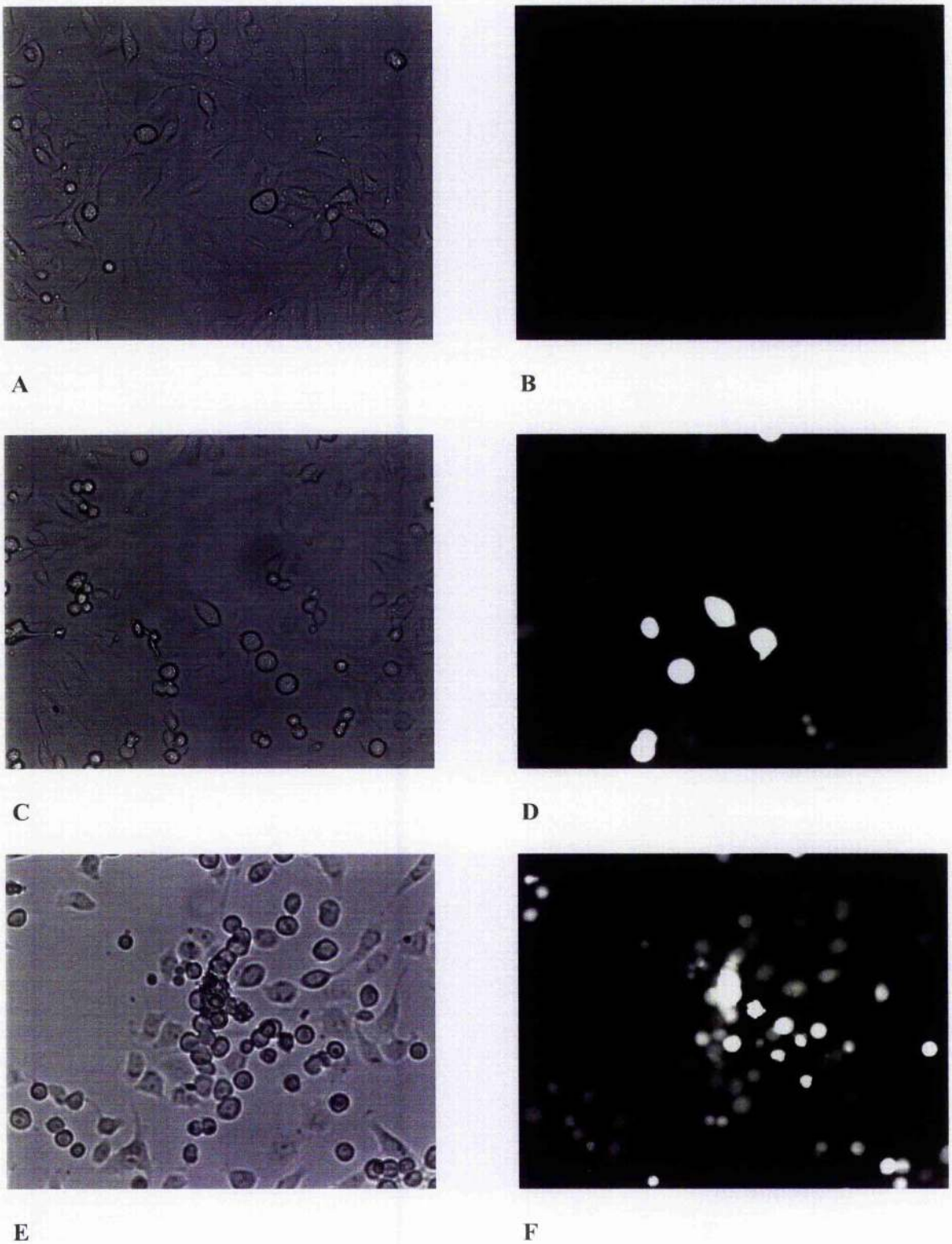


Figure 3.23 GFP Expression in CrFK cells 48 hours post infection
CrFK cells were either uninfected (A and B), infected at a moi of 0.1 with ΔE3GFPCAV-1 (C and D) or infected at a moi of 10 with ΔE3GFPCAV-1 (E and F). The same cells were viewed with both a light microscope (A, C and E) and with a microscope fitted with fluorescence (B, D and F) 8 hours p.i. (see section 3.2.5).

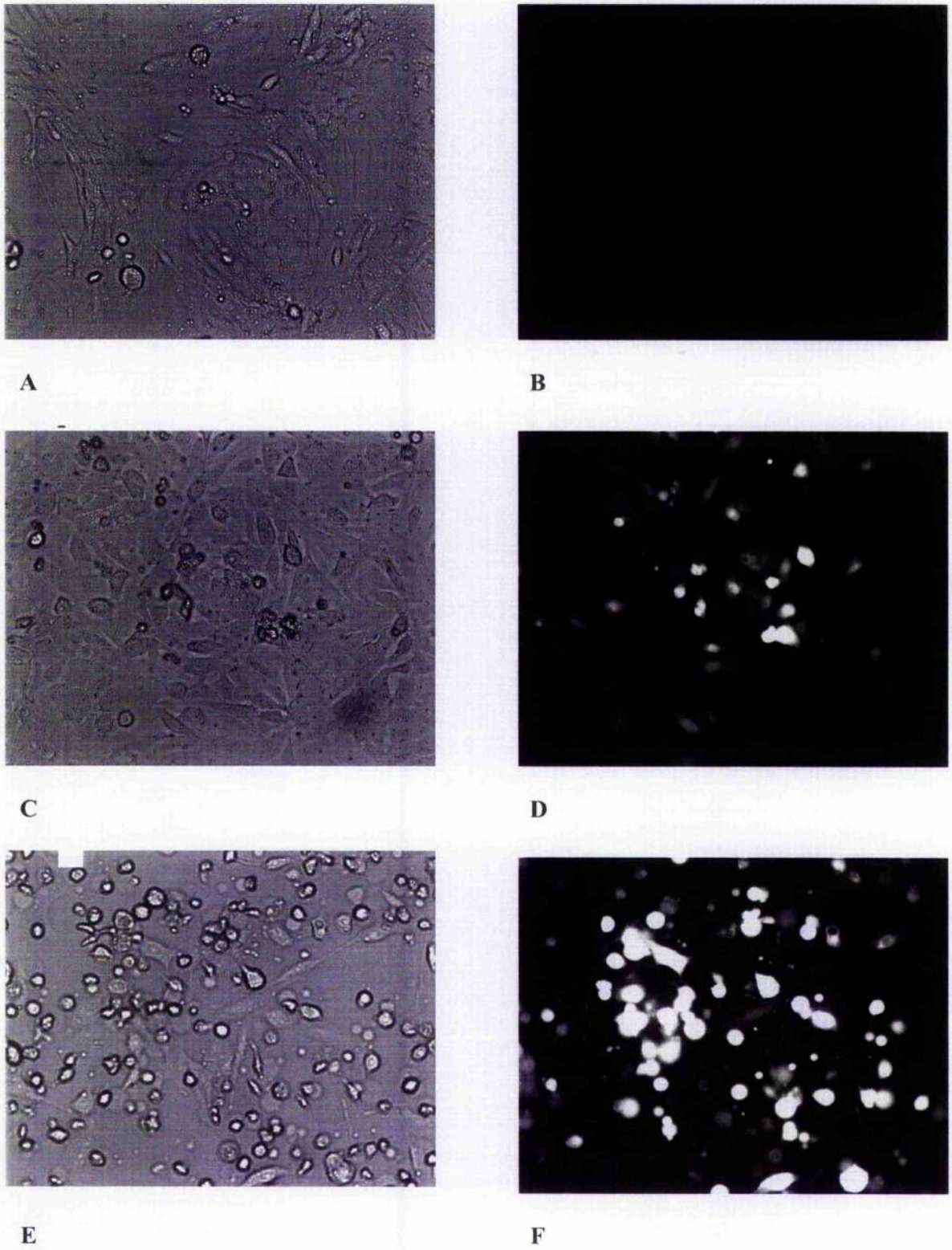


Figure 3.24 GFP expression in CrFK cells 5 days post infection
CrFK cells were either uninfected (A and B), infected at a moi of 0.1 with ΔE3GFPCAV-1 (C and D) or infected at a moi of 10 with ΔE3GFPCAV-1 (E and F). The same cells were viewed with both a light microscope (A, C and E) and with a microscope fitted with fluorescence (B, D and F) 120 hours p.i. (see section 3.2.5).

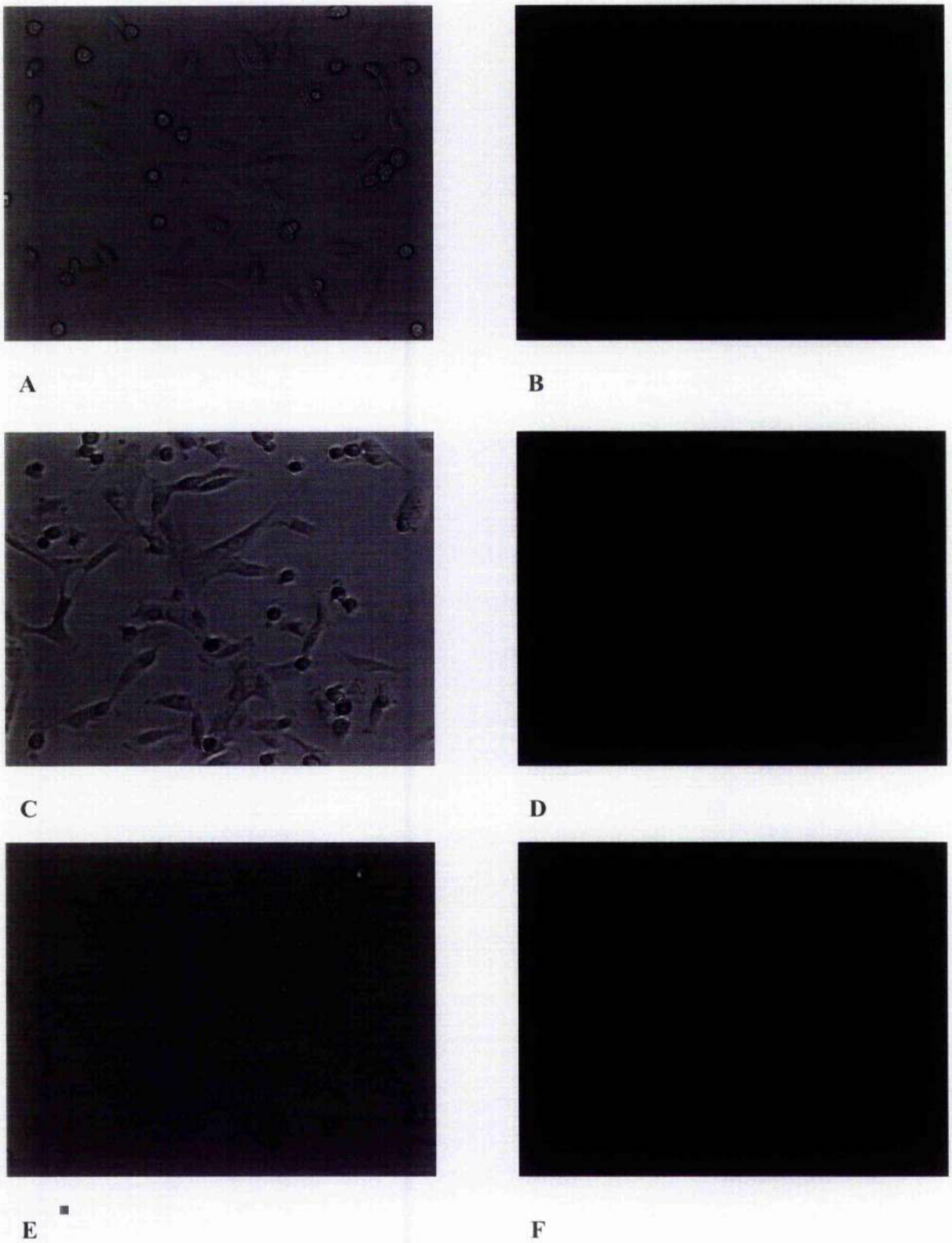


Figure 3.25 GFP expression in FEA cells 8 hours post infection
FEA cells were either uninfected (A and B), infected at a moi of 0.1 with ΔE3GFPCAV-1 (C and D) or infected at a moi of 10 with ΔE3GFPCAV-1 (E and F). The same cells were viewed with both a light microscope (A, C and E) and with a microscope fitted with fluorescence (B, D and F) 8 hours p.i. (see section 3.2.5).

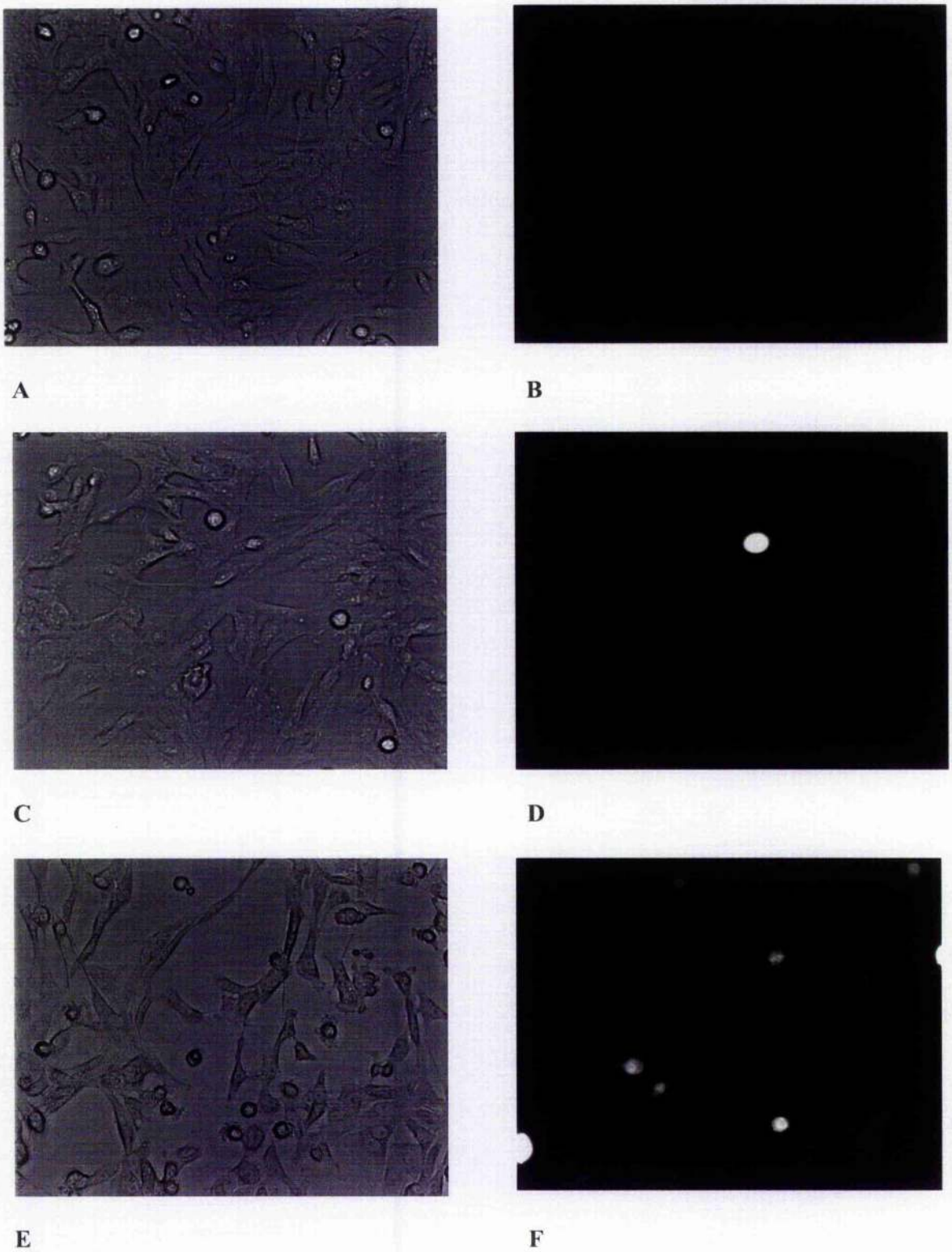


Figure 3.26 GFP expression in FEA cells 24 hours post infection
FEA cells were either uninfected (A and B), infected at a moi of 0.1 with ΔE3GFPCAV-1 (C and D) or infected at a moi of 10 with ΔE3GFPCAV-1 (E and F). The same cells were viewed with both a light microscope (A, C and E) and with a microscope fitted with fluorescence (B, D and F) 24 hours p.i. (see section 3.2.5).

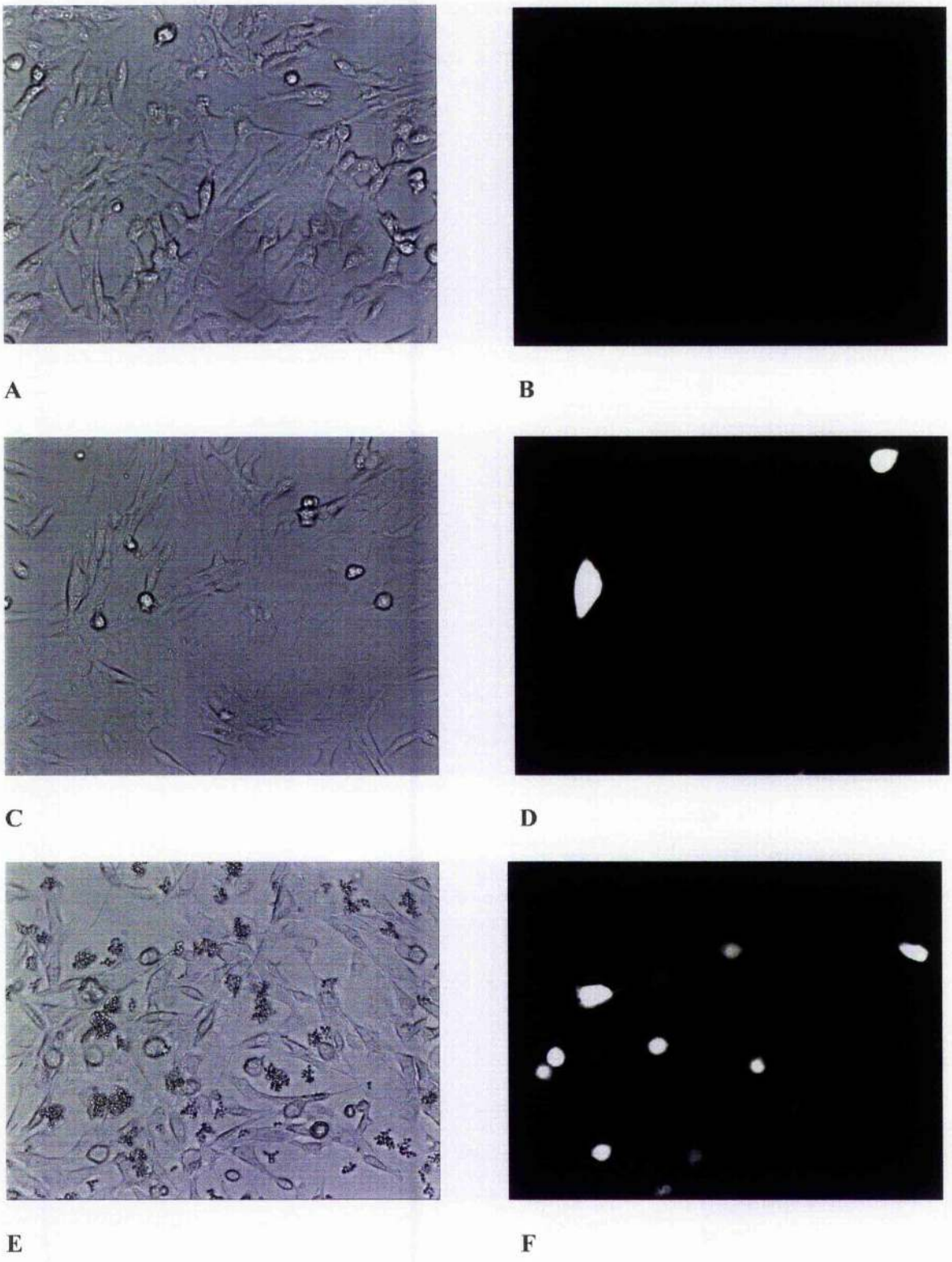
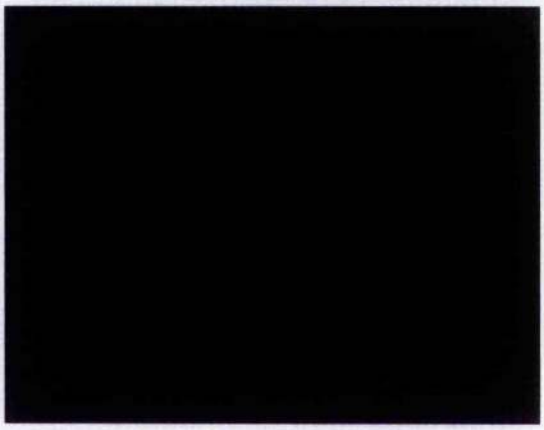
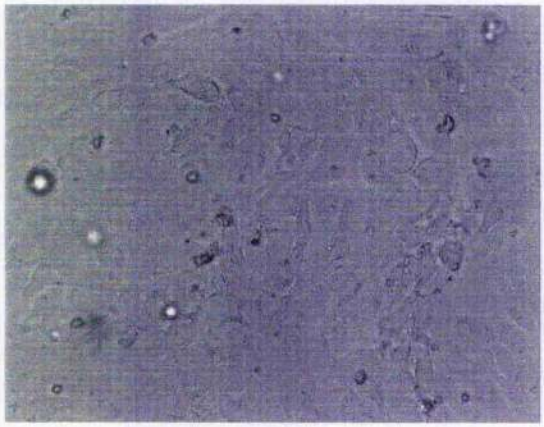


Figure 3.27 GFP expression in FEA cells 48 hours post infection
FEA cells were either uninfected (A and B), infected at a moi of 0.1 with ΔE3GFPCAV-1 (C and D) or infected at a moi of 10 with ΔE3GFPCAV-1 (E and F). The same cells were viewed with both a light microscope (A, C and E) and with a microscope fitted with fluorescence (B, D and F) 48 hours p.i. (see section 3.2.5).

