Development of Packaging Cell Lines For Rescuing BAV2 Viral Vectors

by

Mohamad S. Salamé, B.Sc.
Brock University

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IV. Abstract

The construction of adenovirus vectors for cloning and foreign gene expression requires packaging cell lines that can complement missing viral functions caused by sequence deletions and/or replacement with foreign DNA sequences. In this study, packaging cell lines were designed to provide *in trans* the missing bovine adenovirus functions, so that recombinant viruses could be generated.

Fetal bovine kidney and lung cells, acquired at the trimester term from a pregnant cow, were tranfected with both digested wild type BAV2 genomic DNA and pCMV-E1. The plasmid pCMV-E1 was specifically constructed to express E1 of BAV2 under the control of the cytomegalovirus enhancer/promoter (CMV). Selection for "true" transformants by continuous passaging showed no success in isolating immortalised cells, since the cells underwent crisis resulting in complete cell death. Moreover, selection for G418 resistance, using the same cells, also did not result in the isolation of an immortalised cell line and the same culture-collapse event was observed.

The lack of success in establishing an immortalised cell line from fetal tissue prompted us to transfect a pre-established cell line. We began by transfecting MDBK (Mardin-Dardy bovine kidney) cells with pCMV-E1-*neo*, which contain the bacterial selectable marker *neo* gene. A series of MDBK-derived cell lines, that constitutively express bovine adenoviral (BAV) early region 1 (E1), were then isolated. Cells selected for resistance to the drug G418 were isolated collectively for full characterisation to assess their suitability as packaging cell lines. Individual colonies were isolated by limiting dilution and further tested for E1 expression and efficiency of DNA uptake. Two cell lines, L-23 and L-24, out of 48 generated foci tested positive for E1 expression using Northern Blot analysis. DNA uptake studies, using both lipofectamine and calcium phosphate methods, were performed to compare these cells, their parental MDBK cells,

and the unrelated human 293 cells as a benchmark. The results revealed that the new MDBK-derived clones were no more efficient than MDBK cells in the transient expression of transfected DNA and that they were inferior to 293 cells, when using *lacZ* as the reporter gene.

In view of the inherently poor transfection efficiency of MDBK cells and their derivatives, a number of other bovine cells were investigated for their potential as packaging cells. The cell line CCL40 was chosen for its high efficiency in DNA uptake and subsequently transfected with the plasmid vector pCMV E1-*neo*. By selection with the drug G418, two cell lines were isolated, ProCell 1 and ProCell 2. These cell lines were tested for E1 expression, permissivity to BAV2 and DNA uptake efficiency, revealing a DNA uptake efficiency of 37 % , comparable to that of CCL40.

Attempts to rescue BAV2 mutants carrying the *lacZ* gene in place of E1 or E3 were carried out by co-transfecting wild type viral DNA with either the plasmid pdlE1E-Z (which contains BAV2 sequences from 0% to 40.4% with the *lacZ* gene in place of the E1 region from 1.1% to 8.25%) or with the plasmid pdlE3-5-Z (which contains BAV2 sequences from 64.8% to 100% with the *lacZ* gene in place of the E3 region from 75.8% to 81.4%). These co-transfections did not result in the generation of a viral mutant. The lack of mutant generation was thought to be caused by the relative inefficiency of DNA uptake.

Consequently, cosBAV2, a cosmid vector carrying the BAV2 genome, was modified to carry the *neo* reporter gene in place of the E3 region from 75.8% to 81.4%. The use of a single cosmid vector carring the whole genome would eliminate the need for homologous recombination in order to generate a viral vector. Unfortunately, the transfection of cosBAV2-*neo* also did not result in the generation of a viral mutant. This may have been caused by the size of the E3 deletion, where excess sequences that are essential to the virus' survival might have been deleted. As an extension to this study, the spontaneous E3 deletion, accidently discovered in our viral stock, could be used as site of foreign gene insertion.

V. Acknowledgments

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VI. Literature Review

A. Adenoviruses

Adenovirus (Ad) was first isolated by two independent groups of investigators, Rowe and co-workers in 1953 and Hilleman and Werner in 1954 (reviewed in Fields *et al.*, 1996). Both groups observed the spontaneous degeneration of primary cell cultures established from human adenoid tissue that was removed by tonsillectomy from military recruits with febrile illness. This pathogenic element was shown to be a virus that was not previously identified and it was given several names at first: adenoid degeneration, respiratory illness, adenoidal-pharyngeal-conjunctival and acute respiratory disease agents. Epidemiological studies confirmed that the agents were the cause of acute febrile respiratory syndromes and in 1956 they were named adenoviruses (reviewed in Fields *et al.*, 1996).

In 1962, Trentin and co-workers made a shocking discovery. They showed that newborn hamsters developed tumours upon inoculation with the human adenovirus type 12 (Ad12) (Trentin *et al.*, 1962). However, since then, all studies have failed to detect the presence of adenoviral DNA in human tumours or to link adenoviruses to human malignant diseases.

Currently there are over 100 members of the Ad group which have been identified and isolated from a variety of species. Although interest in these viruses originally centered on their properties as tumour viruses, an important discovery was made revealing mRNA splicing and the presence of introns (Berk *et al.*, 1977; and Chow *et al.*, 1977). The interest in studying Ad pathogenicity continued and with the development of recombinant DNA techniques, adenoviruses became of great interest as viral vectors for gene therapy and viral vaccines.

1. Epidemiology

Adenoviruses are widespread in nature. They infect birds, mammals and humans (Table 1). Most of the infections are subclinical. Although these viruses have a higher tendency to infect the respiratory and gastrointestinal tract, they were also shown to infect the bladder, the eyes, and the central nervous system (very rare) (reviewed in Fields *et al.*, 1996).

Table 1: Diseases caused by human Ads.

Groups at risk and diseases caused by human Ads (compiled from Ford *et al.*, 1993; Grimwood *et al.*, 1995; and Hitt *et al.*, 1997)

Disease:	Groups at Risk:
Acute Respiratory Illness	Military recruits, boarding schools, etc.
Pharyngitis	Infants
Gastroenteritis	Infants
Conjunctivitis	All
Pneumonia	Infants, military recruits
Keratoconjunctivitis	All
Acute Haemorrhagic Cystitis	Infants
Hepatitis	Infants, liver transplant patients

2. Classification

Adenoviruses are non-enveloped double-stranded DNA viruses. The family *Adenoviridae* is divided into the genera *Mastadenovirus* and *Aviadenovirus* (reviewed in Fields *et al.*, 1996). The *Mastadenovirus* genus includes human, simian, bovine, equine, porcine, ovine, canine, caprine, murine, and tree shrew viruses, while the *Aviadenovirus* genus includes five groups of viruses that infect birds (reviewed in Fields *et al.*, 1996). Thus far, 49 human Ad serotypes have been classified into six subgroups according to their haemagglutination properties and their resistance to neutralisation by antiserum to known Ads (Hierholzer *et al.*, 1988).