A

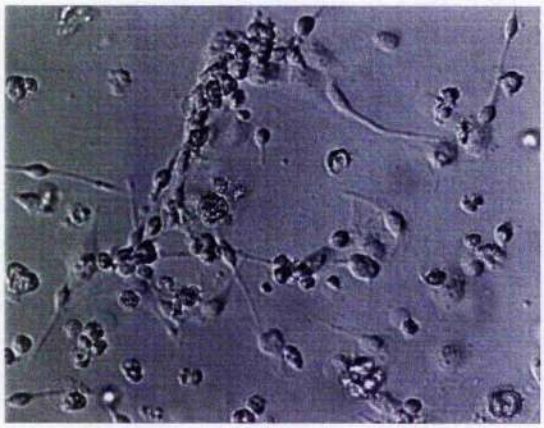


B

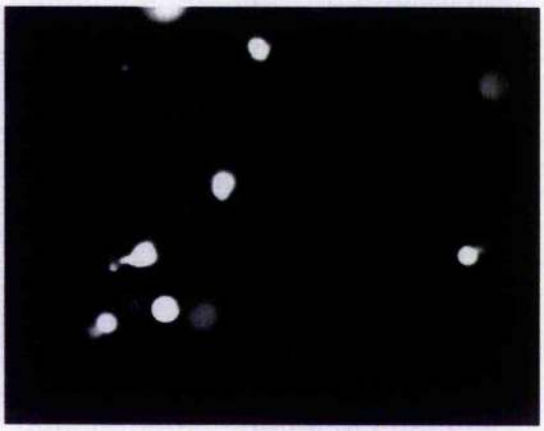


C

D



E



F

Figure 3.28 GFP expression in FEA cells 5 days post infection
FEA cells were either uninfected (A and B), infected at a moi of 0.1 with ΔE3GFPCAV-1 (C and D) or infected at a moi of 10 with ΔE3GFPCAV-1 (E and F). The same cells were viewed with both a light microscope (A, C and E) and with a microscope fitted with fluorescence (B, D and F) 120 hours p.i. (see section 3.2.5).

3.4 DISCUSSION

In 1969 Gehle and Smith reported that CAV-1 infection of human amnion cells resulted in 'progressive disappearance of the virus, or in a low-level production of infectious particles which never exceeded the input'. On the basis of this information the current study investigated the potential of CAV-1 as a vector for gene delivery in humans. In addition, as there is a lack of a widespread adenovirus in domestic cats, CAV-1 may also be a candidate for use as a vector in cats. In order to investigate its suitability as a vector, the wild-type life-cycle of CAV-1 was assessed in two cell-lines from each of the proposed host species.

A549 cells are derived from a human alveolar cell carcinoma (Lieber *et al.*, 1976), whereas 293 cells are human kidney cells that were transformed with the HAd5 E1 region (Graham *et al.*, 1977). Both of these cell-lines are commonly used for the propagation of HAds. CrFK cells are a feline kidney derived cell-line (Crandell *et al.*, 1973) and FEA cells are feline embryonic fibroblast cells (Jarret *et al.*, 1973). When infected with wild-type CAV-1 neither human cell-line developed classical adenoviral induced c.p.e., however, the rounded morphology of a proportion of the cells did suggest a degree of cytotoxicity. Although 100% c.p.e. was not observed in either feline cell-line, CAV-1 infection of both cell-lines was cytotoxic and in CrFK cells was characteristic of adenoviral c.p.e..

One step growth curves were carried out with each cell-line. In 293 cells CAV-1 titres decreased over a 5 day time course and by 5 days there was no detectable infectious CAV-1 in the cell-associated samples. However, infection of A549, FEA and CrFK cells resulted in an overall increase in the yield of infectious CAV-1 over a 5 day period compared to input level (Figures 3.1, 3.2 and 3.7(i)). Replication was delayed in onset in A549 and FEA cells and the amount of cell-associated CAV-1 detected was approximately 1 and 1.5 logs above that at 0 hours, respectively. On the other hand, CAV-1 replication in CrFK cells did not appear to be delayed (Figure 3.5) and a significant increase in titre, almost 3 logs, was detected by 48 hours p.i..

The adenoviral life cycle consists of many different stages, which include attachment, entry, uncoating, transcription, replication, translation, assembly and release. If any of these steps are sub optimal then the subsequent infection of the target cells may be inhibited. CAV-1 infection of 293, A549 and FEA cells is clearly impeded at one or more of these stages and, although there was no apparent delay in CAV-1 replication in CrFK cells, the level of cytotoxicity observed in these cells was lower than in MDCK cells; full c.p.e. was not observed until after 14 days p.i.. It was therefore necessary to investigate different stages of the CAV-1 life-cycle in all these cell-lines.

Based on the number of virus particles recovered at 0 hours p.i., the ability of CAV-1 to bind MDCK, human and CrFK cells appeared to be comparable as the amount of cell-associated CAV-1 was similar in each of the cell-lines (Figures 3.1 and 3.2). However, the reduced CAV-1 titre in FEA cells at 0 hours suggests that CAV-1 may not be as efficient at binding these cells (Figure 3.2). Following entry to the host cell, adenovirus particles are uncoated in order for replication and transcription of the genome to take place. The infection of MDCK cells with CAV-1 showed a decrease in the amount of cell-associated infectious virus at 6 hours p.i. (Figure 3.1) although the amount of DNA detected at this time was similar to that detected at 0 hours p.i. (Figure 3.3), indicating viral uncoating. However, in both human cell-lines the decrease in cell-associated CAV-1 titre at 6 hours p.i. was less than that in MDCK cells. In contrast there was a large decrease (approximately 2-fold) in viral DNA levels in human cell-lines compared to MDCK cells (Figure 3.3). This suggests that in these human cells CAV-1 entry and uncoating is poor relative to canine cells, and that proportionally more of the CAV-1 DNA associated with human cells at 6 hours p.i. is encapsidated and infectious, and most likely still bound at the cell-surface.

Transcription was demonstrated in all of the cell-lines investigated (Figure 3.6). However, the only cell-line that had evidence of CAV-1 transcription at 8 hours was MDCK cells. Transcripts were not detected until the 24 hour time point in 293, A549 and CrFK cells and the 48 hour time point in FEA cells. In addition, transcripts were detected for a shorter interval in 293 cells and, although the RT-PCR carried out was not quantitative, it appeared that the amount of transcripts in the FEA cells at 72 hours p.i. had dropped considerably. Tiemessen *et al.* also found that optimal transcription

was delayed in human conjunctiva cells (Chang), which are semi-productively infected with HAd41, compared with productive infection of 293 cells (De Jong *et al.*, 1983, Tiemessen *et al.* 1996). The same research group found that Chang cells required multiple HAd41 infecting genomes per cell (calculated at 4.32) in order to establish a productive cellular infection, whereas permissive 293 cells only required on infectious particles per cell (Tiemessen & Kidd, 1990). Although DNA replication was evident in human cells infected at a m.o.i. of 0.1 p.f.u. per cell with CAV-1 (Figure 3.4), it may be interesting to investigate transcription and CAV-1 replication in A549, FEA and CrFK cells at a low m.o.i. to determine whether these processes require multiple copies of CAV-1 per cell for productive infection. It may be that the high CAV-1 to cell ratio compensates for deficiencies in transcription, as Tiemessen *et al.*, observed with HAd41 infection of Chang cells (Tiemessen & Kidd, 1990). Silverman and Klessig found that HAd2 infection of African green monkey cells (CV-1) was abortive at least in part because of the under expression of late proteins, including the fibre gene (Silverman & Klessig, 1989, Ross & Ziff, 1992). The level of fibre transcripts detected in CAV-1 infected 293 cells appears to be lower than in the other cell-lines examined, however, quantitative RT-PCR would be necessary to confirm this. A qualitative RT-PCR screen of a wider range of genes may indicate other proteins that are inefficiently transcribed.

In 293 cells CAV-1 entered cells, DNA replication took place and levels remained constant for 12 days (and were comparable to those found in A549 cells). However, there was a reduction in CAV-1 titre in both the cell and extracellular fraction following infection. This indicates that the block to infection occurs at a stage after DNA replication but prior to viral release. These findings bear a resemblance to the infection of hamster cells (BHK) transformed with the left terminal 19.7% of HAd5 (BHK297-C131) with HAd12. Although BHK cells are non-permissive to HAd12, BHK297-C131 cells allowed HAd12 DNA replication and late gene expression (Klimkait & Doerfler, 1985, Schiedner *et al.*, 1994). However, as has been demonstrated in 293 cells, the infection of these cell-lines was not then productive, late mRNAs were not translated and virions were not assembled. There are evidently defects at other stages of the HAd12 life cycle in these cells and this is most likely the case for CAV-1 infection of 293 cells. It would be of interest to investigate the infection of untransformed human kidney cells with CAV-1 to determine whether

complementation of CAV-1 infection is conferred by the HAd5 sequences found in 293 cells, allowing DNA replication and transcription.

Infection of MDCK cells at m.o.i.s of 0.1 and 10 p.f.u./cell resulted in a 24000- and 2600-fold increase in CAV-1 DNA, respectively, relative to input (Figures 3.4 and 3.3). This is to be expected as at a m.o.i. of 0.1 p.f.u./cell only 10% of cells can be infected initially, however virus produced within these cells will spread to the remaining 90% of the population generating further virus. In contrast at a m.o.i. of 10 p.f.u./cell, the majority of cells will be initially infected and in these cells only one round of viral replication will take place. In CAV-1 infection of canine cells at low m.o.i. newly released virus particles infect uninfected cells, starting a new cycle that is repeated until all cells in the culture are lysed. In infected A549 cells , no classical adenoviral c.p.e. is observed and infection at either a m.o.i. of 0.1 or 10 p.f.u./cell leads to similar fold increases relative to input DNA, namely 100- and 50-fold, respectively. Although some virus is released initially, infectious CAV-1 is eventually lost from cells and medium during the long term culture of infected A549 cells (Figure 3.7(i)). There are a number of possible reasons for this. The virus particles produced in A549 cells could be defective for reinfection of A549 cells but allow infection of MDCK cells, although this would be sub-optimal. Alternatively, particles may be inefficiently released to the medium (as evidenced by a lack of c.p.e.) and this combined with repeated subculture might eventually dilute out both extracellular virus and cells infected with virus. As previously mentioned, there may also be a requirement for multiple CAV-1 genomes for productive CAV-1 infection in A549 cells. The passaging of CAV-1 on human amnion cells was previously shown to result in an almost 3-fold reduction in infectious virus yield with each passage, whereas in MDCK cells the yield was either the same or increased (Gehle & Smith, 1969). The integrity of the CAV-1 particles produced during infection of A549 cells could be analysed by electron microscopy and particle counts would allow a comparison between the number of defective and infectious viruses in these cells.

Subsequent to this work a CAV-1 vector carrying a GFP transgene in place of the E3 gene became available. This permitted direct investigation of transgene expression in human cells. This research supported the previous findings with wild-type CAV-1; transgene expression from the CAV-1 vector was delayed in the human and FEA cells

and present in a smaller proportion, compared to canine cells (see Figures 3.9 – 3.28). In addition, classical c.p.e. was not observed. However, in CrFK cells GFP expression was evident from 8 hours p.i. and a higher proportions of the cells by 5 days p.i. and the c.p.e. observed was typical of productive adenovirus infection.

The results presented demonstrate that CAV-1 can infect cells of human origin and that there is moderate replication and transcription of the viral genome. In addition, CAV-1 expressed a transgene in both of the human cell-lines investigated. Consequently, CAV-1 would appear to be a worthwhile candidate for development as a human gene therapy vector. 293 and A549 cells are normally permissive for HAd infection, and as A549 are tumour cells with a respiratory system origin and 293 cells contain part of the HAd5 genome, it would be prudent to investigate the CAV-1 life-cycle of other human cells that would perhaps be less likely to support CAV-1 infection, including primary cell-lines. The availability of a ΔE3-GFP-CAV-1 will facilitate such screening. In addition, investigations with an E1 deleted vector with a GFP transgene will establish whether the expression of transgenes occurs with a replication defective vector.

The investigation into CAV-1 infection of feline cells was less in depth, however it did demonstrate that CAV-1 infects feline cells, DNA replication and transcription occurs, and a productive infection is established. In addition, it appears that the lack of full c.p.e. may allow a persistent CAV-1 infection in feline cells, however, more in depth investigations would be necessary to confirm this hypothesis. Nevertheless, significant levels of transgene expression was demonstrated in both CrFK and FEA cells using a GFP expressing CAV-1 based vector. These features of CAV-1 suggest that it is a worthwhile candidate for development as a vaccine vector for use in cats. An extension of this work would be an investigation of administering wild-type CAV-1 infection in domestic cats. This would provide information on, not only the *in vivo* tropism of CAV-1, but also the immune responses elicited against it in cats.

Possible candidates for the vaccination of cats using CAV-1 based vectors are feline leukaemia virus (FeLV) and feline immunodeficiency virus (FIV). FeLV and FIV cause major diseases in cats and both are transmitted via mucosal epithelium; FIV is shed in the saliva and is mainly spread through bites, FeLV is contracted through

bodily fluids. They are both members of the retrovirus family: FeLV is a gammaretrovirus and FIV is of the genus lenitiviridae. Like HIV, both these viruses infect cells of the immune system causing immune suppression. There is no cure or treatment for either disease, and as such the development of vaccines that prevent infection are currently underway. There is no commercially available vaccine for FIV in the United Kingdom (for a review of FIV and vaccines see Uhl *et al.*, 2002). There are 4 different FeLV vaccines available in the United Kingdom, one is a recombinant peptide vaccine based on the FeLV envelope glycoprotein, another consists of inactivated whole virus, and the third is made up of inactivated subunits prepared from FeLV infected tissue culture filtrate (reviewed by Sparkes, 1997, Harbour *et al.*, 2002. All three of these vaccines are adjuvanted, however, to date none of these have been 100% effective, with readministration commonly necessary (Harbour *et al.*, 2002). In addition, FeLV vaccines have been implicated in the formation of tumours at the site of injection (Kass *et al.*, 1993). The most recent FeLV vaccine available is a canarypox virus vector which expresses the FeLV env and gag genes; canarypox is replication deficient in mammalian cells and expression of these FeLV genes was shown to provide good protection against FeLV when administered intranasally (Poulet *et al.*, 2003). As many diseases are transmitted via mucosal epithelium, application of vaccines via the intranasal route is preferable with the aim of stimulating strong mucosal immunity and eliminating the requirement for adjuvants that might be involved in formation of injection site sarcomas. It would therefore be advisable to investigate the infection of, and transgene expression in, feline cells of the respiratory system, with a view to developing a CAV-1 vector that can be administered intranasally. If the expression of appropriate transgenes is successful *in vitro*, a vaccine trial with experimental domestic cats using a CAV-1 vector expressing immunogens for feline viruses could be carried out.

Finally, the tropism of CAV-1 for feline cells may indicate a potential application as a gene therapy vector in cats in therapeutic or cytotoxic regimes. This would entail development of replication incompetent CAV-1 vectors expressing GFP for transductional studies in a wide range of feline cells.

CHAPTER 4

Fibre Swapping in Canine Adenovirus type 1

4.1 INTRODUCTION

In recent years a vast amount of research has focused on the modification of adenoviral tropism in order to deliver vectors to specific tissues. Adenovirus tropism is somewhat promiscuous, with many tissues expressing the required receptors for viral entry and attachment. However, an obvious drawback of this broad tissue range is the non-specific delivery and expression of transgenes in non-target cells e.g. HAd5 has a natural tropism for the liver, spleen and lungs (Nakamura *et al.*, 2003). The infection of non-target cells leads to decreased bioavailability of the vector for the target tissue, necessitating administration of high titres of the vector. For example, in 1999 an 18-year-old man who had been treated with a replication deficient adenovirus expressing an ornithine-transcarbamylase (OTC) transgene died due to a serious immune reaction to the administered vector (Marshall, 1999). The liver was the target for the transgene, however, as other organs were transduced the vector dose had to be increased to 3.8×10^{13} virus particles; only 1% of these reached hepatic cells. It was this massive dose of vector that stimulated the lethal immune response. An additional concern is that transgene expression at ectopic sites may cause toxicity (Mahasreshti *et al.*, 2003.

By increasing tissue-specificity, lower doses of vector can be used, increasing the safety profile of the vector, as well as production costs and labour. Target tissues that express unique receptors are particularly good candidates for adenoviral targeting. For example, moieties found only on tumour cells can be specifically targeted by adenoviruses (see later).

Complete understanding of the natural tropism of a virus is necessary for the development of a targeted vector based on that virus. The primary determinant of adenoviral tropism is the fibre. The fibres of HAds 2 and 5 have been thoroughly researched and are well characterised. The fibre is associated with the secondary determinant of adenovirus tropism, the penton base. This in turn interacts with hexon proteins to form the viral capsid. These capsid components are the major adenovirus immunogens.

4.1.1 Adenovirus Fibre and Penton: the Main Determinants of Tropism

The fibre is a trimeric protein comprised of an N-terminal tail, a long shaft domain, and a knob domain at the C-terminus. The shaft has a left-handed triple β-helical structure with 3-fold symmetry (Stouten *et al.*, 1992), made up of repeating sequence motifs of 15 amino acids (the number of which varies between serotypes) that each have two short beta-strands and two beta-bends (Green *et al.*, 1983) oriented along the axis (van Raaij *et al.*, 1999). A hydrogen bonding network exists within and between these monomers (van Raaij *et al.*, 1999) and as a result the fibre shaft is stable and rigid. However, a flexible region of 5 amino acids links the shaft and the knob domain and allows movement in the fibre that is proposed to be necessary for receptor binding (van Raajit *et al.*, 1999). Trimerisation of the fibre requires the knob domain and a small C-terminal portion of the shaft domain of at least 15 amino acids in length (Hong & Engler, 1996). In culture, purified fibre knob domains of HAd2 were shown to cross-link into trimers (Louis *et al.*, 1994). Only trimerised fibres are able to bind receptors (Stevenson *et al.*, 1995) and mutations in the HAd5 fibre gene that ablate fibre trimer formation prevent virus infectivity (Santis *et al.*, 1999).

Receptor binding occurs in the fibre knob domain (Stevenson *et al.*, 1995). The knob domain consists of three monomers in a propeller-like structure (Henry *et al.*, 1994). Each of these monomers is composed of two β-sheets that are linked by loops and turns; one β-sheet is involved in receptor recognition and the other is proposed to be involved in trimerisation (Xia *et al.*, 1994). The trimerisation of these monomers leads to the formation of a central depression that was originally hypothesised to be involved in receptor binding (Xia *et al.*, 1994). However, crystallisation of the HAd12 fibre in complex with the N-terminal domain of human CAR revealed that CAR binds the fibre at the interface between adjacent knob monomers (Bewley *et al.*, 1999) and circular dichroism spectroscopy of HAd5 fibre mutants was used to show that the binding sites are positioned on the underside of the knob near the shaft domain (Kirby *et al.*, 2000). The knob domain is also responsible for haemagglutination (Eiz *et al.*, 1997, Eiz & Pring-Akerblom, 1997), the clumping

together of blood cells that occurs when adenovirus suspensions are added to blood. Haemagglutination inhibition tests are used to determine the presence of anti-adenovirus antibodies and to divide the adenoviruses into serologically distinct subgroups; there are 6 subgroups of HAds: A to F (Rosen, 1960). As the knob domain is involved in both receptor and antibody recognition, it is not surprising that it is highly variable, for example, the knob domains of HAd31 (subgroup A) and HAd3 (subgroup B) have only 28.9% identity (Pring-Akerblom & Adrian, 1995). The fibre shaft also influences HAd tropism; in experiments where fibre shafts from different serotypes have been swapped the length of the shaft has been shown to be an important factor in determining tropism, independent of CAR and integrin binding (Shayakhmetov & Lieber, 2000, Koizumi *et al.,* 2003, Nakamura *et al.,* 2003).