Table 2: Ad Classification.

Ad serotypes classified into subgroups based on oncogenicity potential and hemagglutination (Wadell, 1994)

			Hemagglutination		
Sub-Group	Serotypes	Oncogenic Potential	Rhesus	Rat	GC (%)
A	12, 18, 31	High	-	+/-	48-49
B	3, 7, 11, 14, 16, 21, 34, 35	Weak	+	-	50-52
C	1, 2, 5, 6	None	-	+/-	57-59
D	8, 9, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-47	None	+/-	+	57-61
E	4	None	-	+/-	57-59
F-G	40, 41	Unknown	-	+/-	

A recent study by Dan *et al.* (1998) revealed the possible need for a third genus in the *Adenoviridae* family (Figure 1). The proposed name of the new genus was *Atadenovirus* (A-T rich adenoviruses). This members of the new proposed genus are BAV4 to 8, EDS (Duck Ad1) and OAV287 (Ovine Ad isolate number 287). The reason for this proposition is that these members behave differently from the rest of the *Mastadenovirus* genus and that there is high homology of DNA sequence between them. These viruses have 60% A-T rich coding regions, compared to the 42% to 52 % in other *Mastadenovirus* members. They show some degree of heat resistance, provoke inclusion bodies that are structurally different from the inclusion bodies caused by other mastadenoviruses and fail complement fixation that is common to all members of *Mastadenovirus*. Members of the proposed *Atadenovirus* genus can be only propagated in testicular or thyroid cell cultures, however other members of the *Mastadenovirus* genus can be propagated in kidney and testicular cell cultures. Also, protease and hexon sequences were aligned with sequences from other viruses in the *Mastadenovirus* genus to further show a distinctive cluster containing BAV4, OAV287 and EDS.

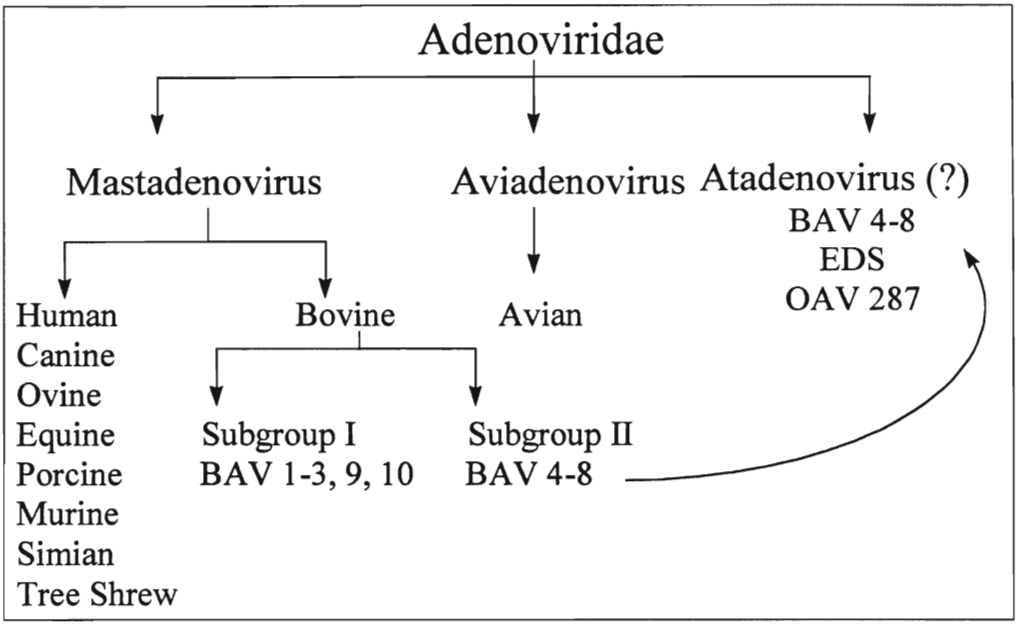


Figure 1: Ad classification.

The figure shows the two genera previously assigned, *Mastadenovirus* and *Aviadenovirus*, plus the new proposed genus, *Atadenovirus*, based on a study by Dan *et al.* (1998).

3. Structure

Adenoviruses are non-enveloped viruses, icosahedral in shape with a diameter of 70-100 nm (Horne *et al.*, 1959). The viral particles contain 13% DNA and 87% protein by weight (Ishibashi *et al.*, 1974).

a) Capsid and Core Protein Organisation

The protein capsid of the virus is composed of 252 capsomeres which include 240 hexons and twelve pentons (Figure 2). Pentons contain a base (part of the capsid) and a projecting fibre protein. The outer capsid is made of seven polypeptides (II, III, IIIa, IV, VI, VIII, IX), while the core structure is made of 4 proteins (V, VII, X, and TP) and the DNA genome (reviewed in Fields *et al.*, 1996)

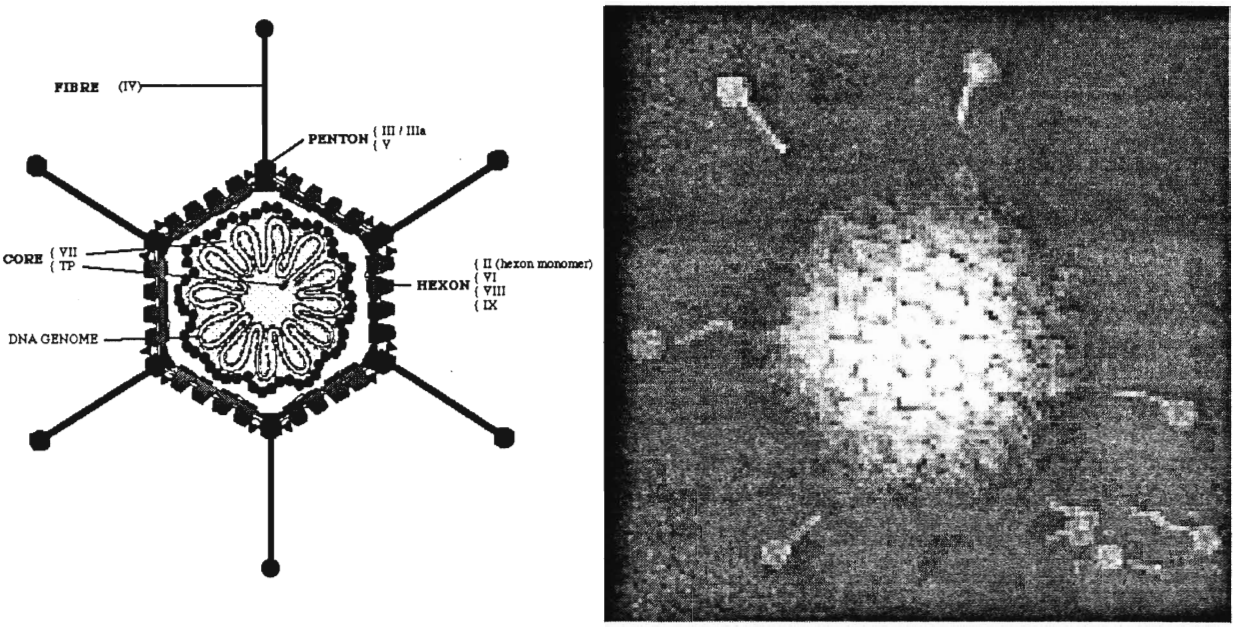


Figure 2: Structure of Ads.

(left) A schematic representation of an Ad showing the fibre protein projections and the viral capsid. The capsid consists of the hexon and penton proteins. Inside the capsid, the figure shows the coiled genome with the terminal proteins bound to its 5′ ends (Stewart *et al.,* 1991). (Right) An x-ray photograph of an Ad showing the capsid and the dissociated fibre proteins (Joklik, 1988).

b) Genome Structure

Adenoviruses have linear, non-segmented, double-stranded DNA, 30 kbp to 44 kbp in length (size differs among groups), with short inverted terminal repeats flanking the coding region. Genome structure (cross-hybridization, restriction map) is one of the characteristics used to assign viruses to groups (70% to 95% homology within groups, 50% to 60% homology between groups). The left and right terminal sequences are short inverted repeats (100 to 140 bp) which play a role in DNA replication. There is a 55kd protein covalently attached to the 5' end of each strand (reviewed in Fields *et al.*, 1996).

The viral genome has been divided into early and late units (Figure 3), with four early units (E1A, E1B, E2A, E2B, E3, and E4) and five late units of mRNAs (L1 to L5). The genes transcribed give rise to multiple RNAs resulting from differential splicing and sometimes from the use of different poly(A) sites (Berget *et al.*, 1977). Structural polypeptides with their proposed functions are listed in Table 2.

Table 3: Ad polypeptides.

List of the known Ad polypeptides, their size and possible functions. These functions were suggested based on studies done on Ad2 and Ad5 (compiled from Fields *et al.*, 1996).

Polypeptide	Size	Function
Polypeptide II	967 amino acids	Associated with the outer capsid; three polypeptide II form one hexon protein
Polypeptide III	571 amino acids	Associated with the outer capsid; five proteins associate to form the penton base protein; plays a role in penetration of the virus.
Polypeptide IIIa	566 amino acids	Associated with the penton base; plays a role in penetration; links hexons to protein VII of the core
Polypeptide IV	582 amino acids	Associated with the outer capsid; forms the trimeric fibre protein; plays a role in receptor binding and haemagglutination
Polypeptide V	368 amino acids	Core protein; associated with DNA and penton base; packaging?
Polypeptide VI	217 amino acids	Associated with the outer capsid; forms a bridge between the capsid and the core structure; associates with and stabilises hexon protein; assembly of viral particle?
Polypeptide VII	174 amino acids	Core protein; serves as a histone-like moleclule for DNA packaging
Polypeptide VIII	134 amino acids	Associated with the outer capsid; forms a bridge between the capsid and the core structure; associates with and stabilises hexon protein; assembly of viral particles?
Polypeptide IX	139 amino acids	Associated with the outer capsid; associates with and stabilises hexon protein
Terminal Protein	55 Kd	Core Protein; covalently bound to the 5′ end of the viral DNA; serves as a primer for DNA replication and serves in attachment of the viral genome to the nuclear matrix
polypeptide mu	19 amino acids	Core protein; Unknown function