The N-terminal of fibre interacts with the penton base specifically and with high affinity, although this interaction is reversible for the disassembly of the virus during infection (Boudin & Boulanger, 1981). As its name suggests, the penton base is made up of 5 monomers and has 5-fold symmetry (Stewart *et al.,* 1991). The penton has two main functions. As part of the capsid, the penton is important in the virion structure and it is also involved in the secondary binding of adenoviruses to cellular receptors. Following fibre binding to primary receptors, penton-receptor interactions mediate internalisation into host cells (Wickham *et al.,* 1993).

Once adenoviruses have gained entry to host cells the fibre is put to further use. Hong & Engler found that the fibre gene houses a nuclear localisation signal (Hong & Engler, 1991). Purified HAd2 fibre proteins were found to accumulate in the nucleus of cells and deletion of 4 amino acids of the nuclear localisation signal from the HAd2 fibre gene resulted in accumulation of mutant fibre proteins in the cytoplasm. Fusion of this nuclear localisation motif to a β-galactosidase gene allowed expression in the nucleus. As a result the fibre is thought to play a role in the intracellular trafficking of adenoviruses (Miyazawa *et al.,* 1999).

The penton also has other roles in adenovirus infection. Domains of HAd2 penton monomers have been identified that are involved in nuclear localisation (Karayan *et al.,* 1997) and the rounding of adenovirus infected cells during c.p.e. has been attributed to the penton (Bai *et al.,* 1993).

4.1.1.1 Adenovirus-Receptor Interactions

The primary receptor for all the HAds except those of subgroup B (Roelvink *et al.*, 1998) is CAR, which is bound by the fibre protein (Bergelson *et al.*, 1997). In addition, a number of animal adenoviruses have been shown to bind to CAR homologues (section 1.4.4). CARs are widespread in the tissues of most species and therefore CAR binding adenoviruses have a broad tissue tropism. Fechner *et al.*, found high levels of variation between CAR levels in different tissues of mice, with the highest levels found in the liver (Fechner *et al.*, 1999). This diversity of CAR expression has also been demonstrated *in vitro* in human cell-lines, with the levels of CAR expression differing between cell types from the same tissue source (Havenga *et al.*, 2002).

It has been shown that the targeting of adenoviruses *in vivo* is not as predicted by *in vitro* results; transgene expression was found to be broader than that predicted by tissue CAR expression (Fechner *et al.*, 1999). As such the influence of other factors, whether cellular or viral, must be seriously considered in the development of gene therapy vectors.

The HAd5 fibre was shown to bind the MHC-I heavy chain by phage display library screening (Hong *et al.*, 1997). MHC-I was therefore proposed to be a primary receptor for HAd5. However, when McDonald *et al.*, investigated HAd5 infection of non-permissive cells that expressed MHC-I allele HLA-A2, no virus uptake was demonstrated. (McDonald *et al.*, 1999). Davison *et al.*, also showed that MHC-I allele HLA-A2 is not a high-affinity receptor for HAd5 in hamster cells (Davison *et al.*, 1999).

Integrins are found on a high percentage of human tissues at comparable levels (Fechner *et al.*, 1999). Normal extracellular proteins use these integrins for cell adhesion (reviewed by Ruoslahti 1996). The HAd2 and HAd5 pentons were found to interact, via an RGD motif, with α_V-integrins (Wickham *et al.*, 1993), which allows internalisation of the virus via endocytic vacuoles. This penton RGD motif is conserved in many adenovirus serotypes (Mathias *et al.*, 1994). Goldman & Wilson

highlighted the importance of penton-integrin interactions by showing that, whereas undifferentiated airways epithelia express high levels of αv-integrins, more mature epithelial cells do not express the αv-integrins and as such are almost completely resistant to HAd transfer (Goldman & Wilson, 1995).

More recently heparan sulphate glycosaminoglycans have been highlighted as mediators of HAd2 and HAd5 binding (Dechecchi *et al.*, 2000, Dechecchi *et al.*, 2001). Binding by both of these viruses was blocked completely by preincubation with both heparin and soluble CAR, whereas only partial inhibition was observed when HAd2 and HAd5 were incubated with either heparin or soluble CAR (Dechecchi *et al.*, 2001).

4.1.2 Modifying Adenovirus Tropism

Tropism modification strategies to date have included redirecting adenoviruses either by modifying adenovirus capsid components or by ablating natural tropism and redirecting vectors with additional molecules.

4.1.2.1 Retargeting using exogenous complexes

Fibre-receptor interactions have been prevented by using either anti-fibre antibodies or synthetic CAR receptors, or by coating adenovirus particles with polymers. These molecules are co-linked to either antibodies that recognise specific ligands or receptor binding ligands that redirect fibre binding. The retargeting of HAd based vectors in this way has been achieved by a number of research groups.

Coating adenovirus vectors with polymers such as poly-[N-(2-hydroxypropyl)methacrylamide] (pHPMA) and polyethylene glycol (PEG) has been shown not only ablate normal viral tropism but also protect the vectors from immune responses (Fisher *et al.*, 2001, Croyle *et al.*, 2001). The chemical incorporation of targeting ligands onto the surface of such polymer coated vectors allows specific binding of vectors to receptor bearing cells (Fisher *et al.*, 2001).

An anti-epidermal growth factor receptor (EGFR) antibody bound to a truncated version of the CAR receptor blocked normal HAd5 binding and redirected binding to EGFR overexpressing cancer cells *in vitro* (Dmitriev *et al.*, 2000). Kashentseva *et al.*, also ablated normal HAd5 tropism by fusing soluble CAR domains with proteins specific for oncoprotein c-erbB-2 (Kashentseva *et al.*, 2002). These bispecific molecules bound to c-erbB-2 and enhanced gene transfer to tumour cell-lines by up to 17-fold compared with HAd alone.

Another group developed a bispecific antibody that consisted of an anti-fibre antibody fused to an anti-EpCAM antibody. EpCAM is expressed on tumours of various tissues to a much higher degree than in normal tissues. Targeting by this method conferred specificity to cancer cell-lines and was independent of CAR expression (Haisma *et al.*, 1999).

The use of polymer coated adenoviruses has great potential, as this not only allows retargeting of the vector to specific cells, but also masks it from the immune system. However, more *in vivo* work is required as clearance of such polymers in non-viral vectors by plasma proteins has been demonstrated (Dash *et al.*, 1999). The use of bispecific molecules to target adenoviral tropism and ablate normal receptor binding is more technically challenging, which increases both labour and costs. Where targeting molecule binding to vectors is incomplete, normal tissue distribution would be observed. As such it would be important that the ratio of adenovirus vectors to bispecific molecule was optimised prior to administration. Another important consideration is that the introduction of another component may evoke further immune responses and therefore clearance of the vector.

4.1.2.2 Retargeting via the insertion of peptides into the HI loop of the fibre knob region

The fibre knob domain consists of two β-sheets; the exposed receptor binding β-sheet consists of β-strands designated G, H, I and D, and the other β-sheet, which is involved in trimerisation, is composed of β-strands J, C, B and A (Xia *et al.*, 1994). These β-strands only make up 35% of the monomer, the other 65% is made up of β-

loops and turns. The 6 main loops are designated AB, CD, GH, HI and IJ. As discussed in the previous section, integrins are receptors found on many cell types and RGD motifs specifically attach to these integrins. Incorporating an RGD motif in the III loop of a HAd5 vector targeted it to integrins on both CAR negative and CAR positive cells (Okada *et al.*, 2001). As such, the transfection efficiency with this vector was increased in dendritic cells, which did not express CAR, as well as CAR expressing colon and melanoma cells when compared with an equivalent untargeted HAd5 vector. Another HAd5 vector with an RGD motif inserted into the HI loop improved gene transfer to ovarian cancer cell-lines by up to 471-fold (Vanderkwaak *et al.*, 1999).

Peptides for binding to specific cell-types can be identified by screening phage display libraries. For example, the SIGYPLP peptide was shown to bind endothelial cells (Nicklin *et al.*, 2000). Inclusion of SIGYPLP in the HAd5 fibre improved the transduction of endothelial cells by 19-fold compared with other non-targeted HAd5 vectors (Nicklin *et al.*, 2001). The screening of phage display libraries was also used to isolate motifs with the ability to bind the human transferrin receptor (hTR), a receptor found on human brain microcapillary endothelium (Xia *et al.*, 2000). Insertion of the isolated motif into the HI loop of the HAd5 knob domain resulted in between 2 and 34-fold increased gene transfer to hTR expressing cell-lines *in vitro*.

4.1.2.3 *Retargeting by fibre or fibre knob swapping between adenoviruses of different serotypes*

Many groups have demonstrated the retargeting of adenoviruses by the swapping of fibre genes, or portions of them, with those of other adenoviruses. The infection profile of group B HAds is distinct from the other HAd groups and group B HAds recognise a receptor other than CAR (Bergelson *et al.*, 1997, Roelvink *et al.*, 1998). Chimaeric HAd5 vectors carrying subgroup B HAd (11, 35, 16 and 51) fibres transduced smooth muscle cells with a higher efficiency than HAd5 vectors (Havenga *et al.*, 2001). *In situ* these chimeras transduced cultured blood vessels (coronary arteries) and the transduction of endothelial cells with an Ad16 fibre carrying HAd5 was much more effective than with the HAd5 based vector. Other HAd5-group B fibre chimeras were able to transduce CAR negative synoviocytes with up to 10-fold efficiency compared with an entirely HAd5 based vectors (Goossens *et al.*, 2001). Dendritic cells are also CAR negative and retargeting of HAd5 with a group B fibre improved transduction efficiency (Rea *et al.*, 2001).

The exchange of fibre genes between HAds and an animal adenovirus has also been investigated. Unlike HAd5, OAV does not have an RGD motif in the penton and therefore does not bind to integrins (Vrati *et al.*, 1996). Whereas OAV is unable to infect human 293 cells, a recombinant ovine adenovirus that housed a chimeric fibre incorporating the CAR binding domain of the HAd5 fibre (Xu & Both, 1998) transduced 293 cells.

4.1.2.4 *Other attempts at retargeting*

Other proteins found on the surface of adenovirus capsids may also be targets for the insertion of retargeting ligands. As a proof of principle, Dmitriev *et al.*, engineered HAd vectors with modifications to the pIX protein, a minor stabilising capsid protein (Dmitriev *et al.*, 2002). The modified pIX was linked at its C-terminal to a Flag epitope and heparan sulphate binding motif, consisting of a polylysine sequence. The modified proteins were incorporated into the capsid and heterologous sequences were

displayed on the capsid surface. These viruses were able to bind to heparin-coated beads and recognise cellular heparan sulphate receptors. As such, this domain has potential for retargeting of adenoviruses if used in conjunction with other modifications.

Another capsid protein that could be modified for adenovirus retargeting is the penton. LDV peptide motifs recognise $\alpha_4\beta_1$ integrins found on lymphocytes and monocytes; the replacement of the RGD motif of the HAd5 penton with the LDV motif redirected HAd5 binding to $\alpha_4\beta_1$ integrin expressing cells (Wickham *et al.*, 1995).

By introducing an RGD motif into a protruding loop of the HAd5 hexon peptide, Vigne *et al.* were able to create a recombinant vector that expressed a transgene in naturally refractory smooth muscle cells at a 10-fold lower concentration than a vector unmodified in the hexon region (Vigne *et al.* 1999). In addition, 293 cells, which are highly permissive to wild-type HAd5, which were saturated with fibre knob domains, were also transduced by this vector and allowed transgene expression.

4.1.3 Objective

It has been demonstrated that CAV-1 can enter both feline and human cells (Chapter 3). However, it has a wide tropism that could prove problematic for tissue-specific administration of CAV-1 based vectors and as such retargeting is required. This retargeting could be achieved by either ligand incorporation into capsid proteins or by blocking the native CAV-1 fibre binding region with bispecific molecules that recognise other cellular receptors. The development of such strategies in HAds 2 and 5 has taken many years and although technology has improved in this time, similar research in CAV-1 would be time consuming and labour intensive. An alternative to this would be to remove the CAV-1 fibre and replace it with other well characterised adenovirus fibres, or recombinant fibres, with known tissue affinities. A fibre swapping vector system was developed in order to investigate the possibility of such an approach. The fibre genes of CAV-2 and HAd5 were incorporated into CAV-1 and the tropism of the resultant vectors was investigated in a range of cell types.

4.2 MATERIALS AND METHODS

4.2.1 Construction of shuttle plasmids

4.2.1.1 Construction of pCR2.1UD(-fib)

PCR amplification of Ufib and Dfib

The regions upstream and downstream of the CAV-1 fibre gene, Ufib and Dfib, respectively, were cloned from cl.111 CAV-1 DNA (Morrison *et al.*, 1997) using *Taq* PCR master mixes (section 2.2.2.2) at annealing temperatures of 55°C with the primers detailed below. The PCR products were analysed by agarose gel electrophoresis (section 2.2.1.5).

Primers for cloning Ufib and Dfib

Primers were designed, based on CAV-1 sequence determined by Morrison *et al.*, in 1997 (Table 4.1). In addition to CAV-1 sequence, Pme I restriction enzyme sites were added onto the 5' ends of ufibpme and dfibpme. The U exon and fibre genes of CAV-1 are on opposite strands and share a start codon. The insertion of a Pme I site between these two genes would have created another start codon 11 bases upstream of the fibre start region, which would possibly have inhibited translation of the fibre gene. Therefore, an extra thymidine nucleotide was incorporated between the Pme I site and the U exon sequence of ufibpme (see Figure 4.1).

Cloning of Ufib and Dfib

Ufib and Dfib were cloned into vector pCR2.1-TOPO® (section 2.1.1.4) (pCR2.1Ufib and pCR2.1Dfib respectively) and transformed into One Shot® cells according to the manufacturer's instructions (section 2.1.2.1). The transformed cells were plated out on LB agar plates and incubated overnight at 37°C. The following day single and STET/lysosyme minipreparations of DNA were prepared (section 2.2.1.10).

Positive clones were screened for by digestion of pCR2.1Ufib were identified by digestion with Bam HI (section 2.2.1.2), which cuts the pCR2.1 vector backbone once and pCR2.1Ufib once producing approximate fragments of either 250 bp and 4650 bp,

CAV-1 region	Primer set	Primer sequence	T_m (°C)	Product size (bp)	Location on CAV-1
Region directly upstream of the fibre gene	ufib	5' AGG GTT TAC GCG CTA ACT GG 3'	59.4	1052	24837-24856
	ufibpme	5' GTT TAA ACT ATG GCT TTG GTG TTG GTG AAC 3'	65.5		25889-25857
Region directly downstream of the fibre gene	dfibpme	5' GTT TAA ACA TAC AGT TTG CCC AAT GTA AAC G 3'	62.9	1031	27519-27541
	dfib	5' ACA ACC TCC CAT GTG ACA GC 3'	59.4		28660-28531

Table 4.1 Primers for amplification of Ufib and Dfib. The thymidine residue introduced in the ufibpme primer sequence is highlighted in blue.

GTTCACCAACACCAAAGCCATAGTTTAAACATGAAGCGGACACGAAGTGCTCT

- - - - - - - - - - U exon - - - - - - - - -> < Pme I > Met <- - - - - - Fibre - - - - - - - - - - - -

Figure 4.1 Mapping of ufibpme primer

The ufibpme primer is highlighted in yellow, with the additional nucleotide highlighted in blue. Start codons of the U exon and Fibre gene are underlined.

or 850 bp and 4050 bp depending on insert orientation. pCR2.1Dfib clones were screened for by Eco RI digestion (section 2.2.1.2), Eco RI cuts the pCR2.1 vector backbone twice on either side of the insert and cuts pCR2.1Dfib once producing fragments of 300 bp, 700 bp and 3900 bp.

50 µl of bacterial culture from positive clones was used to inoculate 2.5 ml of L-broth in bijoux and QIAprep® minipreparations of DNA were prepared (section 2.2.1.10). The authenticity of Ufib and Dfib sequences were confirmed by LiCor (section 2.2.3.1) and ABI Prism (section 2.2.3.2) sequencing, respectively, using T7 forward and M13 reverse primers (Table 4.2). Sequence data generated was analysed using the UW-GCG software package, homology between the nucleotide sequence and the CAV-1 genome was determined using the 'Fasta' program.

Cloning Ufib and Dfib into one vector (pCR2.1UD(-fib))

The regions upstream and downstream of the CAV-1 fibre were linked by a Pme I site, as described below.

pCR2.1Ufib was linearised by Pme I digestion and the DNA was purified using the QIAquick gel purification kit as follows. The agarose gel electrophoresis step was omitted and the DNA solution was diluted with 3 volumes of QG solution. The remainder of the purification was carried out according to the manufacturer's instructions. DNA was eluted in 30ml of dH_2O. The linearised DNA was dephosphorylated (section 2.2.1.3) and then purified once again with the QIAquick gel purification kit as before. Fragment Dfib was isolated from pCR2.1Dfib by digestion with Pme I and Eco RV, visualised by agarose gel electrophoresis (section 2.2.1.5) and gel purified (section 2.2.1.6). A ligation reaction was performed between linearised pCR2.1Ufib and fragment Dfib (section 2.2.1.7). 5 µl of the ligation was transformed into Max Efficiency STBL2 cells according to the manufacturer's protocol. Transformed STBL2 cells were plated out on LB agar plates and incubated at 37°C overnight. The following day single colonies were selected and STET/lysosyme minipreparations of DNA were prepared (section 2.2.1.10). Positive

Target Sequence	Primer	Primer sequence	T_m (°C)	Location on Target Sequence
CAV-1 (upstream of UE3)	E3delup	5' GGG ATG AAT TGG TCA GGA AAG G 3'	62	24689-24710
CAV-1 (within fibre)	26396	5' CTT TAC GCC GAG CTT TC 3'	52.8	26380-26396
CAV-1 (within fibre)	26643	5' GA AAT GCA GTG TCA GTG CG 3'	57.3	26643-26662
CAV-1 (within fibre)	26738	5' TGG ATA GGC GAA GGT CAA 3'	53.7	26721-26738
CAV-1 (within fibre)	27001	5' CAA ACG TTA ACA CCT C 3'	46.6	27001-27015
CAV-1 (within Ufib)	Fib1	5' GTG TCT CTA TGT CCA CAA GG 3'	57.3	25750-25770
CAV-1 (within Dfib)	Fib2	5' GTA GCA TTA CAG CTC GAG TG 3'	57.3	27600-27620
CAV-2 (within fibre)	27181	5' ACG AAC AGT TGC AAG CTG TCC 3'	59.8	27181-27201
CAV-2 (within fibre)	27287	5' GTT GCC ACC AAA GCT CCG CTC 3'	63.7	27287-27307
HAd5 (within fibre)	31587	5' TAC TGC CAC TGG TAG CTT G 3'	62	31587-31605
HAd5 (within fibre)	31731	5' AAT AGT CAC ACC TGG ACC AG 3'	60	31712-31731
HAd5 (within fibre)	32197	5' ACA GGT GCC ATT ACA GTA GG 3'	60	32197-32216
HAd5 (within fibre)	32302	5' GTT TAG CAT CTT TCT CTG C 3'	54	32284-32302
pCR2.1-TOPO	M13 reverse	5' CAG GAA ACA GCT ATG AC 3'	50	pCR2.1-TOPO
pCR2.1-TOPO	T7 forward	5' TAA TAC GAC TCA CTA TAG GG 3'	56	pCR2.1-TOPO

Table 4.2 Sequencing and PCR Primers

pCR2.1UD(-fib) clones were confirmed by Eco RI digestion (section 2.2.1.2). In the correct orientation Eco RI digest of pCR2.1UD(-fib) produces fragments of DNA of approximate sizes 300 bp, 1700 bp and 3900 bp. 50 µl of bacterial culture from positive clones was used to inoculate 2.5 ml of L-broth and QIAprep® minipreparations were carried out (section 2.2.1.10).

4.2.1.2 Construction of pCR2.1UDCm^R

pCR2.1UD(-fib) was linearised by digestion with Pme I (section 2.2.1.2) and purified using the QIAquick™ gel extraction kit (section 4.2.1.1). The linearised DNA was phosphorylated (section 2.2.1.3), followed by purification with the QIAquick™ gel extraction kit (section 4.2.1.1). The chloramphenicol resistance cassette (Cm^R) was excised from vector pSwapmeCm^R (constructed by Dr M.D. Morrison) by Pme I restriction enzyme digestion (section 2.2.1.2) and the appropriate fragment (approximately 1700 bp) was isolated by gel purification (section 2.2.1.6). A ligation reaction was carried out with linearised pCR2.1UD(-fib) vector and the Cm^R fragment (section 2.2.1.7). 5 µl of the ligation was transformed into Max Efficiency STBL2 cells (section 2.2.1.9). Transformed STBL2 cells were plated out on LB agar plates containing 100 µg/ml chloramphenicol and incubated at 37°C overnight. The following day single colonies were selected and inoculated into 2.5ml of L-broth containing 100 µg/ml chloramphenicol and STET/lysosyme minipreparations of DNA were prepared (section 2.2.1.10). Positive pCR2.1UDCm^R clones were selected for by Pme I digestion, which produces fragments of approximate sizes 1700 bp and 5900 bp. 50 µl of bacterial culture from positive clones was used to inoculate 2.5 ml of L-broth containing 100 µg/ml chloramphenicol and QIAprep® minipreparations were carried out (section 2.2.1.10).