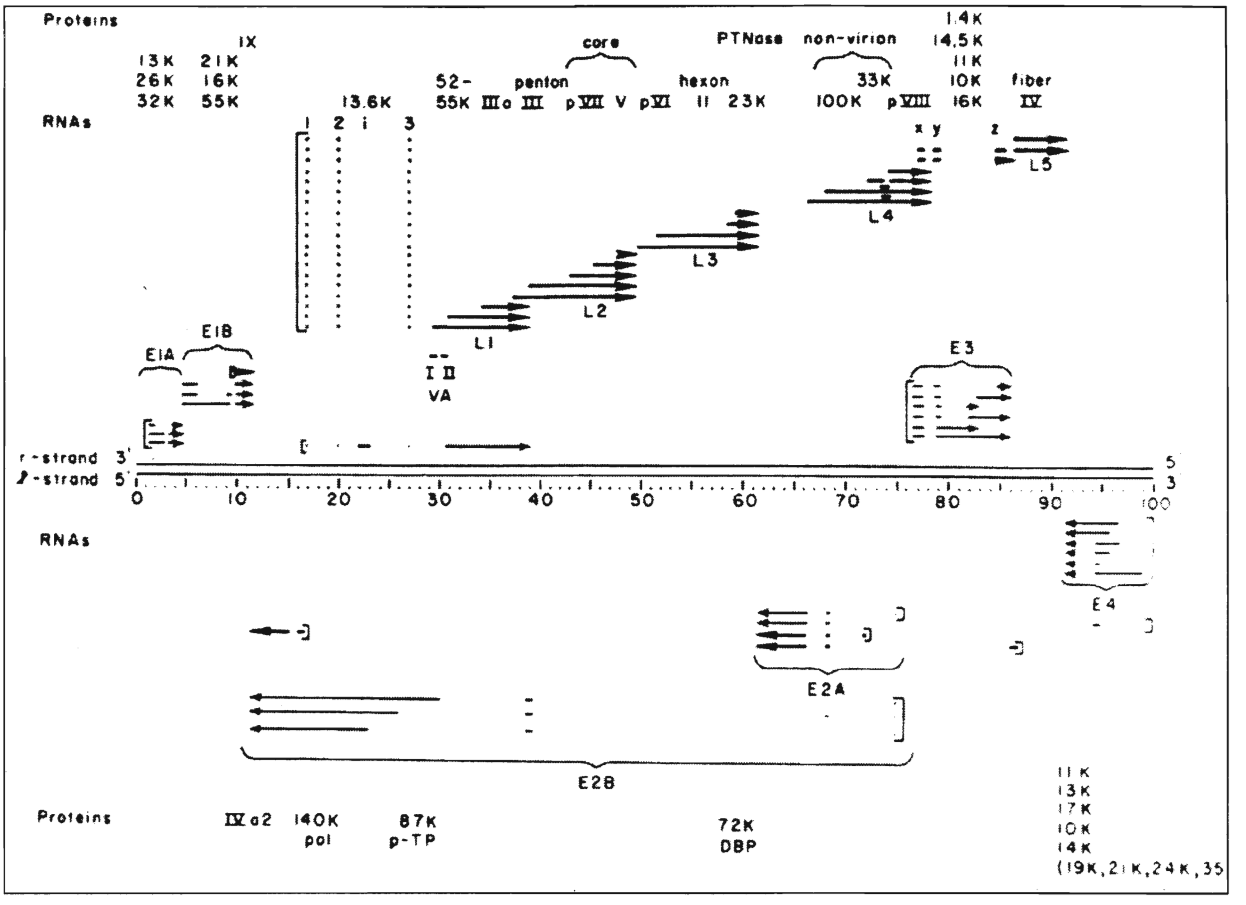


Figure 3: Diagrammatic representation of Ad2 transcription and translation.
Early transcription regions are labelled E1 through E4 and late transcription regions are labelled L1 through L5 (reviewed in Fields *et al.*, 1996).

B. Life Cycle

The life cycle of adenoviruses starts with attachment to the host's cellular receptor, penetration, transport to the nucleus, and finally DNA transcription (reviewed in Pettersson and Roberts, 1986). Transcription of adenoviruses has been assigned to two sections, early and late, which are divided by the start of DNA replication. The early stage immediately follows adsorption of the virus to the cell membrane and penetration, and includes transcription and translation of the four early regions. The onset of DNA replication is accompanied by the initiation of the late gene transcription. Once all the structural proteins are produced, viral assembly begins and, within 36 hours post-infection, cell lysis occurs (Shenk, 1996). The viral progeny repeat the cycle by infecting neighbouring cells.

C. Adsorption and Entry

The "knob" bound to the end of the Ad's fibre protein is thought to mediate viral attachment to the host cell since soluble fibre proteins have been shown to inhibit viral attachment (Defer *et al.,* 1990). The cellular receptor still remains unknown except for Ad2 and Ad5 where the cellular receptor CAR (Coxsakie Ad Receptor) used by the Coxsakie B viruses was shown to be also used by Ad2 and Ad5 (Bergelson *et al.*, 1997). Furthermore, the fact that different serotypes differ considerably in their knob proteins' amino acid sequence and length seems to indicate that different serotypes attach to different receptors on the cell membrane (Kidd *et al.*, 1993). Internalisation of the virus is mediated by a cellular receptor, integrin, which interacts with the RGD (Argenine-Glycine-Aspartate) amino acid conserved sequence on polypeptide III of the penton base (Wickham *et al.,* 1993). Following adsorption, the virus is internalised through receptor-mediated endocytosis (Varga *et al.,* 1991). Adsorption and internalisation is a two-step process, where both interactions of the knob protein and the penton base are required for successful viral internalisation.

Internalisation of adenoviruses is very efficient, where about 85% of the adsorbed viruses are internalised. The viruses contained within the endosomes, then move to the cytosol. The endosomal pH seems to play a role in the viral movement to the cytosol before the formation of a lysosome (Mellman, 1992). Forty-five minutes post-infection, the viral genome, free of hexon proteins, is transferred through the nuclear membrane. Even defective Ad viral particles are able to facilitate non-viral macromolecules into mammalian cell lines through receptor-mediated endocytosis (reviewed in Hitt *et al.*, 1997).

The capsid and the core proteins are dissociated in an orderly manner, where the first proteins to dissociate are the fibre and the penton base; after penetration to the cytoplasm, the virus loses proteins VI and VIII, which provide a bridge between the core and the capsid (Greber *et al.*, 1993). Soon after, the hexons and pIX are destabilised. The DNA binding proteins, V and

VII, are thought to be retained during genome entry into the nucleus, which occurs through nuclear pores (Fredman and Engler, 1993).

D. Transcription

Ad transcription, which occurs within the nucleus of the host cell, has been divided into two phases, early and late (Table 4), and they are separated by the onset of DNA replication. The products of the early regions control the cell cycle, block apoptosis, control gene expression and DNA replication and antagonise the host's immune response to destroy infected cells. The early phase takes between five to six hours in Hela cells (reviewed in Petterson and Roberts, 1986), after which DNA replication is initiated and expression of the late gene can be detected. This is followed by the assembly of progeny virions. The whole cycle takes between 20 to 24 hours for completion, with cell death occuring 30 to 40 hours post infection (Douglas *et al.*, 1997).

Although the early genes are expressed in the "early" stage of infection, they continue to be expressed throughout the "late" stage of the virus life cycle. There is also a low level of expression from the major late promoter soon after infection. A delayed early stage has been suggested for the proteins IVa2 and IX, which begin to be expressed at a stage following early gene expression, but before the initiation of late gene expression (Pettersson *et al.*, 1986).

Table 4: List of known Ad5 transcribed regions.

The stage and direction of transcription of early and late regions are listed (Doerfler, 1996).

Region	Transcription Stage	Transcription Direction
L1	Immediately Early	Rightward direction
E1A	Early	Rightward direction
E1B, E3	Delayed Early	Rightward direction
E2A, E2B, E4	Delayed Early	Leftward direction
IVa2, IX	Intermediate	Rightward direction
L2-L5	Late	Rightward direction

1. Early Transcription

a) E1A

The earliest transcription of the E1 region occurs about one hour post-infection and encondes at least five mRNAs, two expressed in the early stage of transcription and and three in the late stage. The early E1A products, 13S (289 amimo acids) and 12S (243 amino acids) (reviewed in Shenk, 1996), are produced from the same mRNA through differential splicing (Harlow *et al.*, 1985). These proteins play a wide range of roles during infection, such as induction of DNA synthesis, induction of mitosis (transformation), and *trans*-activation of viral genes. E1A controls E1B, E2, E3, and E4 mRNA accumulation. However, it cannot itself bind DNA; instead, it seems to activate cellular proteins which bind to viral sequences within the early region promoters.

In vitro, E1A proteins co-operate with E1B proteins to immortalise primary cells (reviewed in Fields *et al.*, 1996). E1A expression, on its own, is not sufficient to immortalise primary cells. E1A products induce apoptosis by stabilising p53 protein and causing it to accumulate inside the nucleus. The high levels of p53 protein inside the nucleus prevent cell cycle progression and cause cell death (Donehower *et al.*, 1992). The specific mechanism by which p53 causes apoptosis is not well understood.

E1A 12S and 13S mRNA contain 3 highly conserved regions among different adenoviruses, namely CR1 to 3. These products have been shown to interact with a cellular protein of the p300 family (Cook *et al.,* 1996). This "p300 family" of proteins interacts with a variety of enhancer-binding transcription factors. E1A appears to act by linking these factors to components of the transcription machinery, therefore promoting DNA transcription.

b) E1B

Early region 1B codes for two proteins: a 19kd and a 55kd protein. The 19kd product is needed for preventing cellular apoptosis, since it inhibits p53 induced cell death and co-operates with E1A. The cellular phosphoprotein p53 is a sequence-specific DNA binding protein and a transcription factor. P53 is capable of both *trans*-activating and repressing cellular DNA transcription. It can also suppress oncogenic transformation, negatively regulate cell cycle progression and induce apoptosis (Vogelstein and Kinzler; 1992). All of these roles have been shown to participate in the tumour suppressor function of p53.

E1B has also been shown to co-operate with E1A products to oncogenically transform primary cells *in vitro* (Finlay *et al.*, 1989). E1A and E1B are both required for full transformation and tumour formation caused by Ad12 in rodents. The E1B 55kd product promotes the cell to go into the S phase of the cell cycle, which enhances viral DNA replication. Transformation is accomplished by inhibiting E1A-induced p53-dependent apoptosis. The 55kd protein of E1B binds to the *trans*-activation domain at the N-terminal of p53 and prevents its transcription activation property (Sabbatini *et al.*, 1995) . Mutant analysis of the E1B 55kd protein revealed that binding affinity to p53 correlates with the ability of E1B to transform primary cells in culture. In a study by Bishchoff *et al.* (1996), an Ad deficient in the E1B region was able to selectively replicate in p53-deficient human tumour cells. Therefore, the virus was able to grow in these cells and cause their lysis.

c) E2

E2 codes for proteins needed for DNA replication and packaging. E2A codes for the 72 kd DNA-binding protein (DBP), a histone-like protein, thought to play a role in DNA packaging. E2B codes for the 140kd DNA polymerase (Ad-pol) and the 80 kd terminal protein precursor (pTP) (reviewed in Hitt *et al.*, 1997). E2 transcription switches between two promoters, "early" and "late", where the "early" promoter is used in the early phase of transcription and the "late" promoter is used during the late phase of transcription. The reason for the switch between promoters remains to be identified (reviewed in Swaminathan and Thimppaya, 1995).

d) E3

E3 gene products interfere with immuno-surveillance of the host's immune system. E3 codes for 9 overlapping mRNA's that are generated by differential splicing and the use of different poly-A sites (reviewed in Hitt *et al.*, 1997). Eight proteins have been identified. Two proteins, the 19kd and 14.7 kd proteins, have been studied in more detail. The 19kd protein is one of the most abundant early proteins and consists of 142 amino acids. It interacts with and prevents class I major histocompatibility complex (MHC I) from leaving the rough endoplasmic reticulum, thereby preventing infecting cells from presenting viral antigens on the cell surface (Deryckere *et al.*, 1995). This process prevents Tc cytotoxic cells from recognising infected cells and consequently they never initiate MHC-dependent cell lysis. In contrast, the 14.7 kd protein protects infected cells from cytolysis triggered by tumour necrosis factor α (TNFα).

The interaction between MHC class I and the E3 19kd is not covalent and does not involve disulfide bonds or carbohydrates; however, the internal disulfide bonds present in the E3 19kd protein are necessary for binding (Sparer and Gooding, 1998). This protein is able to bind to a wide range of alleles in human and mouse hosts with differing affinities (Cox *et al.*, 1990). The initial speculation was that the 19kd protein binds to one of the conserved regions of MHC

class I proteins; however, the binding regions were mapped to α_1-and α_2-helices of the variable domain in MHC class I proteins (Beier *et al.*, 1994). E3 products are not required by the virus to grow in cell culture.

e) E4

The E4 transcription region is less characterised than the other early regions. At least seven open reading frames have been attributed to this region (reviewed in Hitt *et al.*, 1997). E4 has been shown to play a role in promoting primary cell transformation *in vitro* by co-operating with E1A and E1B. Moreover, E4 products play a role in preventing cellular mRNA transport from the nucleus to the cytoplasm of infected cells. E4 is also involved in enhancing viral DNA transcription by co-operating with a 19kd DNA binding protein. A recent study by Nevels *et al.* (1997) revealed a 34 kd protein encoded by open reading frame 6 (ORF 6) of the E4 that stably binds the carboxy-terminus of the cellular protein p53 and that also interacts with the 55 kd E1B protein. The carboxy terminus of p53 protein plays a role in regulating DNA binding, nuclear localisation, recognition of DNA damage; it may also have a role in causing cellular apoptosis (Soussi and May, 1996).

f) VA RNA

The adenoviral genome codes for the so-called "VA" RNAs (virion associated RNAs). However, contradictory to what the name indicates, the VA RNA molecules are not part of the virus structure, but they have been detected in large amounts in infected cells (reviewed in Fields *et al.*, 1996). These RNAs are thought to act as translational activators. They block the protein kinase that phosphorylates and inactivates the eukaryotic transcription factor eIF-2, thereby blocking the virus-inhibition activity of interferons, which activate the protein kinase (reviewed in Hitt *et al.*, 1997).

E. DNA Replication

Adenoviral DNA replication begins about 5 hours post-infection in Hela cells (Challberg and Kelly, 1989) and requires both viral encoded and cellular factors. Only one of the strands in the double stranded DNA genome serves as a template, resulting in a displaced single strand and a double stranded hybrid duplex. The displaced single stranded DNA then forms a "pan-handle" structure through the annealing of its inverted terminal repeats (Figure 4); this double stranded structure is then recognized by the replication proteins and allowed to go through the replication cycle, resulting in a full double stranded hybrid DNA duplex structure (reviewed in Van der Vliet, 1995).