4.2.1.3 Construction of pCR2.1UDfibCAV-1, pCR2.1UDfibCAV-2 and pCR2.1UdfibHAd5

PCR amplification of Adenovirus Fibre Genes

Fibre genes were amplified from CAV-1, CAV-2 and HAd5 using *Taq* PCR as described in section 2.2.2.2. The appropriate primers are detailed in Table 4.3. These corresponded to the 5' and 3' ends of the appropriate fibre gene and included Pme I

Virus	Primer	Primer Sequence	T_m (°C)	Fibre location on virus	Product size (bp)
CAV-1	CAV-1 Fib5	5' GTT TAA ACC GCC ATG AAG CGG ACA CGA AGT GC 3'	70	25889-27520	1643
	CAV-1 Fib3	5' GTT TAA ACT CAT TGA TTT TCC CCC ACA TAG 3'	64		
CAV-2	CAV-2 Fib5	5' GTT TAA ACC GCC ATG AAG CGG ACA CGA AGA GCT CTA 3'	72	26592-28220	1640
	CAV-2 Fib3	5' GTT TAA ACT TAT TGA TTT TCG CCT ACA TAG G 3'	61		
HAd5	HAd5 Fib5	5' GTT TAA ACC GCC ATG AAG CGC GCA AGA CCG TCT GAA GAT AC 3'	74	31042-28220	1757
	HAd5 Fib3	5' GTT TAA ACT TAT TCT TGG GCA ATG TAT G 3'	59		

Table 4.3 Primers for the amplification of CAV-1, CAV-2 and HAd5 fibre genes.

sites at either end. Four extra nucleotides (CGCC) were included between the 5' Pme I site and start codon to create a consensus Kozak sequence (CC(A/G)CCATGG) (Kozak 1986) (Figure 4.2). The PCR products were visualised by agarose gel electrophoresis (section 2.2.1.5).

CAV-1 Fib5 5'-GTTTAAACCGCCATGAAGCGGACACGAAGTGC-3'
CAV-2 Fib5 5'-GTTTAAACCGCCATGAAGCGGACACGAAGAGCTCTAC-3'
HAd5 Fib5 5'-GTTTAAACCGCCATGAAGCGCGCAAGACCGTCTGAAGATAC-3'

< Pme I > Met < - - - - - - - - - - - - fibre DNA - - - - - - - - - - - - >

< KOZAK >

Figure 4.2 Fibre 5' primers

Cloning of Adenovirus Fibre Genes into pCR2.1-TOPO®

PCR amplified CAV-1, CAV-2 and Had5 fibre genes were cloned into vector pCR2.1-TOPO® (pCR2.1FibCAV-1, pCR2.1FibCAV-2 and pCR2.1FibIIAd5) and transformed into One Shot® cells (section 2.2.1.9). The transformed cells were plated out on LB agar plates and incubated overnight at 37°C. The following day single colonies were selected and STET/lysosyme minipreparations were prepared (section 2.2.1.10). Positive clones were screened for by digestion with restriction enzyme Pme I (section 2.2.1.2), which releases each of the fibre genes from the pCR2.1-TOPO® backbone producing fragments of approximately 3900 base pairs (pCR2.1) and 1600 (CAV-1 and CAV-2) or 1700 (HAd5) base pairs. 50 µl of bacterial culture from positive clones was inoculated into 2.5 ml of L-broth and DNA was prepared by QIAprep® minipreparation (section 2.2.1.10). The authenticity of the fibre sequences was confirmed by ABI sequencing (section 2.2.3.2) using T7 forward and M13 reverse primers and the appropriate internal primers. Internal primers for were 26396, 26643, 26738 and 27001 for pCR2.1FibCAV-1, 27181 and 27287 for pCR2.1FibCAV-1, and 31587, 31731, 32197 and 32302 for pCR2.1FibHad5 (see Table 4.2). Sequence data generated was analysed using the UW-GCG software package, homology between the nucleotide sequence and the appropriate adenovirus genome was determined using the 'Fasta' program.

Insertion of adenovirus fibre genes into pCR2.1UD(-fib)

pCR2.1UD(-fib) was linearised with Pme I, phosphatased and purified as described in section 4.2.1.2. Fibre genes were excised from the appropriate vector, pCR2.1CAV-1fib, pCR2.1CAV-2fib or pCR2.1HAd5fib, by Pme I restriction enzyme digestion (section 2.2.1.2) and the appropriate fibre fragments were isolated and purified by gel extraction (section 2.2.1.6). Ligation reactions were performed with linearised pCR2.1UD(-fib) vector and fibre fragments (section 2.2.1.7). 5 µl of the ligation was transformed into Max Efficiency STBL2 cells (section 2.2.1.9). Transformed STBL2 cells were plated out on LB agar plates and incubated at 37°C overnight. The following day single colonies were selected and STET/lysosyme minipreparations were performed (section 2.2.1.10). Positive clones were selected for by digestion with the appropriate enzymes (section 2.2.1.2). Digestion of pCR2.1UDFibCAV-1 with Afl II yields bands of approximate size 1200 and 6800 bp in the correct orientation.

Digestion of pCR2.1UDFibCAV-2 with Sal I yields bands of approximate size 600 and 7100 bp in the correct orientation. Digestion of pCR2.1UDFibHAd5 with Hpa I yields bands of approximate size 900 and 6700 bp in the correct orientation. 50 µl of bacterial culture from positive clones was used to inoculate 2.5 ml of L-broth and QIAprep® minipreparations were carried out (section 2.2.1.10).

4.2.1.4 Construction of pCR2.1UE3Dfib

pCR2.1Dfib was linearised by digestion with Pme I and purified using the QIAquick gel extraction kit (section 4.2.1.1). Linearised pCR2.1Dfib was then phosphatased (section 2.2.1.3), followed by purification with the QIAquick gel extraction kit (section 4.2.1.1). Fragment UE3 was isolated from plasmid $KXCm^R$ (Morrison *et al.*, 2002) by digestion with KpnI and Pme I restriction enzymes (section 2.2.1.2). The KpnI overhangs were blunted as described in section 2.2.1.4 and the appropriate fragment was isolated by gel extraction (section 2.2.1.6). Linearised pCR2.1Dfib vector DNA and UE3 fragments were ligated together according to section 2.2.1.7. 5 µl of the ligation was transformed into Max Efficiency STBL2 cells. Transformed STBL2 cells were plated out on LB agar and incubated at 37°C overnight. The following day single colonies were selected and STET/lysosyme minipreparations were performed (section 2.2.1.10). Digestion with Hpa I was performed to select for positive clones (section 2.2.1.2), Hpa I digestion of pCR2.1UE3Dfib yields bands of approximately 1300 and 4900 base pairs.. 50 µl of bacterial culture from positive clones was used to inoculate 2.5 ml of L-broth and QIAprep® minipreparations were carried out (section 2.2.1.10).

4.2.2 Construction of Cosmids Containing Recombinant CAV-1 Genomes

4.2.2.1 Construction of cosmid cΔFibCAV-1Cm^R

A cosmid that contained the CAV-1 genome with the fibre gene deleted and replaced by a chloramphenicol resistance cassette (Cm^R), flanked by Pme I restriction enzyme sites (Figure 4.3), was generated as follows.

Fragment UD(-fib)Cm^R (section 4.2.1.2) was excised from pCR2.1UD(-fib)Cm^R by Pst I and Spe I restriction enzyme digestion (section 2.2.1.2). Pst I and Spe I digestion of pCR2.1UD(-fib)Cm^R yields bands of approximately 1200, 2700 and 3700 base pairs. The 3700 bp fragment was isolated by gel extraction (section 2.2.1.6). Electrocompetent BJ5183 cells were cotransformed with cosmid FLR-CAV DNA and UD(-fib)Cm^R by electroporation (section 2.2.1.9). Electroporated cells were plated out on L broth plates containing 100 μg/ml choramphenicol and incubated at 37°C overnight. The following day individual colonies were selected and suspended in 2.5 ml of L broth containing 100 μg/ml choramphenicol. STET/lysosyme minipreparations were performed (section 2.2.1.10). Positive clones were selected for by Hpa I restriction enzyme digestion (section 2.2.1.2). As BJ5183 cells are unstable for the maintenance of cosmid DNA, DNA from positive clones was transformed into Max Efficiency STBL2 cells (section 2.2.1.9). Transformed STBL2 cells were plated out on LB agar plates containing 100 μg/ml chloramphenicol and incubated at 37°C overnight. The following day single colonies were selected and inoculated into 2.5 ml of L broth containing 100 μg/ml chloramphenicol. Cosmid DNA was prepared from the bacterial cultures by the STET/lysosyme minipreparation method (section 2.2.1.10). Positive clones were selected for by restriction enzyme digestion with Hpa I (section 2.2.1.2). 1 ml of a fresh overnight culture from a positive clone was used to inoculate 400 ml of L broth containing 100 μg/ml chloramphenicol and DNA was prepared using the using Qiagen plasmid Mega Kit (section 2.2.1.11) following the instructions of the manufacturer. Glycerol stocks of the final construct were prepared and stored at -70°C (section 2.2.1.12).

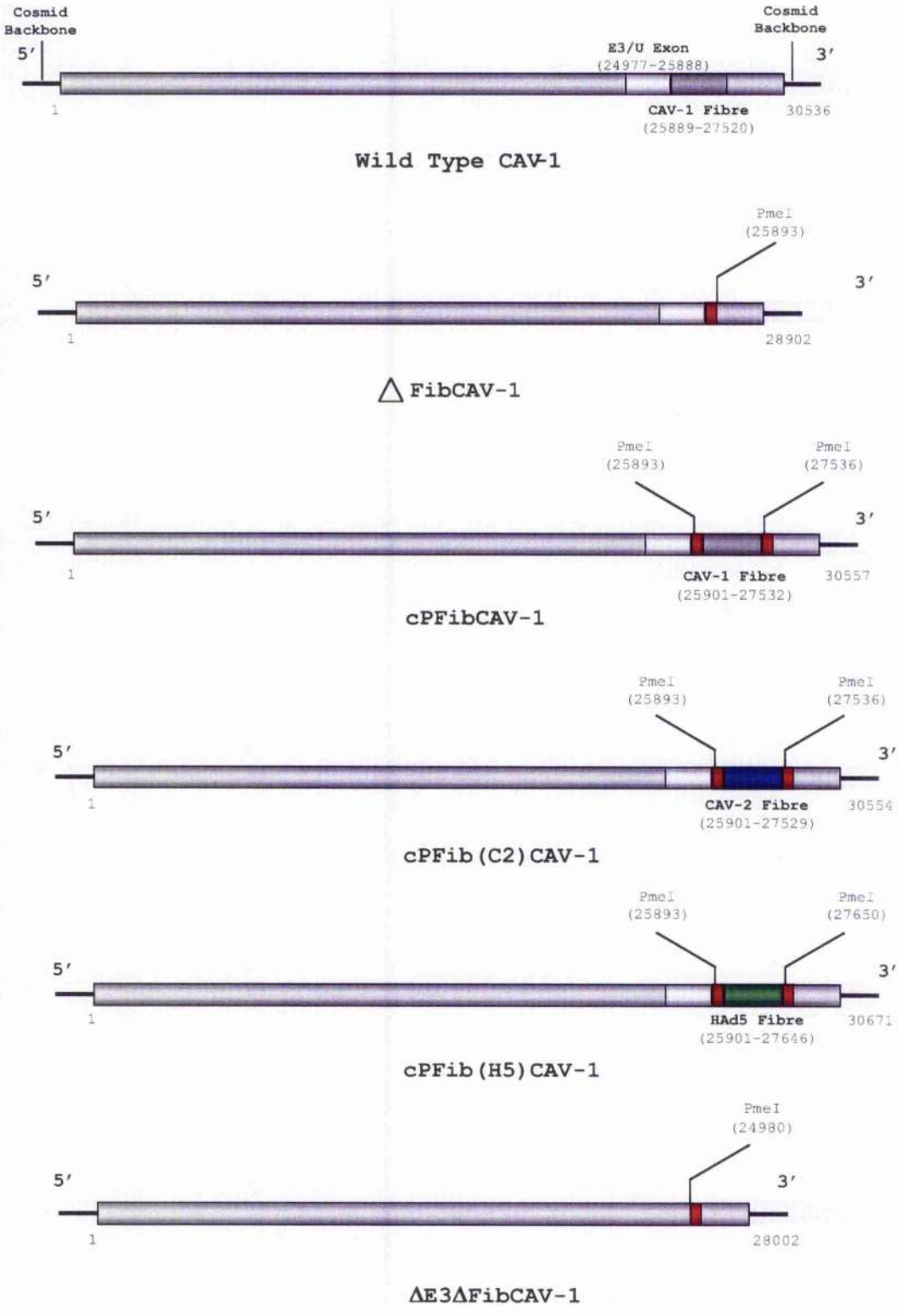


Figure 4.3 Maps of CAV-1 recombinant cosmid constructs

4.2.2.2 Construction of cosmid cΔFibCAV-1

A cosmid that had the fibre gene replaced by a unique Pme I restriction enzyme site (Figure 4.3) was generated as follows.

Cosmid cΔFibCAV-1Cm^R was digested with Pme I (section 2.2.1.2) to remove the chloramphenicol resistance gene and purified using alcohol precipitation as follows. DNA in solution was precipitated by the addition of 0.1 volumes of 5 mM sodium chloride and 2 volumes of 100% ethanol, and incubation at -20°C for 30 minutes. The DNA was then pelleted by centrifugation at 14K for 15 minutes in a microcentrifuge. The surrounding liquid was removed and the DNA was air dried, then resuspended in 20 µl of dH_2O. A ligation reaction was carried out on the purified DNA (section 2.2.1.7). Electrocompetent BJ5183 cells were cotransformed with cosmid ΔFibCAV-1Cm^R DNA and fragment UD(-fib) as described in section 2.2.1.9. Electroporated cells were plated out on L broth plates and incubated at 37°C overnight. The following day individual colonies were selected and STET/lysosyme minipreparations were performed (section 2.2.1.10). Positive clones were selected for by Hpa I restriction enzyme digestion (section 2.2.1.2).

DNA from positive clones was transformed into Max Efficiency STBL2 cells. Transformed STBL2 cells were plated out on LB agar plates and incubated at 37°C overnight. The following day single colonies were selected and used to inoculate 2.5 ml of L broth. Cosmid DNA was prepared from the bacterial cultures by the STET/lysosyme minipreparation method (section 2.2.1.10) and digested with Hpa I (section 2.2.1.2) to check for positive clones. 1 ml of a fresh overnight culture from a positive clone was used to inoculate 400 ml of L broth containing 100 µg/ml chloramphenicol and DNA was prepared using the using Qiagen plasmid Mega Kit (section 2.2.1.11) following the instructions of the manufacturer. Glycerol stocks of the final construct were prepared and stored at -70°C (section 2.2.1.12).

4.2.2.3 Construction of Cosmids cPFibCAV-1, cPFib(C2)CAV-1 and cPFib(H5)CAV-1)

The cotransformation of linearised FibCAV-1 with fibre recombinant shuttle vectors was used to generate cosmids containing fibre recombinant CAV-1 genomes (Figure 4.3) as follows.

Cosmid ΔFibCAV-1 was linearised by digestion with Pme I (section 2.2.1.2) and purified by alcohol precipitation follows. DNA in solution was precipitated by the addition of 0.1 volumes of 5 mM sodium chloride and 2 volumes of 100% ethanol, and incubation at -20°C for 30 minutes. The DNA was then pelleted by centrifugation at 14K for 15 minutes in a microcentrifuge. The surrounding liquid was removed and the DNA was air dried, then resuspended in 20 µl of dH2O. Fragments UDfibCAV-1, UDfibCAV-2 and UDfibHAd5 were excised from pCR2.1UDfibCAV-1, pCR2.1UDfibCAV-2 and pCR2.1UdfibHAd5 by Pst I and Spe I restriction enzyme digestion (section 2.2.1.2). Pst I and Spe I digestion of both pCR2.1UDfibCAV-1 and pCR2.1UDfibCAV-2 yields bands of approximately 1200, 2700 and 3600 base pairs. Pst I and Spe I digestion of pCR2.1UdfibHAd5 yields bands of approximately 1200, 2700 and 3800 base pairs. The 3600 and 3800 fragments were isolated from UDfibCAV-1 and UDfibCAV-2, and UDfibHAd5, respectively, by gel extraction (section 2.2.1.6). Electrocompetent BJ5183 cells were cotransformed with linearised cosmid ΔFibCAV-1Cm^R DNA and either UDfibCAV-1, UDfibCAV-2 or UdfibHAd5 fragments by electroporation, as described in section 2.2.1.9. Electroporated cells were plated out on L broth plates and incubated at 37°C overnight. The following day individual colonies were selected and STET/lysosyme minipreparations were performed (section 2.2.1.10). Positive clones were selected for by Hpa I restriction enzyme digestion (section 2.2.1.2).

DNA from positive clones was transformed into Max Efficiency STBL2 cells. Transformed STBL2 cells were plated out on LB agar plates and incubated at 37°C

overnight. The following day single colonies were selected and used to inoculate 2.5 ml of L broth. Cosmid DNA was prepared from the bacterial cultures by the STET/lysosyme minipreparation method (section 2.2.1.10) and digested with Hpa I (section 2.2.1.2) to check for positive clones. 1 ml of a fresh overnight culture from a positive clone was used to inoculate 400 ml of L broth containing 100 μg/ml chloramphenicol and DNA was prepared using the using Qiagen plasmid Mega Kit (section 2.2.1.11) following the instructions of the manufacturer. Glycerol stocks of the final construct were prepared and stored at -70°C (section 2.2.1.12).

4.2.2.4 Construction of Cosmid cΔE3ΔFibCAV-1

A cosmid that contained the CAV-1 genome with the E3, U exon and fibre genes deleted and replaced by a Pme I restriction enzyme site (Figure 4.3) was generated as follows.

Cosmid ΔFibCAV-1 was linearised by digestion with Pme I and purified by alcohol precipitation (section 4.2.2.3). Fragment UE3Dfib was isolated from pCR2.1UE3Dfib by Pst I and Spe I restriction enzyme digestion (section 2.2.1.2). Pst I and Spe I digestion of pCR2.1UE3Dfib yields bands of approximately 1200, 2700 and 2700 base pairs. The 2700 base pair fragment was isolated by gel extraction (section 2.2.1.6). Electrocompetent BJ5183 cells were cotransformed with linearised cosmid ΔFibCAV-1 DNA and fragment UDE3Dfib as described in section 2.2.1.9. Electroporated cells were plated out on L broth plates and incubated at 37°C overnight. The following day individual colonies were selected and STET/lysosyme minipreparations were performed (section 2.2.1.10). Positive clones were selected for by Hpa I restriction enzyme digestion (section 2.2.1.2).

DNA from positive clones was transformed into Max Efficiency STBL2 cells. Transformed STBL2 cells were plated out on LB agar plates and incubated at 37°C overnight. The following day single colonies were selected and used to inoculate 2.5 ml of L broth. Cosmid DNA was prepared from the bacterial cultures by the STET/lysosyme minipreparation method (section 2.2.1.10) and digested with Hpa I (section 2.2.1.2) to check for positive clones. 1 ml of a fresh overnight culture from a positive clone was used to inoculate 400 ml of L broth containing 100 μg/ml

chloramphenicol and DNA was prepared using the using Qiagen plasmid Mega Kit (section 2.2.1.11) following the instructions of the manufacturer. Glycerol stocks of the final construct were prepared and stored at -70°C (section 2.2.1.12).

4.2.3 Generation of MDCK Fibre Complementation (pCI-NeoFib) Cell-Line

4.2.3.1 Cloning of CAV-1 Fibre Gene into an Expression Vector

The PCR amplified CAV-1 fibre gene was isolated by Pme I digestion of pCR2.1FibCAV-1 and purified as described in section 4.2.1.2. Expression vector pCI-Neo (section 2.1.1.4) was linearised by digestion with Sma I (section 2.2.1.2) and purified using the QIAquick™ gel extraction kit (section 4.2.1.1). Linearised pCI-Neo was dephosphorylated as described in section 2.2.1.3 and purified using the QIAquick™ gel extraction kit (section 4.2.1.1). Linearised pCI-Neo DNA and the CAV-1 fibre fragment were ligated according to section 2.2.1.7. 5 µl of the ligation was transformed into Max Efficiency STBL2 cells and cells were plated out on LB agar plates and incubated at 37°C overnight. The following day single colonies were selected and STET/lysosyme minipreparations were performed (section 2.2.1.10). Positive clones were screened for by digestion with restriction enzyme Avr II (section 2.2.1.2) (AvrII digestion of pCI-NeoFib in the correct orientation yields bands of approximate size 2800 and 4400 base pairs). 50 µl of bacterial culture from positive clones was used to inoculate 2.5 ml of L-broth and QIAprep® minipreparations were carried out (section 2.2.1.10). 1 ml of a fresh overnight culture from a positive clone was used to inoculate 400 ml of L broth containing 100 µg/ml chloramphenicol and DNA was prepared using the using Qiagen plasmid Mega Kit (section 2.2.1.11) following the instructions of the manufacturer. Glycerol stocks of the final construct were prepared and stored at -70°C (section 2.2.1.12).