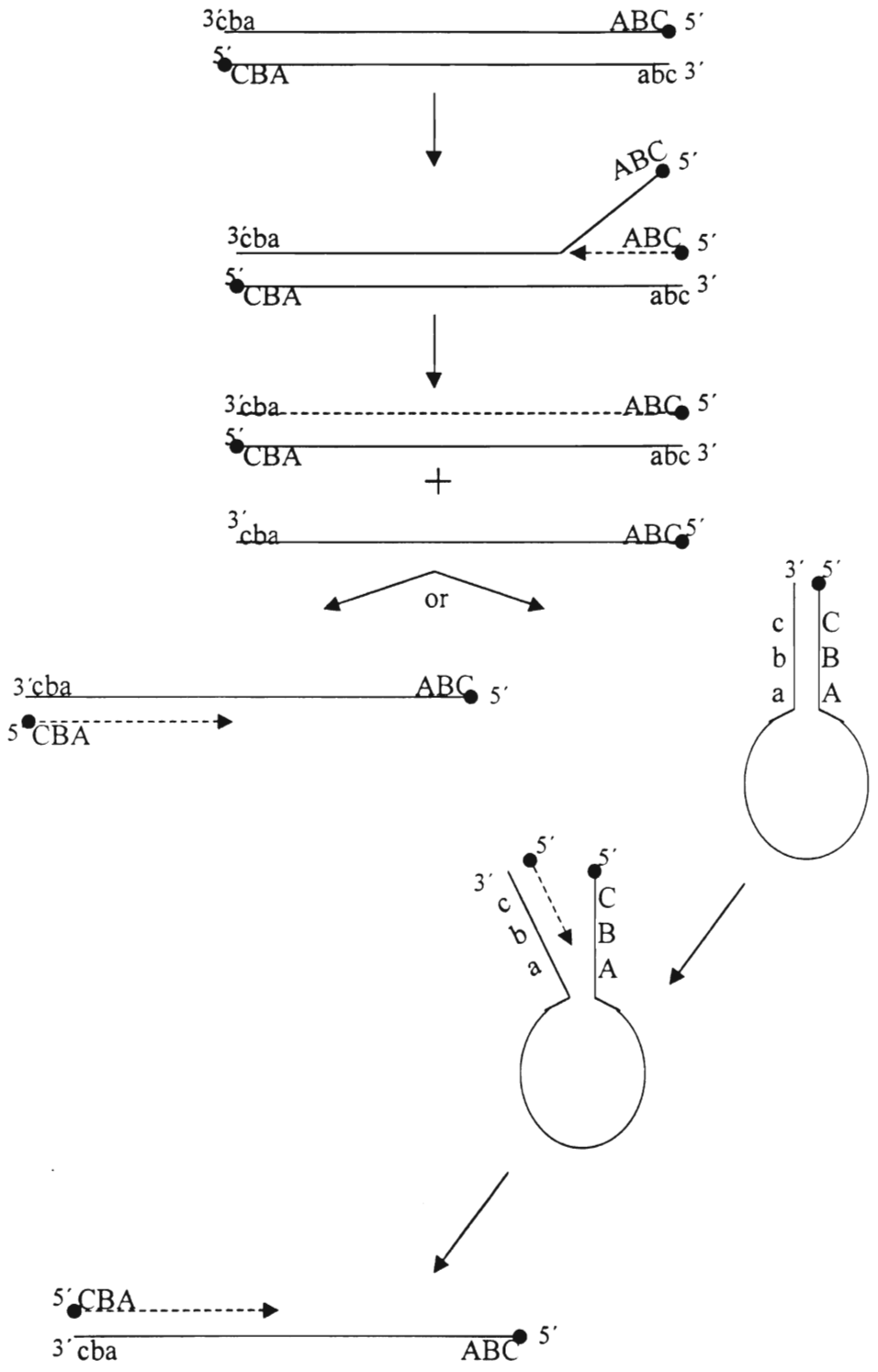


Figure 4: Schematic diagram showing Adenoviral DNA replication.

One of the strands serves as a template, which results in a displaced single strand. The displaced strand forms a panhandle structure through the annealing of inverted terminal repeats (ITR's). The double stranded structure is then recognised by the transcription machinery. Upper and lower case letters are used to indicate complementarity of the ITR's where capital letter orientation will bind to the lower case orientation only (reviewed in Van der Vliet, 1995)

F. *Late Transcription*

Late transcription is initiated subsequent to DNA replication. The late region is comprised of five families, namely: L1 to L5.

1. Late region

The late regions' proteins make up the capsid components. Transcription of the late region starts from the MLP (major late promoter) and produces a single mRNA product that is differentially spliced into the five different families of mRNAs (Shaw and Ziff, 1980). The activity of the major late promoter (MLP) increases 100-fold after the onset of DNA replication and it is thought to be activated by the MLP transcription factor (reviewed in Fields *et al.*, 1996).

2. Virus Assembly

Virus assembly starts with the formation of pentons and hexons capsomeres in the cytoplasm. The process continues in the nucleus through a series of intermediate structures that consist of the DNA genome, TP, pV, pVII, and the polypeptide mu. Proteolytic processing results in the final mature virus particles which are released through cell induced lysis (reviewed in Shenk, 1996).

G. *Bovine adenovirus*

Most of what is konwn about Ads is based on studies done with Ad2 and Ad5. BAVs on the other hand, are less studied and this section will summarize what is currently known about them.

Bovine adenoviruses (BAVs) were first isolated in 1959 (Klein *et al.*,1959). They have been classified into two subgroups, which are further divided into nine serotypes based on the presence of complement-fixing antigen, growth rate, and their ability to grow in different primary and established cell lines. Subgroup 1 contains serotypes 1, 2, 3, and 9 while subgroup 2

contains serotypes 4 to 8. The organisation of the BAV genome resembles that of Ads. BAVs are spread worldwide causing respiratory and gastrointestinal illnesses in bovine species (Mattson *et al.*, 1988).

1. Bovine adenovirus type 2

BAV2 is divided into two subtypes, namely A and B (Belak and Palfi, 1974). These two subtypes differ in their permissivity in cattle and sheep. Subtype A can only infect cattle, while subtype B can infect both cattle and sheep. They also differ in their restriction endonuclease pattern and in hemagglutination properties where subtype A hemagglutinate bovine erythrocytes and subtype B hemagglutinate rat erythrocytes (Belak *et al.*, 1983).

H. Current Uses of Adenoviruses

Most adenoviral vectors are based on Ad5. Adenoviral vectors are either helper independent (replication-competent) or conditional helper-independent (replication-deficient in non-complementing cells) recombinants. Replication-competent vectors have been successfully used in animal models to induce antibody, secretory and cell-mediated immunity against HBV, CMV, and RSV. Replication-deficient vectors, missing the E1 region ($E1^-$), are an attractive delivery tool in gene therapy, since the virus' only purpose is to deliver the gene to the target cell (reviewed in Hitt *et al.*, 1997).

The most common Ad vectors carry deletions in either E1, E3 or both. Other vectors such as E2 and E4 have also been generated (Amalfitano *et al.*, 1996); however, they are not as common as the E1/E3 deletion viruses. Recent studies suggest keeping E3 intact to allow the virus to hide from the immune system, therefore giving it a better chance of delivering the gene it is carrying. The E1 deletion vectors are favoured, since they have the added safety feature of lacking the E1 region that in Ad12 can cause oncogenic transformation of primary cells in rodents.

Today's generation of gene therapy viral vectors is limited in that they are being destroyed by the humoral and cellular immune response of the patients. This greatly affects the re-administration of the same viral vector. The other limitation of adenoviruses is that they infect a wide range of cell types, which limits the targeting of specific cell types or organs (Douglas, 1997).

1. First Generation Adenoviral Vectors

First generation adenoviral vectors were based on human Ad2 and Ad5. These viruses have been well studied and characterised. They have been shown to share close to 95% homology (Chroboczek *et al.*, 1992). Human Ad2 and Ad5 belong to group C, which does not cause tumours in rodents. Viral vectors based on these serotypes carrying E1 deletions have an extra safety feature, as previously described.

Ad viruses can carry up to 105 % of the wild type genome size (Bett *et al.*, 1993). Therefore, Ad vectors can accept up to 2 kb of foreign DNA without any deletions. Deletions in both E1 and E3 regions allow for up to 5 kb-inserts. The largest E3 deletion accomplished in Ad5 was 3.1 kb (Bett *et al.*, 1994). E1 deleted viruses require the 293 packaging cell line (Figure 5), a human embryonic kidney cell line transformed with the E1 region of Ad5 (Graham *et al.*, 1977).

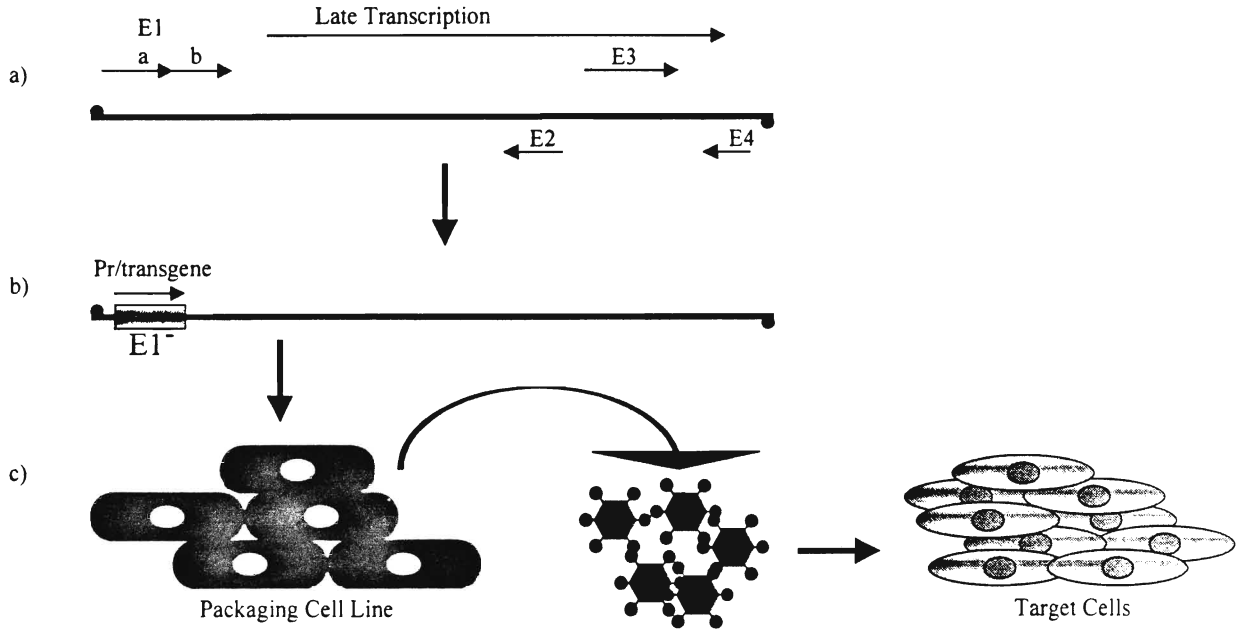


Figure 5: Schematic representation of the generation of E1-deleted Ads.

(a) Ad genome with 0% at the left end and 100% at the right end. The diagram indicates the direction of the Early and the Late region transcriptions. (b) A diagrammatic representation of an adenoviral mutant's genome. The genome carries an E1 deletion with a transgene inserted in its place. The transgene is controlled under a constitutive promoter to control the gene's expression. The figure also shows the absence of Early or Late region expressions, caused by the absence of the E1 region. (c) This part of the figure represents the process of rescuing a recombinant mutant virus, where the modified genome from part (b) can be transfected into a packaging cell line, which provides the E1 products in *trans*. DNA transcription and translation of the viral DNA results in the formation of a complete assembled virus, which carries the transgene and its promoter. The resulting virus can then be used to infect target tissues, or cells in order to transfer a gene for the sake of gene therapy or vaccination.

The attenuated E1-deleted Ad vectors have been shown in several studies to result in lower levels of foreign gene expression *in vitro* when compared to E3-deleted replication-competent vectors (reviewed in Hitt *et al.*, 1997), with the exception of one study by Levrero *et al.* (1991) where both types of vectors resulted in equal levels of expression. E1-deleted Ad vectors are more suitable for *in vivo* gene therapy. However, for *in vitro* recombinant gene expression, E3-deleted replication-competent viruses have been shown, by most studies, to result in higher levels of recombinant protein expression.

E1 replacement genes have been shown to be expressed when present in both leftward and rightward orientation, although the rightward orientation has been shown to result in a higher

level of expression (reviewed in Hitt *et al.*, 1995). In replication competent E3-deleted vectors, genes lacking strong promoters were shown to be expressed mainly in the rightward orientation (Mittal *et al.*, 1993; Yarosh *et al.*, 1996).

There are two main problems associated with the first generation of Ad vectors, when used in gene therapy. The first problem is the presence of low levels of DNA viral transcription in attenuated E1-deleted vectors observed both *in vivo* (Yang *et al.*, 1994) and *in vitro* (Spergel *et al.*, 1992). This low level of viral protein expression is partly responsible for activation of the immune system, which results in the elimination of infected cells in gene therapy trials (Rich *et al.*, 1993). Other contributors to immune system activation are the presence of adenoviruses themselves in the initial inoculum (McCoy *et al.*, 1995) and, in some instances, the foreign gene product itself (Tripathy *et al.*, 1996). The second problem concerns with the propagation of the E1-deficient Ad vectors. These viruses must be grown in the packaging cell line 293, which provides the E1 products in *trans*. However, the cell line also provides enough overlap between the Ad5 DNA in the cells and the vector's DNA to generate replication-competent Ad viruses through homologous recombination. The wild type revertants, depending on the replication rate of the mutant vector, might be able to outgrow the viral vector of interest (Lochmuller *et al.*, 1994). The presence of wild type revertant viruses, even at low levels, may pose a serious problem in gene therapy by activating the immune system or by acting as a helper virus for the replication of the attenuated viral vector. That could result in inappropriately high levels of transgene expression which may be harmful to the patients (reviewed in Hitt *et al.*, 1997).