4.2.3.2 *Transfection of pCI-NeoFib into MDCK cells*

MDCK cells in 25 cm^2 tissue culture flasks were transfected with 0.5, 2 or 5 μg of the pCI-NeoFib plasmid using lipofectAMINE as detailed in section 2.2.4.5. The following day the transfected cells were subcultured using 2 ml of trypsin, and diluted with 8 ml of 5% MDCK medium containing 0.6mg/ml G418 to select for cells expressing neo^R. A G418 kill curve for MDCKs revealed that the minimum concentration necessary to kill these cells is 0.6 mg/ml (unpublished data Dr M. D. Morrison, University of Glasgow. This was diluted 1:10 with 5% MDCK medium containing 0.6mg/ml G418, and 10 ml of this dilution was subcultured into 10cm diameter tissue culture dishes. G418 was added to medium to select for stably transfected cells. After 12 days isolated colonies of cells were trypsinised and cultured in 6 well plates, and once confluent further subcultured into 25cm^2 tissue culture flasks, in 5% MDCK medium containing 0.6 mg/ml G418. RNA was isolated from each cell-line using Gentra's Purescript® RNA isolation kit according to the manufacturer's protocol. Positive cell-lines were identified by RT-PCR of RNA isolated from each cell-line using primers Fib1 and Fib2 (Table 4.2) and Promega's "Access" kit as described in section 2.2.2.3.

4.2.4 Generation of Recombinant Canine Adenoviruses

Fse I digestion of cosmids cΔFibCAV-1, cΔE3ΔFibCAV-1, cPFibCAV-1, cPFib(C2)CAV-1 and cPFib(H5)CAV-1 releases the recombinant virus genome from the cosmid backbone (see Figure 4.4). Between 0.5 and 5 µg of each vector was transfected into the appropriated cell-line as described in section 2.2.4.5. ΔFibCAV-1, ΔE3ΔFibCAV-1, PFibCAV-1, PFib(C2)CAV-1 and PFib(H5)CAV-1 were transfected into MDCK cells. ΔFibCAV-1 and ΔE3ΔFibCAV-1 were also transfected into pCI-NeoFib cells. The transfected cells were then monitored for c.p.e..

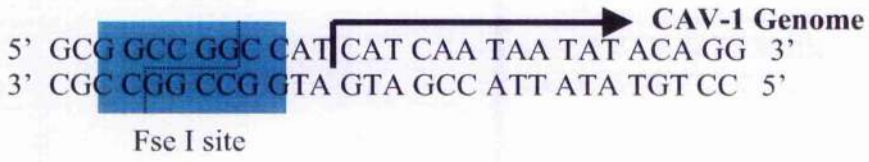


Figure 4.4 Mapping of the junction between the cosmid backbone and the CAV-1 genome in FLR-CAV. This map applies to both ends of the CAV-1 genome.

Transfection of cosmid cPFib(H5)CAV-1 and the following experiments with PFib(H5)CAV-1 were carried out in Category III Containment according to the standard operating procedures of the University of Glasgow and under GM (contained use) regulations..

4.2.4.1 Validation of Recombinant Canine Adenoviruses by PCR

Medium was removed from transfected cells exhibiting c.p.e. and placed in screw-cap 1.5 ml eppendorf tubes. DNA was denatured by boiling the samples for 10 minutes. The denatured samples were diluted 1:100 in ddH_2O and PCR and restriction enzyme digestion was carried out on samples and control samples as follows.

PFibCAV-1

Taq PCR of virus isolated from 3 transfections of cosmid cPFibCAV-1, and pCR2.1UDFibCAV-1 DNA (positive control), was carried out using master mixes (section 2.2.2.2) with primers Fib1 and Fib2 (Table 4.2). The amplified DNA was purified using the QIAquick gel extraction kit (section 4.2.1.1) and a 5 µl aliquot of each sample was digested with Pme I (section 2.2.1.2). Samples of the amplified DNA and Pme I digested DNA were analysed by agarose gel electrophoresis (section 2.2.1.5).

PFib(C2)CAV-1

Taq PCR using master mixes (section 2.2.2.2) with Fib1 and Fib2 primers was carried out on virus isolated from 4 transfections of cosmid cPFib(C2)CAV-1, pCR2.1UDCAV-2Fib DNA (positive control) and pCR2.1UDFibCAV-1 DNA (negative control). The amplified DNA was purified using the QIAquick gel extraction kit (section 4.2.1.1) and a 5 µl aliquot of each sample was digested with each Pme I, Sal I and Nar I restriction enzymes (section 2.2.1.2). Samples of the amplified DNA and restriction digested DNA were analysed by agarose gel electrophoresis (section 2.2.1.5).

PFib(H5)CAV-1

Taq PCR using master mixes (section 2.2.2.2) with Fib1 and Fib2 primers was carried out on virus isolated from 4 transfections of cosmid cPFib(H5)CAV-1, pCR2.1UDHAd5Fib DNA (positive control) and pCR2.1UDFibCAV-1 DNA (negative control). The amplified DNA was purified using the QIAquick gel extraction kit (section 4.2.1.1) and a 5 µl aliquot of each sample was digested with each Afl II, Hpa I and Nco I restriction enzymes (section 2.2.1.2). Samples of the amplified DNA and restriction digested DNA were analysed by agarose gel electrophoresis (section 2.2.1.5).

ΔFibCAV-1

Taq PCR of cΔFibCAV-1 virus, pCR2.1UD(-fib) DNA (positive control) and CAV-1 (negative control) was carried out using master mixes (section 2.2.2.2) and Fib1 and Fib2 primers (Table 4.2). The amplified DNA was analysed by agarose gel electrophoresis (section 2.2.1.5).

ΔE3ΔFibCAV-1

Taq PCR of isolated cΔE3ΔFibCAV-1 virus, pCR2.1UE3Dfib DNA (positive control), ΔE3Fib plasmid DNA and pCR2.1UD(-fib) DNA (negative control) was carried out using master mixes (section 2.2.2.2) and primers Fib2 and UEdelup (Table 4.2). The amplified DNA was analysed by agarose gel electrophoresis (section 2.2.1.5).

4.2.4.2 Validation of PFibCAV-1,PFib(C2)CAV-1 and PFib(H5)CAV-1 by sequencing

The sequence authenticity of Ufib, Dfib and all fibre cassettes were confirmed by sequence analysis. No further PCR steps were involved in the derivation of recombinant viruses and all the subsequent steps were performed by traditional cloning without associated *Taq* error potential.

3 x 10^6 MDCK cells were subcultured in 175 cm^2 tissue culture flasks and incubated at 37°C overnight. The following day the cells were infected with a sample of either PFibCAV-1, PFib(C2)CAV-1 or PFib(H5)CAV-1 from transfected cells exhibiting c.p.e.. Once the infected cells had reached 100% c.p.e. the cells were harvested and DNA was isolated using the DNeasy® Tissue Kit (Qiagen, UK) according to the manufacturer's instructions. ABI Prism sequencing was performed on the DNA samples (section 2.2.3.2) using Fib1 and Fib2 primers (Table 4.2) to confirm the DNA sequence at the junctions between cloned fibres and the CAV-1 backbone of the recombinant vectors. Sequence data generated was analysed using the UW-GCG software package, homology between the nucleotide sequence and the appropriate adenovirus genome was determined using the 'Fasta' program.

4.2.4.3 Production of virus stocks

MDCK or pCI-NeoFib cells were subcultured in 175 cm^2 tissue culture flasks until they had reached approximately 90% confluency. The cells were infected with samples of the medium from transfected cells exhibiting c.p.e. Once the infected cells

had reached 100% c.p.e. they were scraped into the culture medium. The medium containing cells was placed in 50 ml falcon tubes and centrifuged at 1 K for 5 minutes at room temperature. The supernatant, except for 5 ml, was removed and retained. The cell pellets were resuspended in the remaining 5 ml of medium and subjected to 3 freeze thaw cycles, followed by further centrifugation at 1 K for 5 minutes at room temperature. The supernatant was added to the initial retained medium, mixed, and 1 ml aliquots were stored at -70°C. One vial was thawed and used to titre virus aliquots by the $TCID_{50}$ method (section 2.2.4.6).

4.2.5 One-Step Growth Curves

MDCK were subcultured at 2 x 10^5 cells, in 2 ml of medium, per well of a six-well tissue culture plate and incubated at 37°C overnight. The following day each well was infected with either CAV-1, PFibCAV-1, PFib(C2)CAV-1 or PFib(H5)CAV-1 at m.o.i. of 10 in 0.5 ml of the appropriate medium and incubated at 37°C for 1 hour. The medium containing virus was then removed, the cells were washed twice with PBS and 2 ml of the appropriate medium was added to each well. This was considered to be the 0 hour time point. At each time point the medium was removed and 1 ml aliquots were retained, the cells were washed twice with PBS and trypsinised with 0.5 ml of trypsin. 2 wells were pooled for each sample. All samples were centrifuged at 14K for 10 minutes in order to pellet cell debris and then stored at -70°C. Cell-associated virus was isolated by subjecting the cellular samples to three cycles of freeze thawing, followed by centrifugation at 14K for 10 minutes. Infectious virus was titred using the $TCID_{50}$ method detailed in section 2.2.4.6.

4.2.6 Cytotoxicity Assay

MDCK, A549, 293, CrFK, FEA, D17, pCI-NeoFib, CML10 and A72 cells (see section 2.1.3.1 for cell origins) were subcultured at 2 x 10^5 cells, in 2 ml of medium, per well of a six-well tissue culture plate and incubated at 37°C overnight. The

following day each well was infected with either CAV-1, PFibCAV-1, PFib(C2)CAV-1 or PFib(H5)CAV-1 at m.o.i. of 10 in 0.5 ml of the appropriate medium and incubated at 37°C for 1 hour. The medium containing virus was then removed and the cells were washed twice with PBS. The cells were then overlaid with 2 ml of 1% low melting temperature agarose in 5% MDCK growth medium and incubated at 37°C for 5 days. After 5 days viral plaques could be seen at each dilution of CAV-1 infected MDCK cells. Cells were stained overnight with 2 ml of 0.5% (w/v) crystal violet in 9% (v/v) formaldehyde in dH_2O at room temperature. The agarose was removed and excess stain was removed by washing the cells with H_2O. The cells were examined by light microscopy and appropriate plates were photographed using a PC scanner (Primax 9600 Profi) and MGI® Photosuite™ Starter Edition version 1.04.

4.3 RESULTS

4.3.1 Construction and Production of Fibre Mutant CAV-1 Viruses

Fibre mutant CAV-1 based viruses were generated using the cotransformation strategy shown in Figure 4.5. The CAV-1 fibre gene was deleted from the CAV-1 genome in a cosmid backbone (FLR-CAV) and replaced with a chloramphenicol acetyltransferase (Cm^R) gene flanked by Pme I restriction enzyme sites (cΔFibCAV-1Cm^R) by homologous recombination of shuttle vector pCR2.1UDCm^R (see section 4.2.1.2) with FLR-CAV. cΔFibCAV-1, a cosmid with a unique Pme I restriction enzyme site replacing the fibre gene, was generated by Pme I digestion of cΔFibCAV-1Cm^R, followed by religation. A cosmid containing the CAV-1 with deletions in the E3, U exon and fibre genes was also generated (cΔE3ΔFibCAV-1).

The fibres of CAV-1, CAV-2 and Had5 were amplified by PCR and used in the construction of shuttle vectors pCR2.1UDfibCAV-1, pCR2.1UDfibCAV-2 and pCR2.1UdfibHAd, respectively. Homologous recombination of these with cΔFibCAV-1 generated cosmids cPFibCAV-1, cPFib(C2)CAV-1 and cPFib(H5)CAV-1, respectively, which contain Pme I sites flanked cloned CAV-1, CAV-2 and HAd5 fibres at the CAV-1 fibre locus, respectively.

Cosmids were digested with restriction enzyme FseI to release the chimeric genome from the cosmid backbone (section 2.2.1.2). Transfection of FseI digested cosmids PFibCAV-1, PFib(C2)CAV-1 and PFib(H5)CAV-1 into MDCK cells lead to the development of viral c.p.e. and the recovery of infectious particles. The stock titres of PFibCAV-1, PFib(C2)CAV-1 and PFib(H5)CAV-1 were 6.1 x 10^7 p.f.u./ml, 3.3 x 10^7 p.f.u./ml and 1.2 x 10^7 p.f.u./ml, respectively. Transfection of ΔFibCAV-1 and ΔE3ΔFibCAV-1 into MDCK cells resulted in no viral plaques. A cell-line that expressed the CAV-1 fibre stably was created by transfection of MDCK cells with pCI-NeoFib plasmid. This cell-line supported the transfection of FseI digested cosmids cΔFibCAV-1 and cΔE3ΔFibCAV-1 and allowed the growth of the recovered

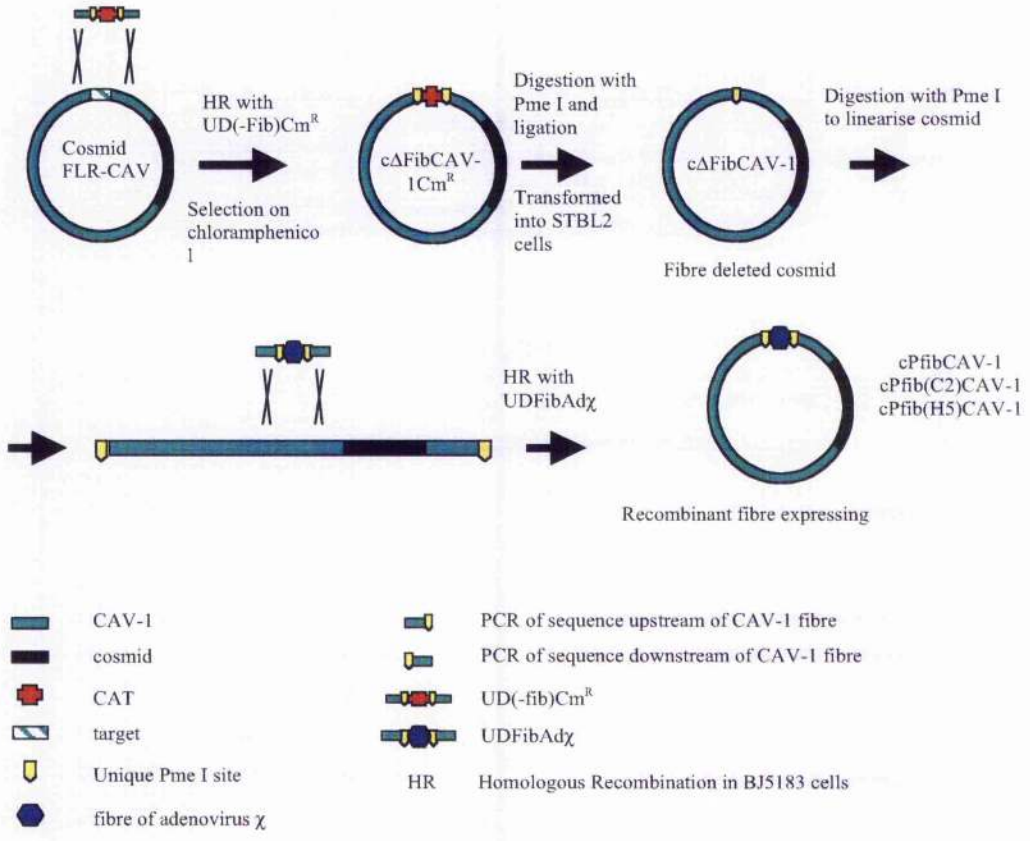


Figure 4.5 Strategy for production of CAV-1 recombinants (adapted from Chartier *et al.*, 1996).

Regions flanking the target were amplified by PCR and cloned into a "shuttle" vector with the chloramphenicol acetyltransferase (CmR) gene, flanked by unique restriction endonuclease (RE) sites (Pme I), inserted between them. This tripartite insert was cotransformed with cosmid FLR-CAV (contains wild-type CAV-1 genome flanked by FseI sites) into recombinase proficient BJ5183 cells. Homologous recombination replaced the target with CmR and recombinants were selected for on chloramphenicol containing LB agar plates. The CmR gene was removed by PmeI digestion, followed by ligation and transformation into STBL2 cells. The appropriate fibre gene was then inserted into the Pme I site using shuttle vectors.

fibre-deleted CAV-1 viruses. Stock titres of 7 x 10^3 and 2.9 x 10^4 p.f.u./ml were obtained for fibre mutant viruses ΔFibCAV-1 and ΔE3ΔFibCAV-1, respectively.

4.3.1.1 Validation of Recombinant Canine Adenoviruses

The junctions between cloned fibres and CAV-1 backbone of PFibCAV-1, PFib(C2)CAV-1 and PFib(H5)CAV-1 viruses were sequenced to confirm sequence authenticity.

The correct insertion or deletion in each vector was validated by PCR of viral DNA with the appropriate primers and, where appropriate, restriction digestion of the amplified DNA.

Validation of PFibCAV-1

PCR of virus isolated from 3 transfections of cosmid PFibCAV-1 and pCR2.1UDFibCAV-1 DNA (positive control) with primers flanking the fibre region was used to verify the presence of the fibre gene. Pme I digestion of the amplification products verified the presence of Pme I restriction enzyme sites (Figure 4.6).

Validation of PFib(C2)CAV-1

Primers that flank the CAV-1 fibre region were used to amplify the fibre region of virus isolated from 4 transfections of cosmid PFib(C2)CAV-1, pCR2.1UDCAV-2Fib DNA (positive control) and pCR2.1UDFibCAV-1 DNA (negative control). Pme I digestion of the amplification products verified the presence of Pme I restriction enzyme sites. Digestion with restriction enzyme Pme I was used to verify the presence of Pme I sites and digestion with Sal I and Nar I was used to verify the presence of the CAV-2 and CAV-1 fibres, respectively (Figure 4.7).

Validation of PFib(H5)CAV-1

Primers that flank the CAV-1 fibre region were used to amplify the fibre region of virus isolated from 4 transfections of cosmid PFib(H5)CAV-1, pCR2.1UDHAd5Fib DNA (positive control) and pCR2.1UDFibCAV-1 DNA (negative control). Digestion

with restriction enzymes Afl II, Hpa I and Nco I was used to verify the presence of the Had5 or CAV-1 fibres (Figure 4.8).

Validation of ΔFibCAV-1

PCR of ΔFibCAV-1 virus, pCR2.1UD(-fib) (positive control) and CAV-1 virus (negative control) with primers Fib1 and Fib2 was used to validate the deletion of the fibre gene from ΔFibCAV-1 (see Figure 4.9). Fib1 and Fib2 are positioned approximately 200 bases on either side of the fibre gene in wild type CAV-1, therefore, in the absence of the fibre gene a product of approximately 400bp product was amplified. Wild type CAV-1 was used as a negative control to demonstrate the products expected from PCR with the same primers in the presence of the fibre gene.

Validation of ΔE3ΔFibCAV-1

PCR of isolated ΔE3ΔFibCAV-1 virus, pCR2.1UE3Dfib DNA (positive control), pCR2.1UE3Dfib plasmid DNA and pCR2.1UD(-fib) DNA (negative control) with primers Fib2 and UEdelup was used to validate the deletion of the fibre gene from ΔE3ΔFibCAV-1 (Figure 4.10). Fib2 and UEdelup are positioned approximately 200 bases from the fibre and E3 genes, respectively, in wild type CAV-1, therefore, in the absence of these genes a product of approximately 400bp product was amplified.

4.3.2 One-Step Growth Curves

To investigate the growth kinetics of fibre recombinant viruses PFibCAV-1, Pfib(C2)CAV-1 and PFib(H5)CAV-1, MDCK cells were infected at a m.o.i. of 10 and incubated at 37°C over a period of 48 hours. Virus was isolated from the cells and the culture medium at the time points indicated in the figure legends. The titres were determined by the $TCID_{50}$ method. Figures 4.11 and 4.12 show the total log $TCID_{50}$ values at each time point.

Although the amount of cell-associated PFib(C2)CAV-1 virus that accumulated was 1 log lower than CAV-1 and PFibCAV-1 over the time course, ANOVAs between the cell associated and extracellular growth curves of CAV-1, PFibCAV-1 and

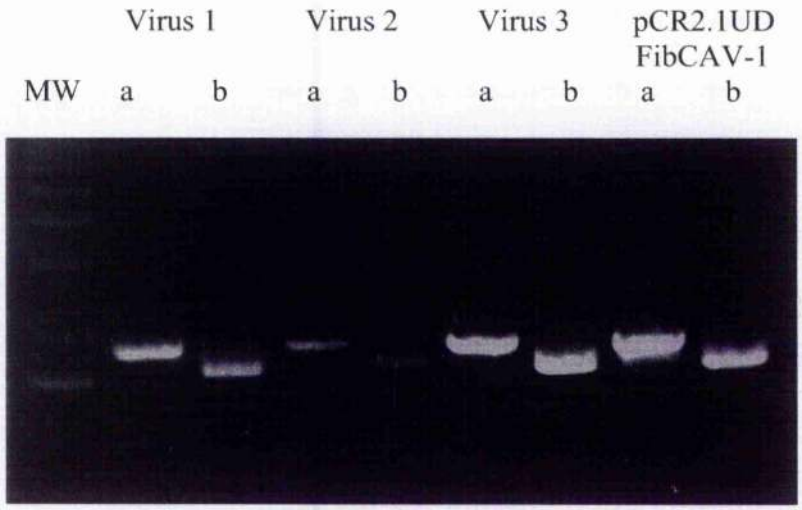


Figure 4.6 PCR of pFibCAV-1 virus. DNA was isolated from MDCK cells that showed viral c.p.e. after transfection with cosmid pFibCAV-1. The DNA was subjected to PCR with primers Fib1 and Fib2 and digested with PmeI. PCR and PmeI digestion was carried out on pCR2.1UDFibCAV-1 DNA (positive control) for comparison. MW = 1 Kb molecular weight marker.

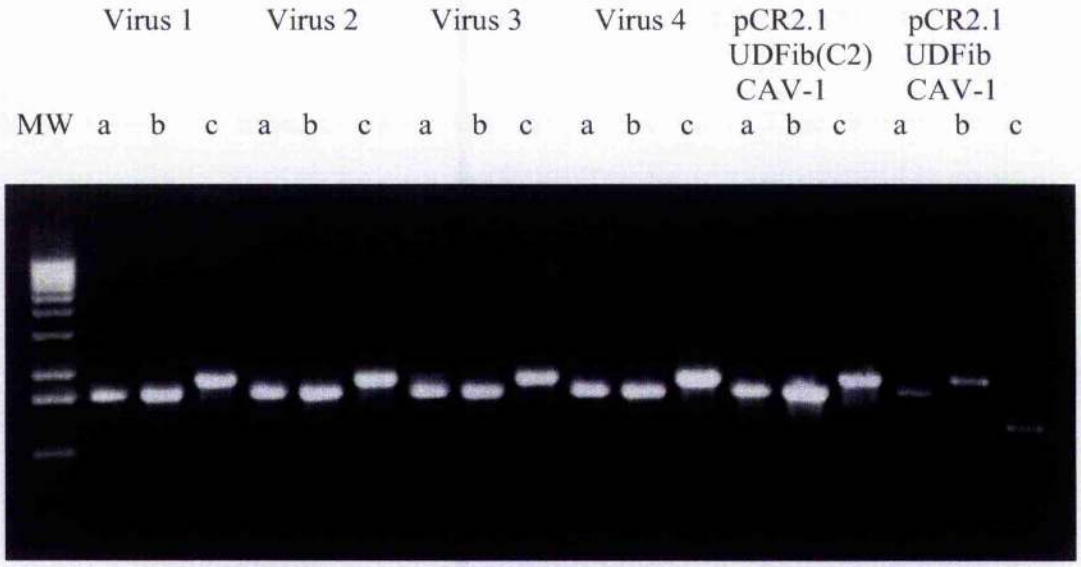


Figure 4.7 PCR of pFib(C2)CAV-1 virus. Virus was isolated from MDCK cells that showed viral c.p.e. after transfection with cosmid pFib(C2)CAV-1. Four isolates of pFib(C2)CAV-1 (Virus 1-4) were denatured subjected to PCR with primers Fib1 and Fib2. The amplicons were digested with (a) PmeI, (b) SalI or (c) NarI restriction enzymes. PCR and restriction enzyme digests were also carried out on pCR2.1UDFibCAV-2 DNA (positive control) and pCR2.1UDFibCAV-1 DNA (negative control) for comparison. MW = 1 Kb molecular weight marker.

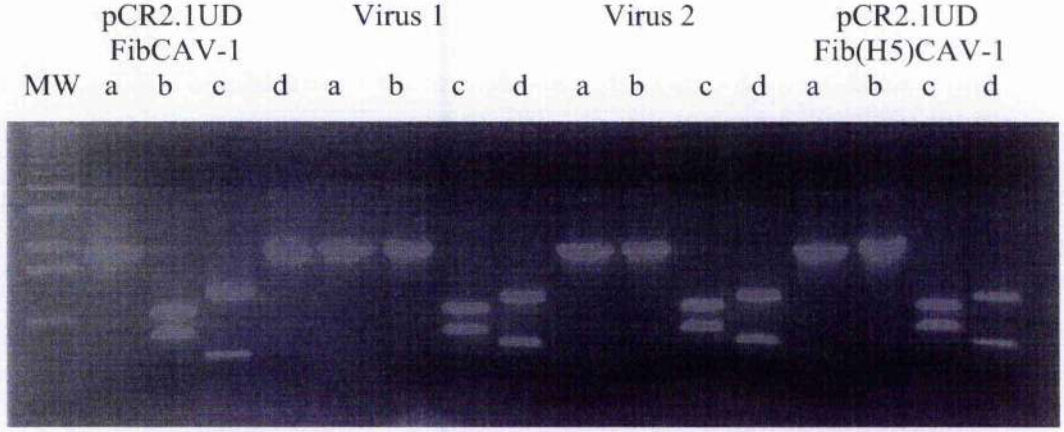


Figure 4.8 PCR of pFib(H5)CAV-1 virus. Virus was isolated from MDCK cells that showed viral c.p.e. after transfection with cosmid pFib(H5)CAV-1. Two isolates of pFib(H5)CAV-1 (Virus 1 and Virus 2) were denatured and subjected to PCR with primers Fib1 and Fib2. The amplicons (a) were digested with (b) AflII, (c) HpaI or (d) NcoI restriction enzymes. PCR and restriction enzyme digests were also carried out on pCR2.1UDFibCAV-1 DNA (negative control) and pCR2.1UDFibHAd5 (positive control) for comparison. MW = 1 Kb molecular weight marker.

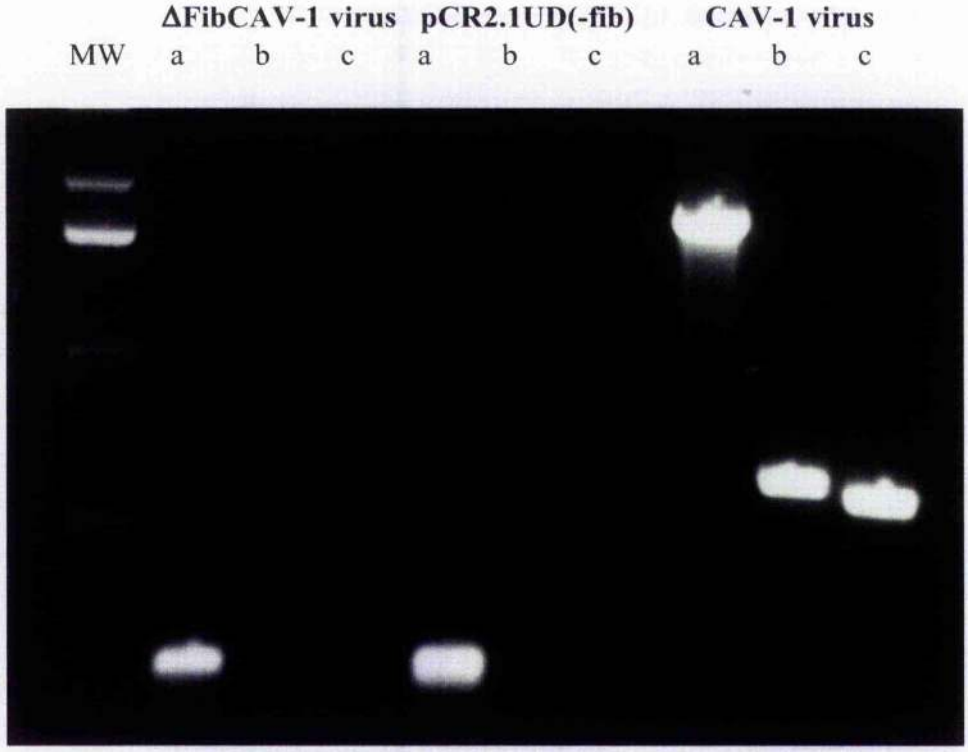


Figure 4.9 PCR of ΔFibCAV-1 virus. DNA was isolated from MDCK cells that showed viral c.p.e. after transfection with cosmid ΔFibCAV-1. The DNA was subjected to PCR with primers (a) Fib1 and Fib2, (b) Fib1 and 26396, and (c) Fib 2 and 27001. PCR with the same primers was also carried out on pCR2.1UD(-fib) DNA (positive control) and CAV-1 virus (negative control) for comparison. MW = 1 Kb molecular weight marker.

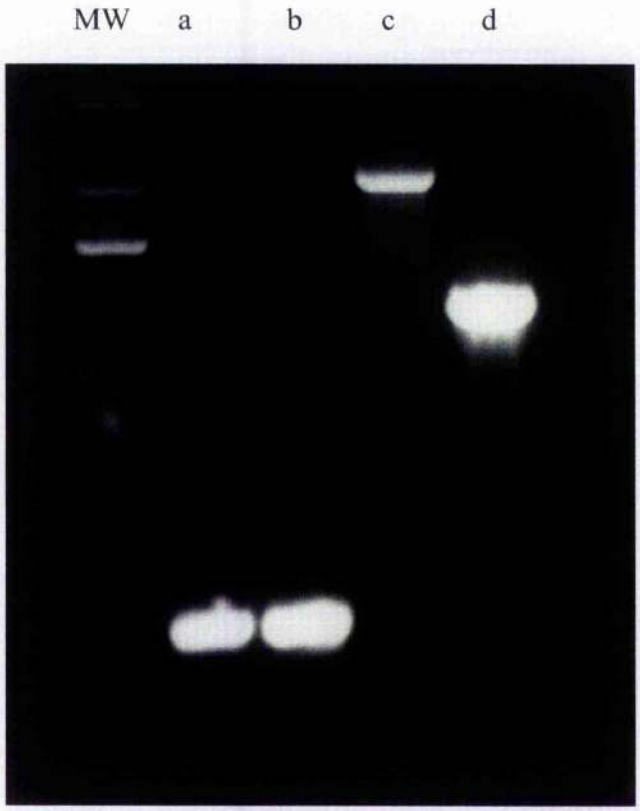
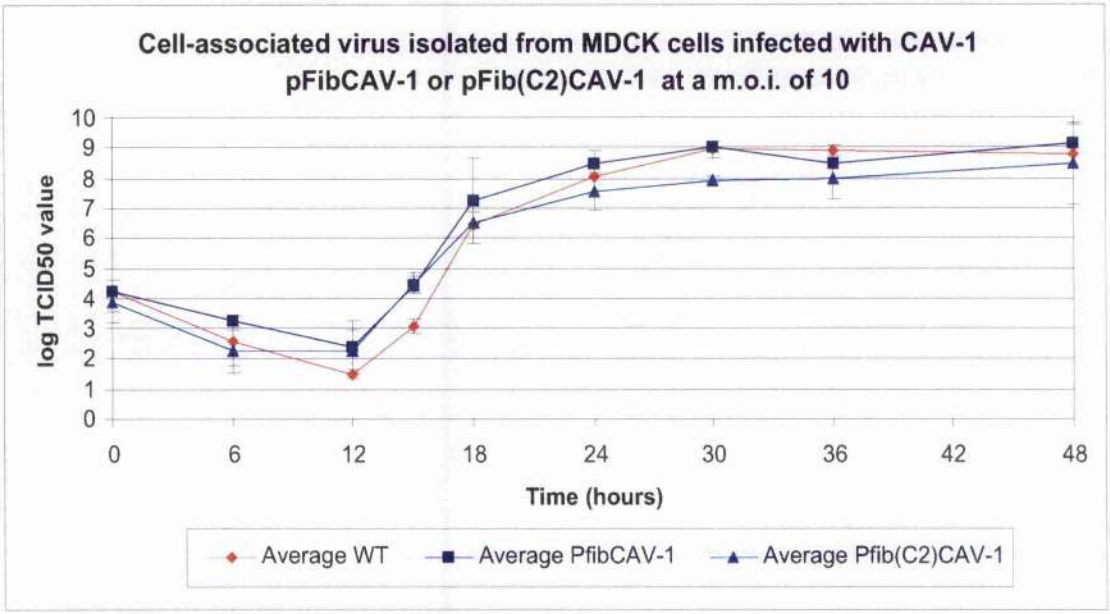


Figure 4.10 PCR of ΔE3ΔFibCAV-1 virus. DNA was isolated from MDCK cells that showed viral c.p.e. after transfection with cosmid ΔE3ΔFibCAV-1. PCR was also carried out on (a) ΔE3ΔFibCAV-1 virus (b) pCR2.1UE3Dfib DNA (positive control), (c) ΔE3Fib plasmid DNA (negative control), and (d) pCR2.1UD(-fib) DNA (negative control) with primers Fib2 and UE3delup. MW = 1 Kb molecular weight marker.



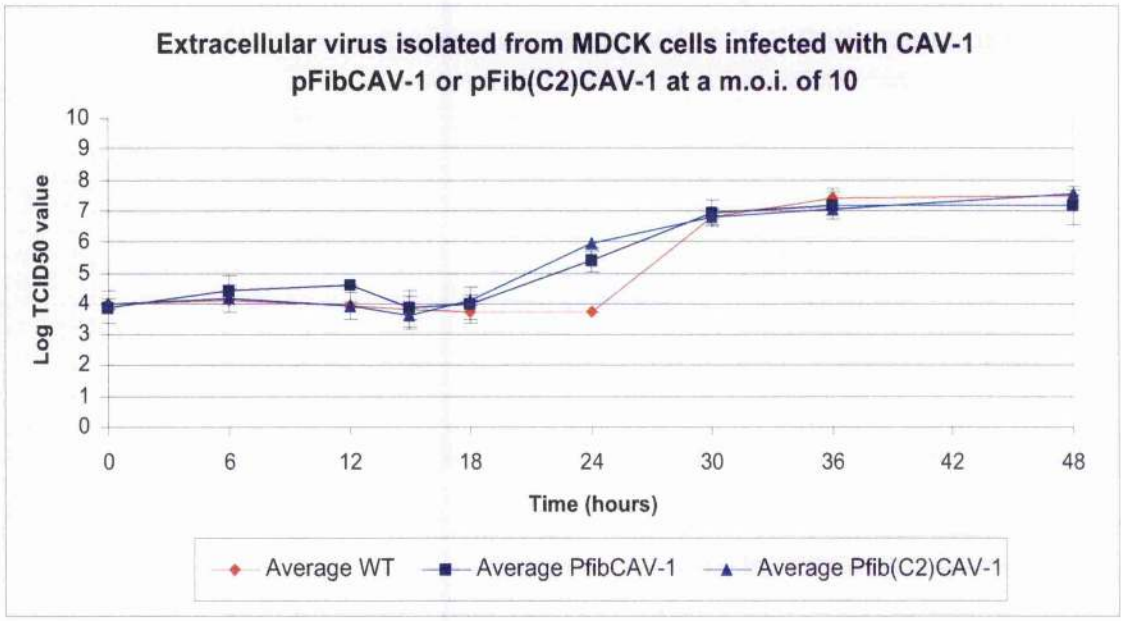
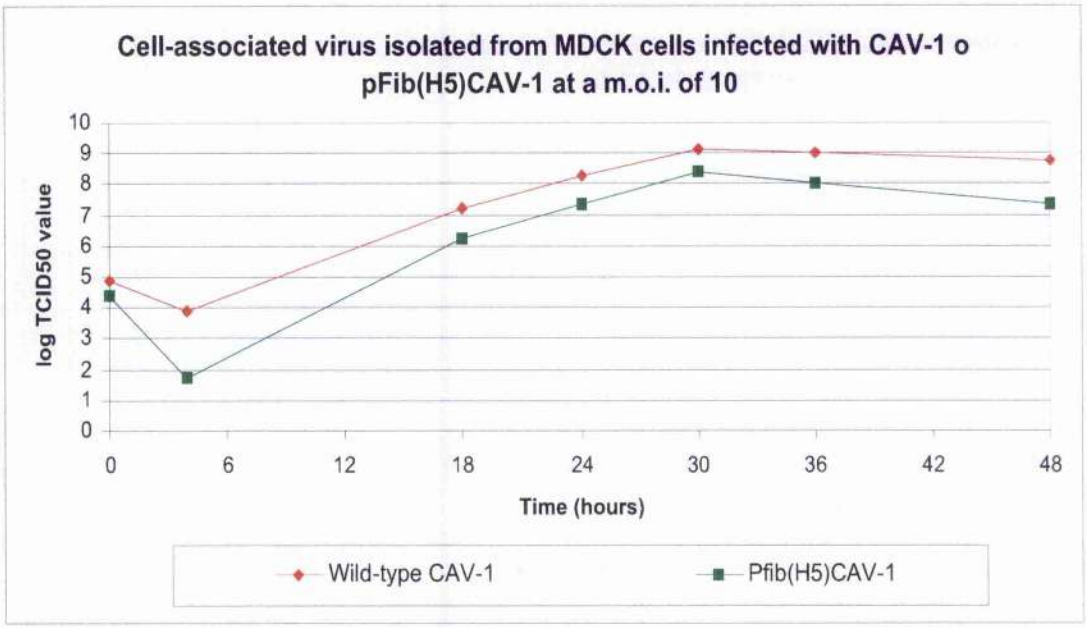


Figure 4.11 One Step Growth Curves for CAV-1, PFibCAV-1 and PFib(C2)CAV-1. MDCK cells were infected at a m.o.i. of 10 with either CAV-1, PFibCAV-1 or PFib(C2)CAV-1. Cell-associated and extracellular virus was isolated at 0, 6, 12, 15, 18, 24, 30, 36 and 48 hours p.i., $TCID_{50}$ values are presented.



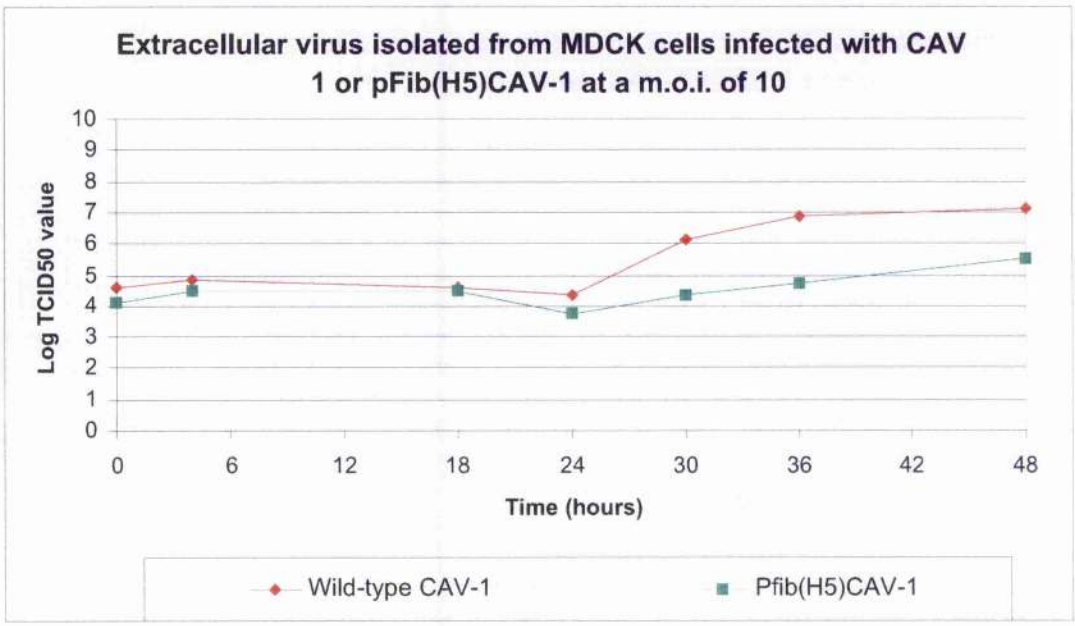


Figure 4.12 One Step Growth Curves for CAV-1 and PFib(H5)CAV-1. MDCK cells were infected at a m.o.i. of 10 with either CAV-1 or PFib(H5)CAV-1. Cell-associated and extracellular virus was isolated at 0, 4, 18, 24, 30, 36 and 48 hours p.i., $TCID_{50}$ values are presented.

PFib(C2)CAV-1 returned P-values of 0.894 and 0.926, respectively, indicating no significant difference (Figure 4.11).

The growth kinetics of PFib(H5)CAV-1 were also the same as CAV-1 (Figure 4.12). However, at 4 hours p.i. the decrease in cell-associated virus titres was 2 logs lower than CAV-1 and the accumulated cell-associated PFib(H5)CAV-1 48 hours p.i. was approximately 1.5 logs lower than CAV-1. The titre of PFib(H5)CAV-1 in the growth medium 48 hours p.i. was 1.5 logs less than with CAV-1.

4.3.3 Tropism of CAV-1 Recombinants

CAV-1 fibre recombinants that express GFP would be of benefit in order to fully investigate the effect of fibre pseudotyping. In the absence of such constructs preclinical investigations of CAV-1 recombinant tropism were performed through analysis of c.p.e. in various cell-lines.

There was no difference observed visually in the infection of human A549 and 293 cells, feline FEA cells or canine D17, CML10 or A72 cells (cell origins detailed in section 2.1.3.1) with PFib(C2)CAV-1 and PFib(H5)CAV-1, compared with wild type CAV-1 (data not shown). At a m.o.i. of 0.1, there appeared to be approximately 10-fold decrease in amount of c.p.e. observed in PFib(C2)CAV-1 infected MDCK cells, compared with CAV-1 infection (Figure 4.13). This was true of infection at m.o.i.s of 0.01 and 0.001. The findings were similar in CrFK and pCI-NeoFib cells with PFib(C2)CAV-1 (Figures 4.14 and 4.15).

Infection of MDCK cells with PFib(H5)CAV-1 at a m.o.i. of 0.1 resulted in an approximate 100-fold decrease in the amount of c.p.e. compared with CAV-1 (Figure 4.13). This was also true of the MDCK cells infected at 0.01 m.o.i. with PFib(H5)CAV-1. At a m.o.i. of 0.001 no viral c.p.e. was detected. However, there was only an approximately 10-fold decrease in the amount of c.p.e. observed in PFib(H5)CAV-1 infection of both CrFK and pCI-NeoFib cells at each m.o.i. (Figures 4.14 and 4.15).

pCI-NeoFib cells were investigated to determine whether expression of the CAV-1 fibre would complement any deficiency in the recombinant viruses. Each of the viruses, including wild-type CAV-1, demonstrated increased levels of c.p.e. in pCI-NeoFib cells compared to MDCK cells.

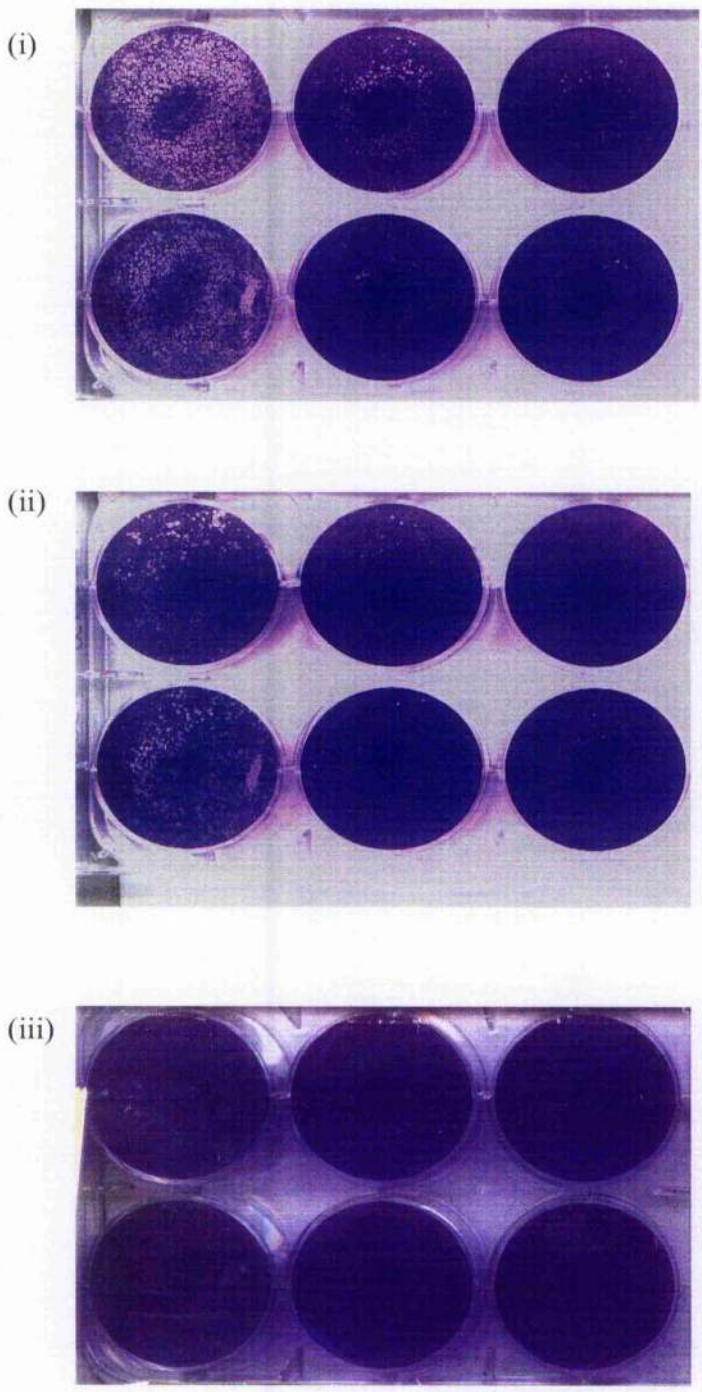


Figure 4.13 Cytotoxicity assay with fibre recombinant CAV-1 viruses in MDCK cells. Cells were infected at a m.o.i. of 0.1, 0.01 or 0.001 with (i) CAV-1, (ii) pFib(C2)CAV-1 and (iii) pFib(H5)CAV-1.

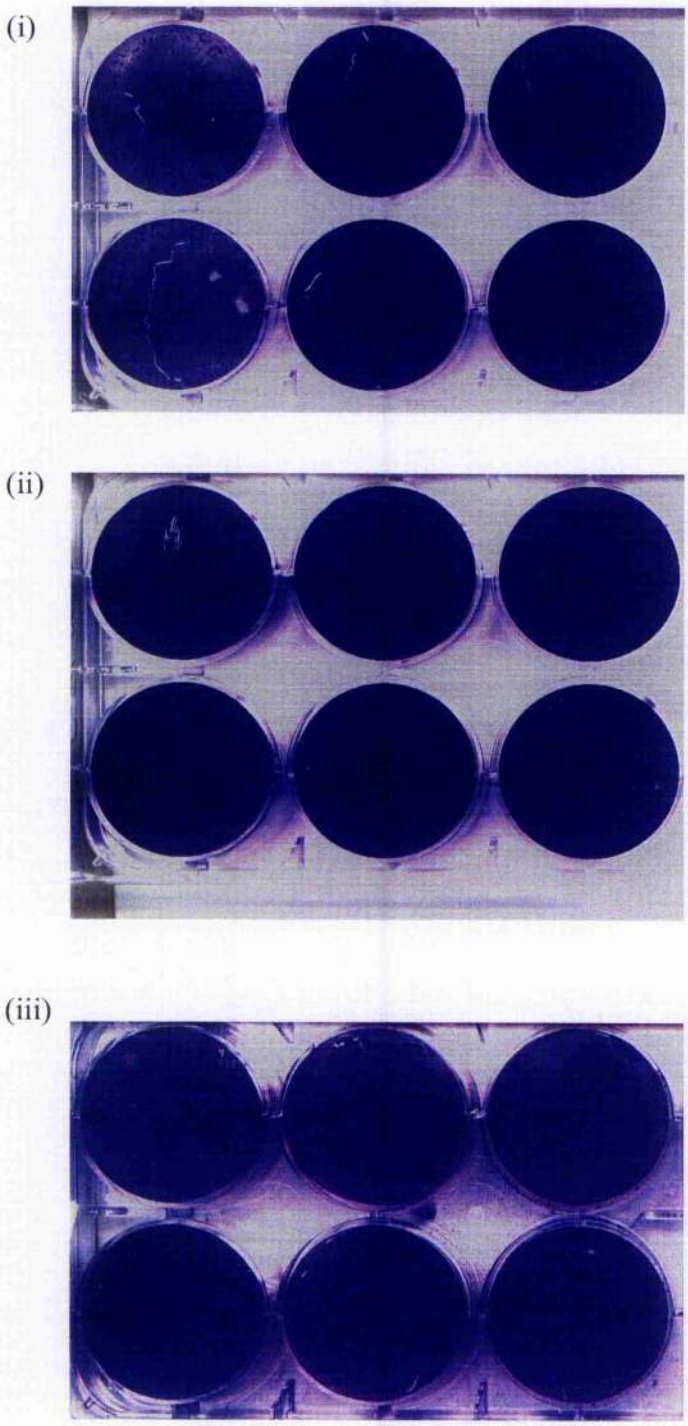


Figure 4.14 Cytotoxicity assay with fibre recombinant CAV-1 viruses in CrFK cells. Cells were infected at a m.o.i. of 0.1, 0.01 or 0.001 with (i) CAV-1, (ii) pFib(C2)CAV-1 and (iii) pFib(H5)CAV-1.

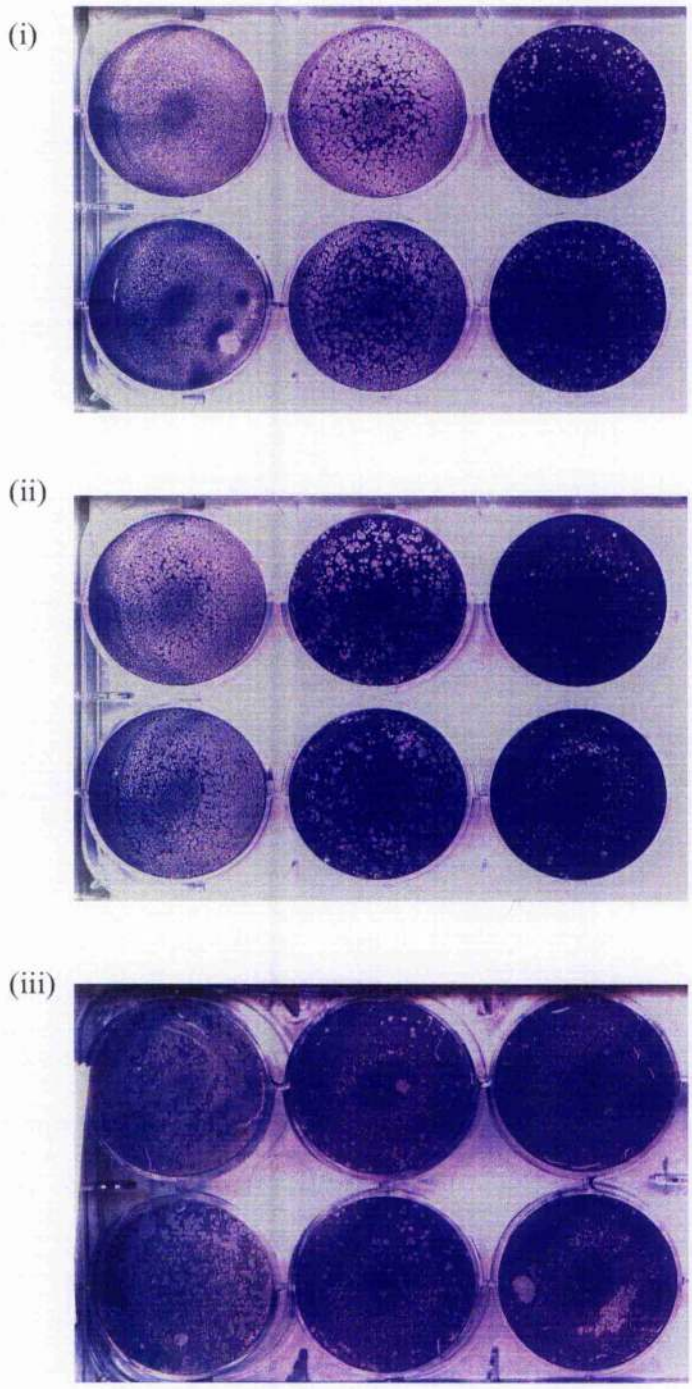


Figure 4.15 Cytotoxicity assay with fibre recombinant CAV-1 viruses in pCI-NeoFib cells. Cells were infected at a m.o.i. of 0.1, 0.01 or 0.001 with (i) CAV-1, (ii) pFib(C2)CAV-1 and (iii) pFib(H5)CAV-1.

4.4 DISCUSSION

Like the HAds, CAV-1 has a broad tissue tropism in its canine host. It naturally replicates in endothelial cells of the vascular system and has been shown to cause disease in a wide range of tissues, primarily the respiratory system and liver, but including others such as the nervous system and eyes (Koptopoulos & Cornwell, 1981). The ability to transduce and express genes in a wide range of cell and tissue types is undoubtedly one of most important features adenoviruses offer as gene therapy vectors. Nevertheless, the majority of proposed gene therapy protocols aim to treat diseases that require the delivery of vectors to specific target sites. The targeting of adenoviruses can be achieved through the modification of proteins involved in tropism.

The major determinants of adenoviral tropism are the fibre and penton proteins, which interact with each other specifically and with high affinity. It was demonstrated in 1982 that isolated fibre and penton base proteins from different serotypes could interact with each other (Boudin & Boulanger, 1982). Several groups have since showed the assembly of infectious adenovirus particles with fibre-penton chimeras (see section 4.1.2.3). It has also been shown that by the exchange of fibres, or domains of fibres, between HAds of different serotypes it is possible to significantly alter the tropism of the resultant vectors. Xu and Both described an OAV chimera that had the knob domain replaced by that of HAd5 (Xu & Both, 1998). The resultant OAV chimera could infect previously non-permissive human cells (Xu & Both, 1998). To date there is no published data on whole fibre swapping between adenoviruses native to different host species.

The addition of restriction enzyme sites on either side of the fibre gene did not significantly alter the growth kinetics of PFibCAV-1 compared with wild-type CAV-1 and, as such, a vector system that facilitates fibre swapping between CAV-1 and heterologous adenoviruses was produced. This vector system will potentially allow the insertion of fibres, both wild-type and modified, from a wide range of adenovirus serotypes into the fibre locus of CAV-1. The fibre of CAV-2 has 80.6% amino acid identity with that of CAV-1 and incorporation of this fibre into CAV-1 resulted in an

infectious virus that had growth kinetics with no significant difference to CAV-1. Insertion of the HAd5 fibre, which has only 26% amino acid identity with that of CAV-1, also yielded an infectious virus, although there was a significant reduction in both extracellular and cell-associated titres. Nevertheless, infectious PFib(H5)CAV-1 particles were recovered to relatively high titres and the growth kinetics were comparable to those of CAV-1.

The tropism of CAV-1 chimeras with CAV-2 and HAd5 fibres was assessed in various cell-lines. In particular, the exchange of the CAV-1 fibre with that of HAd5 did not result in detectable cytotoxicity within A549 cells. Whereas HAd5 productively infects A549 cells, wild type CAV-1 does not (Chapter 3). Presumably the block to PFib(H5)CAV-1 infection of A549 cells is a consequence of events after the fibre-receptor interaction, as might occur in wild-type CAV-1 infection of these cells. It is possible that a secondary interaction analogous to penton-integrin interactions in HAds is required by CAV-1 for entry into the host cell and is unachievable in A549 cells. Perhaps irregular capsid assembly compromises A549 infection by PFib(H5)CAV-1 as there is only 50% identity between the CAV-1 and HAd5 tail domains. It is also known that HAd fibres are important in the intracellular trafficking of adenoviruses (Hong & Engler, 1991, Miyazawa *et al.*, 1999), however, the intracellular trafficking requirements of the CAV-1 fibre are unknown and these too may have an influence in this situation.

Plaque assays demonstrated that the tropism of CAV-1 was modified by the replacement of the native fibre with the fibre of either CAV-2 or HAd5. However, more in depth investigations are necessary to verify the integrity of modified viruses and their tropism. Similar chimeric CAV-1 vectors expressing GFP would be an invaluable tool in future work. *In vivo* investigations would also be necessary before considering the use of a fibre modified CAV-1 vector, as tropism *in vitro* is not always indicative of tropism *in vivo* (see section 4.1.1.1).

Transfection of MDCK cells with cosmid ΔFibCAV-1 yielded no viral plaques, therefore an MDCK-based cell-line was produced that expressed the CAV-1 fibre under the control of a CMV promoter (pCI-NeoFib). Transfection of this cell-line allowed the propagation of ΔFibCAV-1 virus and stocks were produced containing 7

x 10^3 p.f.u./ml, suggesting that the fibre supplied *in trans* by this method was functional. The production of ΔFibCAV-1 in pCI-NeoFib cells indicates that the CAV-1 fibre gene does not have to be supplied *in cis* for the assembly and encapsidation of infectious viral particles. However, the $TCID_{50}$ method used to titre this virus does not provide information regarding the production of non-infectious particles. Two groups have reported the generation of fibreless HAd5 vectors in fibre complementing cell-lines and both found that there was a high particle to infectious virus ratio in stocks of these viruses (only one of every 10^5 particles were infectious) (Von Seggern *et al.*, 1999, Legrand *et al.*, 1999). Analysis of virus particles by caesium chloride gradients and electron microscopy showed that capsid assembly was normal, however, maturation of virions was impaired and this suggested that additional roles of the fibre were retarded by its removal. Such investigations with ΔFibCAV-1 should provide more information about the reduced numbers of infectious virions in stocks generated compared with wild type CAV-1 as a result possibly offer further insights into the functions of the CAV-1 fibre in the normal life cycle. In addition, Von Seggern *et al.* found that the RGD protrusions were angled more inwardly with fibreless HAd5 vectors than in wild type HAd5, this may have contributed to the decrease in infectivity as the removal of the fibre results in these viruses having an integrin-dependant entry pathway. The mechanisms of secondary interactions with CAV-1 in its host species are unknown, therefore the mechanism of entry of ΔFibCAV-1 could further this knowledge. Although cytotoxicity was noted, the cytopathic effect observed in ΔFibCAV-1 infected pCI-NeoFib cells was not typical of adenovirus infection; the infected cells rounded but no clumping was observed. Viral packaging of ΔFibCAV-1 in pCI-NeoFib cells is clearly functional, however, infection with the resultant virus is impaired. The low titres of ΔFibCAV-1 obtained restricted the investigations possible in this study. It would be necessary to determine whether alternative cell-lines could be produced to increase virus yield, which would allow further investigations into the mechanisms that are affected by the fibre deletion in ΔFibCAV-1. Virus stocks could also be concentrated and purified using caesium chloride gradients. The development of a ΔFibCAV-1 based vector carrying a reporter gene would also aid these studies.

Like HAds 2 and 5, the E3 region of CAV-1 is non-essential for replication in tissue culture (Morrison *et al.*, 2002). In HAds 2 and 5 this region functions in immune

modulation of the host by several mechanisms. It blocks both MHC transport to host surface of the host cell (Bennet *et al.*, 1999) and expression of the TNF receptor (Gooding *et al.*, 1991, Shisler *et al.*, 1997). E3 is also involved in internalisation of the cellular growth factors by endosomes (Tollefson *et al.*, 1991) and lysis of host cells for release of the progeny virus (Tollefson *et al.*, 1996). The adenovirus U exon gene is found between the E3 and fibre encoding regions (Davison *et al.*, 1993). The function of the U exon is undetermined, although it has been proposed to encode the N terminus of an, as yet, undefined protein (Davison *et al.*, 2000). ΔE3FibCAV-1 has a deletion that spans the E3, fibre and U exon genes of CAV-1. Like cosmid ΔFibCAV-1, transfection of MDCK cells with cosmid ΔE3FibCAV-1 did not result in the formation of viral plaques but transfection of pCI-NeoFib cells resulted in the isolation of ΔE3FibCAV-1 virus particles. ΔE3FibCAV-1 infection of MDCK cells showed no cytotoxicity and the cytopathic effect observed in ΔE3FibCAV-1 infected pCI-NeoFib cells was similar to that of ΔFibCAV-1. It is not known whether the deletion of the U exon would affect either wild-type or heterologous fibre expression, this could be investigated by the production of an E3 and U exon deleted CAV-1 vector with a functional fibre gene.

It has been demonstrated that the CAV-1 fibre can be replaced with heterologous adenovirus fibres and that tropism of these CAV-1 chimeras is altered. This work was intended as a proof of principle. The vector system developed could be applied in order to exchange the CAV-1 fibre with a wide range of adenovirus fibres. It would also allow the exchange of knob domains between CAV-1 and other adenoviruses. Human adenovirus tropism is well researched and modified fibre and knob domains have been created which allow specificity of binding *in vivo*. At present, there is little known about the domains within the CAV-1 fibre that determine tropism, and as such the use of modified human adenovirus fibres might allow the targeting of CAV-1 to selected cell types. Redirection of fibre-deleted CAV-1 vectors with exogenous molecules is also a possible method of CAV-1 retargeting.

CHAPTER 5

Neutralising Antibodies to CAV-1 and HAd5 Pseudotyped CAV-1 in Human Serum

5.1 INTRODUCTION

The host immune response to adenoviruses is highly efficient. Infected cells are rapidly destroyed by the cellular response and the humoral response protects against re-infection with the same adenovirus serotype.

The intracellular expression of adenovirus gene products leads to the activation of cytotoxic T lymphocytes, which kill infected cells (Chirmule *et al.,* 1999). Capsid proteins have also been shown to stimulate an anti-adenovirus cellular immune response (Yang *et al.,* 1995). The action of CD4 helper cells is essential in the immune response to adenoviruses and their exclusion was found to prevent responses by CTLs and B cells, however, CD4 cells alone are insufficient in the clearance of adenovirus infected cells.

Neutralising antibodies are also elicited in response to capsid proteins, preventing re-infection by that serotype. Detailed immunological testing in HAd pre-immunised lung cancer patients showed that anti-fibre antibodies emerged first followed by anti-penton base and then anti-hexon antibodies (Gahery-Segard *et al.,* 1998). Anti-fibre antibodies were found to have no neutralising effect on their own, however patients with anti-fibre and anti-penton base antibodies had significant neutralising activity and it was suggested that these antibodies had a synergistic effect. The mechanism suggested for this synergism was an initial uncoating of the adenovirus particles by anti-fibre antibody that then allowed access to the anti-penton base antibodies, and presumably anti-hexon antibodies. High proportions of the human population harbour neutralising antibodies to both HAd2 and HAd5 since they are endemic in the population, however, the data with regards to human adenoviruses neutralisation is conflicting (Horwitz, 1990). When Hoffman *et al.,* analysed 15 serum samples from the USA for anti-HAd5 antibodies they found that they all had neutralising antibodies (Hofman *et al.,* 1999) compared to a second American study that revealed that, although 97% of the population had antibodies to HAd5 capsid proteins, serum from only 55% of the subjects actually neutralised HAd5 infection *in vitro* (Chirmule *et al.,* 1999). Similar to the results found by Chirmule *et al.,* a French group found that 52% of human samples had neutralising antibodies to HAd5, although this was investigated

using an E1 deleted vector (Kremer *et al.,* 2000). It is difficult to compare the published results as the experiments were carried out using different criteria and the materials and methods are often incomplete in the published papers.

This complex immune response to adenoviruses is the main limiting factor in the development of adenovirus based gene therapy vectors.

5.1.1 Sequential Administration of Adenovirus Vectors

It has been proposed that the problems posed to gene therapy by the endemic presence of the common HAds, and the immune response to HAds, may be overcome by the sequential administration of vectors based on non-cross-reactive adenovirus serotypes. Baboons that were administered with an E1 deleted, hAAT expressing HAd5, followed by the equivalent HAd2, after expression from the HAd5 vector had been extinguished, showed no increase in the rate of elimination of transduced cells. This indicated that the HAd2 vector was able to evade the humoral response mounted against the HAd5 vector (Morral *et al* 1999). The sequential delivery of helper dependent HAds based on non-cross-reactive serotypes has also been demonstrated (Parks *et al* 1999). The immune responses elicited against a hdHAd2 were unable to neutralise a hdHAd5, whereas repeated treatment with the same hdHAd2 reduced transgene expression by up to 100-fold. Non-cross-reactive serotypes might also include both chimeric and non-human adenoviruses. There is less chance of a pre-existing immune response to animal adenoviruses in humans and in recent years this realisation has encouraged several research groups to investigate adenoviruses that infect different species as human gene therapy vectors. The sequential delivery of a range of attenuated vectors of different serotypes should provide an adenovirus based gene therapy protocol that allows extended expression of transgenes in patients.

Moffatt *et al.,* investigated cross-reactivity between BAV-3, PAV-3 and HAd5 vectors (Moffatt *et al.,* 2000). Mice immunised with BAV-3 and/or PAV-3 demonstrated over 5000-fold transgene (β-gal) expression from a HAd5 based vector compared with mice immunised with HAd5 (Moffatt *et al.,* 2000). Cross-

neutralisation assays with sera from mice immunised either intranasally or intraperitoneally with BAV-3 and/or PAV-3 or HAd5 showed at least 30-fold higher antibody titres to homologous viruses compared with heterologous virus. The administration of OAV vectors to HAd5 immunised mice was also successful (Hofmann *et al.*, 1999).

5.1.2 Immune Responses to Animal Adenoviruses in Humans

Immune responses to CAV-2 and OAV have been investigated using human sera. Kremer *et al.*, showed that 98% of subjects had no detectable neutralising antibodies to an E1 deleted GFP expressing CAV-2, compared with 52% exhibiting neutralising antibodies against the E1 deleted GFP expressing HAd5 vector (Kremer *et al.*, 2000). 15 random samples of human sera, which each contained antibodies against HAd5, contained no cross-reactive antibodies against OAV (Hofmann *et al.*, 1999).

5.1.3 Objective

The primary aim of this investigation was to establish whether antibodies present in human sera were capable of neutralising CAV-1. A secondary aim was to establish whether a HAd5 pseudotyped CAV-1 vector (described in Chapter 5) was more sensitive to neutralisation as anti-fibre antibodies can play a role.

In addition, feline sera was analysed for both anti-CAV-1 and anti-HAd5 antibodies with a view to the use of CAV-1 as a vaccine vector in domestic cats (see Chapter 3 for a review of published investigations regarding adenovirus infection in cats).

Anti-HAd5 antibody levels were determined for comparative purposes.

5.2 MATERIALS & METHODS

5.2.1 Adenovirus Neutralisation with Human Sera

50 serum samples from healthy control subjects were obtained from the Leukaemia Research Fund, Department of Veterinary Pathology, University of Glasgow. Neutralisation assays were carried out on these samples to detect neutralising antibodies against CAV-1, HAd5 and pFib(H5)CAV-1 as detailed in section 2.2.4.7.

5.2.2 Adenovirus Neutralisation with Feline Sera

50 random feline serum samples that had been routinely submitted to the Feline Diagnostic Laboratory, Department of Veterinary Pathology, University of Glasgow, were analysed to detect neutralising antibodies against CAV-1 and HAd5 as detailed in section 2.2.4.7.

5.3 RESULTS

5.3.1 Pre-existing Neutralising Antibodies to CAV-1, HAd5 and pFib(H5)CAV-1 in Human Sera

50 samples of human sera were examined for neutralising activity against CAV-1, HAd5 and pFib(H5)CAV-1. 50% of these had a degree of anti-CAV-1 activity, and 74% had a neutralising effect on HAd5 (Table 5.1). However, neutralising antibody titres of less than 64 are considered to be 'low' and only subjects with titres of 64 or over are considered to be immune (based on CAV-1 vaccination trials, unpublished data from Professor H Thomson, Department of Veterinary Pathology, University of Glasgow). Considering this information, only 22% had significant anti-CAV-1 activity compared with 46% anti-HAd5. There appeared to be no relationship between the samples that had anti-CAV-1 activity and those that had anti-HAd5 activity.

74% of the samples demonstrated neutralising activity against pFib(H5)CAV-1, however, only 44% of the samples had neutralising antibody titres over 64. With the exception of one sample, all the serum samples that neutralised CAV-1 also neutralised pFib(H5)CAV-1, whereas only 60% of the samples that neutralised HAd5 neutralised pFib(H5)CAV-1. 13% of the samples that neutralised pFib(H5)CAV-1 had both CAV-1 and HAd5 neutralising activity.

	Titre of Neutralising Antibodies to :				Titre of Neutralising Antibodies to :		
Sample No.	**CAV-1**	**HAd-5**	**PFib(H5) CAV-1**	**Sample No.**	**CAV-1**	**HAd-5**	**PFib(H5) CAV-1**
1	45	256	128	**26**	0	91	32
2	23	32	23	**27**	0	0	0
3	0	0	0	**28**	724	362	512
4	128	181	512	**29**	0	128	64
5	0	128	181	**30**	0	32	0
6	23	0	0	**31**	181	512	512
7	181	32	128	**32**	32	724	512
8	512	16	128	**33**	0	32	0
9	0	128	32	**34**	0	181	91
10	0	181	91	**35**	16	23	23
11	16	128	32	**36**	128	23	64
12	0	128	23	**37**	0	11	0
13	45	32	128	**38**	0	91	128
14	32	45	11	**39**	45	91	32
15	362	0	128	**40**	0	128	45
16	0	0	0	**41**	60	23	23
17	0	181	128	**42**	0	128	181
18	0	362	512	**43**	16	0	0
19	0	0	0	**44**	16	0	0
20	0	128	32	**45**	32	0	11
21	0	91	91	**46**	256	0	128
22	0	0	0	**47**	0	0	0
23	256	32	256	**48**	0	128	256
24	181	16	128	**49**	0	0	0
25	362	32	91	**50**	32	181	32

Table 5.1 Neutralising Antibodies in Human Serum Samples

50 human serum samples were analysed to determine the anti-CAV-1, HAd5 and PFib(H5)CAV-1 neutralising antibodies. Shadowed cells contain antibody titres of 64 or less, only sera with titres over 64 are considered to indicate immunity to the appropriate virus.

5.3.2 Pre-existing Neutralising Antibodies to CAV-1 and HAd5 in Feline Sera

50 random samples of feline sera were analysed for neutralising antibodies against CAV-1 and HAd5. With the exception of samples 12, 26 and 35, none of the samples had detectable neutralising antibodies against CAV-1 or Had5. Samples 12 and 35 had neutralising titres of 23 and 91, respectively, against HAd5. Sample 26 had a neutralising antibody titre of 23 against CAV-1. However, only animals with a titre of 64 or over are considered immune (based on unpublished data from Professor H Thomson, Department of Veterinary Pathology, University of Glasgow) and as such only one sample demonstrated the presence of any significant levels of neutralising antibodies and these were directed against HAd5.

5.4 DISCUSSION

A high proportion of the population have neutralising antibodies to HAds 2 and 5, which could limit their use as gene therapy vectors. The use of less ubiquitous HAds and animal-derived adenoviruses may offer a solution to this problem. These adenoviruses are antigenically distinct from HAds 2 and 5 and exposure to them is limited, therefore humans are less likely to have immunity to them. There is variation between the published results regarding percentages of the population that have neutralising antibodies against the most ubiquitous of the human adenoviruses, type 5 (section 5.1). Analysis of 50 samples of human sera revealed the presence of neutralising antibodies against HAd5 at significant levels in only 46% of the samples. Only 22% of the same samples had significant levels of neutralising antibodies against CAV-1.

Antibodies to CAV-1 and CAV-2 have been shown to be cross-reactive (Koptopoulos & Cornwell, 1981, unpublished data). Kremer *et al.*, reported that 98% of subjects had no detectable neutralising antibodies to CAV-2 (Kremer *et al.*, 2000). As such it might have been expected that a lower proportion of the sera tested in the presented study would have neutralising antibodies against CAV-1. However, the experimental protocol in Kremer's experiment was based on GFP expression from a partially E1 attenuated CAV-2 vector administered at high titres to human 911 cells. In essence, Kremer *et al.* investigated how CAV-2 vectors may be blocked by neutralising antibodies in a gene therapy setting. The neutralisation of CAV-1 demonstrated may not have been achieved by CAV-1 specific neutralising antibodies, for example, anti-HAd8 antibodies have previously been shown to neutralise CAV-1 (Smith *et al.*, 1970). It is also unknown whether the level of CAV-1 neutralisation shown here would affect vector efficacy *in vivo*. Further immunological investigations of human sera would provide more accurate information regarding CAV-1 neutralisation, for example, binding assays with the CAV-1 capsid proteins should determine whether the immune response in the tested sera is directed to specific proteins in these samples. Further work to determine the cross-neutralisation of CAV-1 by antibodies of other adenoviruses is also necessary.

The fibre recombinant pFib(H5)CAV-1 was neutralised by almost the same percentage of serum samples as HAd5. All but one of the samples that neutralised CAV-1 were shown to neutralise pFib(H5)CAV-1 whereas only 12 of the 22 samples that had anti-HAd5 neutralising activity neutralised pFib(H5)CAV-1. This is to be expected as the hexon has been shown to be a major immunogen of adenoviruses (Gahery-Segard *et al.,* 1998), although these results also confirm that fibre immunity is relevant to adenovirus neutralisation *in vitro.* Although anti-fibre antibodies are involved in neutralisation in culture (Wolfhart, 1988) there have been reports of their ineffectiveness *in vivo* (Gall *et al.,* 1996). It has been shown in humans that anti-fibre antibodies are not sufficient to neutralise adenovirus on their own, and that they require the presence of anti-penton antibodies (Gahery-Segard *et al.,* 1998). The hypothesis is that adenovirus particles are initially uncoated by anti-fibre antibodies and this uncoating allow access to anti-penton antibodies, however, only 2 of the anti-HAd5 antibody containing samples that neutralised pFib(H5)CAV-1 also contained significant levels of anti-CAV-1 antibodies. As such, *in vivo* immunological analyses of the pre-existing immunity of target patients would be necessary prior to the administration of any fibre recombinant vectors.

Another future field of investigation should be the determination of p.f.u. to particle ratios in each of the viruses investigated. It is possible that stocks of pFib(H5)CAV-1 contain a higher proportion of defective particles (discussed in more detail in Chapter 4). If this was the case, these particles would affect the titres returned by the neutralisation assay.

In conclusion, there is evidence that CAV-1 vectors are less likely to be neutralised than HAd5 vectors in patients. Further studies would determine whether the level of CAV-1 neutralisation demonstrated here would be significant in a gene therapy setting.

It was expected that there would be little pre-existing immunity to CAV-1 found in the cat population, as it has no reservoir in the domestic canine community (see Chapter 1). It is therefore necessary to investigate the pre-existing immune response to CAV-1 in the feline population prior to the development of a CAV-1 based vaccine. Of 50 cats investigated, none had significant levels of neutralising antibodies

against CAV-1 and only one cat had immunity to HAd5. In chapter 3 it was demonstrated that CAV-1 infects feline cells, DNA replication and transcription occurs, and a productive infection is established. Further to this, transgene expression was demonstrated in both feline cell-lines investigated using a GFP expressing CAV-1 based vector. These features of CAV-1, in conjunction with the lack of CAV-1 neutralisation by feline sera demonstrated here, suggest that it is a worthwhile candidate for development as a vaccine vector for use in cats.

CHAPTER 6

General Discussion

Much of the development of adenoviruses as vectors has focused on the common HAds 2 and 5, mainly because there is so much known about these serotypes as they have been used as models for the elucidation of many eukaryotic cellular processes including DNA replication and transcription. However the development and application of these vectors has highlighted certain limitations including pre-existing immunity to these serotypes and a wide tropism hampering specific tissue targeting.

This work has investigated the suitability of CAV-1 as an alternative vector to HAd 2 and 5 for the delivery of transgenes to cats and humans, for the purposes of vaccination and gene therapy. The essential criteria for any such vector include the following :

1. It must enter cells of the target host.
2. It must express transgenes within these cells.
3. It must be capable of targeting transgene expression to certain cells.
4. There must be low pre-existing immunity to the vector within the target host.

CAV-1 had been demonstrated to infect cells of heterologous host species (pig, racoon, ferret) (Emery & York, 1958, Bolin *et al.*, 1958, Fieldsteel & Yoshihara, 1957, reviewed in Koptopoulos & Cornwell, 1981). In the studies presented here more sensitive molecular techniques were used to investigate CAV-1 infection of both feline and human cell lines, to explore modification of tropism and to determine levels of neutralising antibodies to CAV-1 within feline and human serum.

Only circumstantial evidence for adenovirus infection of cats has been published and although CAV-1 infection of feline cells is productive *in vitro*, the results in Chapter 3 demonstrate that there are no neutralising antibodies against CAV-1 in feline sera. There is clearly a block to natural CAV-1 infection in cats. At present in the UK, the routine vaccination of dogs with live CAV-2 may indirectly prevent CAV-1 infection of cats, however, adenovirus infection of cats was not documented before vaccination became commonplace. Wild type CAV-1 infection in the canine host begins in the respiratory epithelium. Viral replication leads to the release of progeny virus into the blood stream, which infect cells of the vascular endothelium and a range of organs. The feline cells investigated here were of kidney and embryonic fibroblast origin.

Productive infection may not occur in other feline cells, for example, if CAV-1 infection of feline respiratory epithelium was non-productive the spread of CAV-1 to permissive cells would be prevented. As such further investigations of wild-type CAV-1 infection in cats via natural routes would be necessary to determine suitable routes of administration of a CAV-1 based vaccine.

CAV-1 infection of human cells was studied in cells derived from tissues that one might expect to be infected by CAV-1 i.e. lung epithelium and kidney cells. However, despite entering these cells, the CAV-1 infectious cycle was incomplete. Infection of A549 cells was semi-productive, however, no infectious virus was generated in 293 cells. The block(s) to CAV-1 replication clearly occur at different stages of the virus life cycle in each of these cell-lines, and it is tempting to speculate that the block in 293 cells is at or before the packaging stage, while in A549 cells it might be that virus release from or lysis of cells is inefficient. Nevertheless, transgene expression was evident from ΔE3-GFP-CAV-1 in both cell-lines.

Like HAds, the application of CAV-1 for human gene therapy would require targeting of vectors. The CAV-1 genome was able to accommodate fibre genes from both CAV-2 and HAd5 and preliminary investigations suggested that the pseudotyping of CAV-1 altered its native tropism. The available vector system will allow the insertion of fibre genes from a catalogue of HAd5 fibre constructs, with known receptor binding capabilities, which could be used in conjunction with replication deficient CAV-1 vectors to target specific cell types.

An alternative to using heterologous adenovirus fibre constructs would be to modify the fibre of CAV-1, which would also be aided by the vector system described here. Inserting peptides with known receptor affinities into the receptor binding site of the fibre may confer tissue specificity onto the CAV-1 vector. In order to make such modifications, it will be necessary to establish the sites within the CAV-1 fibre that tolerate peptide insertion and permit effective expression of ligand binding properties of the peptide. The effect of such insertions on primary receptor binding would have to be established, for example, if native CAR binding is retained studies will be required to determine how native tropism might be ablated through further knob mutagenesis.

Assuming that the modification of the CAV-1 fibre would allow the binding to specific receptors, the nature of CAV-1 penton interactions with cells must still be determined. The penton plays a critical role in both internalisation of adenoviruses and the efficient lysis of cells following virus replication and packaging into new particles. The results in Chapter 4 suggest that both of these events are diminished in human cells. Understanding the role of the penton in CAV-1 infection will therefore be important in the development of it as a vector. The penton can mediate infection in HAds with normal fibre binding ablated (Mizuguchi *et al.*, 2002). It has also been shown that fibre-less HAd particles can be recovered that are still infectious (albeit it at a much lower efficiency) with cell entry achieved through penton interactions with cellular integrins (Von Seggern *et al.*, 1999). These interactions are mediated through an RGD motif in the penton, however the CAV-1 penton lacks such a motif and as a result CAV-1 may employ a different strategy for internalisation. As it has been demonstrated that the penton contributes to the wide tissue tropism of HAd 2 and 5 (due to widespread integrin expression), it will be important to determine if this is the case for CAV-1 based vectors. Future investigations with labelled fibre-less CAV-1 will be important to determine the role of the penton base in viral infectivity.

Although it was demonstrated that CAV-1 can enter and express transgenes in cells of human origin, and has the potential to be targeted, this would be of little use if the vector encountered neutralising antibodies upon administration. Whereas 46% of human serum samples neutralised HAd5, only 22% of the same samples neutralised CAV-1. The hexon is the major adenovirus immunogen, and as such it was hypothesised that the immune profile of the HAd5 pseudotyped vector would be similar to that of CAV-1. All but one of the anti-CAV-1 samples neutralised the pseudotyped vector, however, a further 24% of samples also neutralised this vector. These findings indicate that anti-fibre antibodies may be a limiting factor in the use CAV-1 vectors pseudotyped with HAd fibres, although studies have shown that antibodies specific to fibre proteins are unable to neutralise HAds *in vivo*. As such *in vivo* immunological studies would be necessary to determine whether anti-HAd5 fibre antibodies are significant to prevent CAV-1 based vector activity. In addition, it is likely that the neutralisation of this vector would be reduced in a gene therapy setting as the assay used here was extremely sensitive. The use of CAV-1 vectors that

maintain the majority of the CAV-1 fibre would further reduce the likelihood of vector extinction by anti-HAd5 fibre antibodies.

In conclusion, CAV-1 is an excellent candidate for development as vector for use in both cats and humans. However for vaccination in cats it will be important to determine if CAV-1 can use natural routes for infection. For gene therapy purposes, which are presented here primarily from the perspective of human use but could equally apply to use in cats, future work on secondary interactions of the CAV-1 penton and target cells will be necessary to determine whether this has to be addressed as far as targeting is concerned. Finally, the studies described here, although directed towards feline and human cells, have broader implications for CAV-1 vector use in other species.

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