2. Second Generation Adenoviral Vectors

The second generation Ad vectors carry modifications in order to correct the flaws of the first generation vectors. One of these modifications is the development of added deletions, such as the one developed by Armentano *et al.* (1995) which contained a deletion within E4. It is still

not known how this modification to the E4 region will affect the basal level expression of the late regions, but such modifications will not eliminate the immune response against the original inoculum or to subsequent administrations of the vector (Kaplan *et al.*, 1996).

E2A products play a role in viral DNA replication and packaging. In a study by Engelhardt *et al.* (1994), an Ad carrying a mutation in the E2A region resulted in a temperature sensitive E2A 72kd DNA binding protein. This mutant showed a significant decrease in late gene expression levels when grown at 37°C. In addition, homologous recombination in the 293 cell line, which would normally result in wild type revertants, resulted in a virus that was completely impaired in expression of the late genes.

Wild type Ad viruses can complement and allow for the replication of attenuated E1-deleted vectors. This characteristic can pose a potential hazard, especially *in vivo*, when used as a gene therapy tool (reviewed in Hitt *et al.*, 1997). In a study by Imler *et al.* (1995), an E1-deleted Ad5 vector was further attenuated by introducing a mutation in the *cis*-acting encapsidation signal in the left end of the genome (270 bp to 346 bp). The mutation resulted in an Ad5 vector with impaired incapsidation by a factor of three to ten, when compared to the wild type Ad5. Consequently, in the case of mixed infection with a wild type virus, the recombinant virus will be outgrown by the wild type, thereby, eliminating the chance of spreading the recombinant virus. Although careful monitoring must be exercised when growing this virus in 293 cells, it has an added safety feature to prevent the inadvertant dissemination of recombinant Ad viruses to other tissues within the patient or to other individuals.

Alternative packaging cell lines that would prevent reversions to wild type have been proposed as a modification of the first generation Ad viral vectors (discussed under "Packaging Cell Lines"). One of the major areas of research of the use Ad viral vectors is in treatments of cystic fibrosis.

Cystic fibrosis results from a mutation in the cystic fibrosis transmembrane conductance regulator gene (CFTR). Mutation in this gene results in a non-functional ion channel that leads to an imbalance of chloride ion concentration in the epithelia (Collins, 1992).

Replication defective Ad2 and Ad5-based viral vectors carrying the CFTR cDNA showed various degrees of success in delivering the healthy copy of the gene. The problems encountered were caused mainly by the humoral and cellular immune responses to the vector and to the infected cells. Although the current generation of gene therapy vectors is thought to be completely crippled in terms of replication, a low level of *trans*-complementation still occurs, resulting in low levels of DNA replication. Another possibility is that the gene being delivered itself, when expressed could, elicit an immune response causing the destruction of the infected cell carrying it. However, the most obvious obstacle is the priming disposition of an immune response that would neutralise any subsequent administrations (Bout *et al.*, 1994).

Throughout the studies, several observations were made, such as: the occurrence of inflammation and transient expression of the transferred CFTR gene; subsequent attempts to restore expression by a second administration was usually unsuccessful (reviewed in Johnson, 1995).

3. Generating wild type-free Adenovirus vectors

Recombinant Ad vectors were initially developed and used for eukaryotic gene expression and vaccine development *in vitro* (Gomez-Foix *et al.*, 1992). However, animal studies revealed the possibility of using recombinant vectors for gene therapy, with the first trial in humans utilising a vector carrying the CFTR gene (Crystal *et al.*, 1994). Recombinant vectors, especially those carrying E1 deletion and E1 substitutions, are grown in 293 cells (human embryonic kidney cells transformed with fragmented Ad5 DNA and determined to be carrying 0% to 12.5% of the genome).

Viral vectors used for gene therapy *in vitro* evaluation and clinical studies must be wild type-free. However, as previously mentioned, growing E1 deletion Ad mutants in 293 cells results in reversion to wild type Ad through homologous recombination between the viral DNA and the integrated Ad5 left end segment. A study by Zhang *et al.* (1995) resulted in the development of a PCR-based method, which can be used to detect 1 pfu of wild type Ad virus in 10^9 pfu of recombinant virus. Another method for detecting contaminants was based on the fact that only wild-type Ad viruses are able to grow in Hela cell lines. Therefore, Dion *et al.* (1996) used a stock of recombinant Ad vectors to infect Hela cells and, based on the amount of virus produced, they were able to extrapolate the amount of virus present in the original stock. This system can also be used to detect contaminants that result from laboratory handling or air-borne spreading.

To avoid having to deal with revertants, the alternative route would be to develop a packaging cell line that complements the missing E1 proteins, but without allowing any extra DNA sequences, so as to prevent overlapping and subsequent homologous recombination. This is part of the objective of this study.

In recent studies, new cell lines were developed to complement the E1, E4 (Brough *et al.*, 1996) and E1, E4, pIX (Krougliak and Graham, 1995) of Ad5. These cell lines were based on the established 293 cell line. Although these cell lines still contain the overlapping E1 region, they cannot allow for full wild-type revertants, since the E4 and pIX regions do not allow for homologous recombination. Ad vectors missing E1, E3, E4, and pIX are able to grow in the newly established cell lines and they can take up to 11 kb of inserted foreign DNA. This new modification of the 293 cell line allows for the production of Ad vectors that can carry larger size foreign DNA fragments, that are safer and that lack helper function for adeno-associated virus.

In another similar study (Amalfitano *et al.*, 1996), a 293-derived cell line was constructed to complement E1 products and Ad2 DNA polymerase. The enhanced feature of this cell line is

the inability of the virus to replicate its DNA *in vivo*. This is desirable, since, otherwise, cells that contained the inserted gene of interest would be eliminated. Human gene therapy trials have shown that the transient expression of foreign DNA is correlated with low levels of viral DNA expression, which then leads to an immune response and eventually the elimination of the infected cells.

A new cell line, designated 911 (Fallaux *et al.*, 1996), was produced by transfection of human embryonic retinoblast cells with an Ad5 carrying plasmid vector. The new cell line prevents reversions to wild type, since it only carries DNA from nt79 to nt5789, but it still shares many of the properties of the 293 cell line, such as its ability to support a high level of recombination and recombinant virus production. Other cell lines, based on human lung carcinoma (Imler *et al.*, 1996) and designated as PTG6564-17 and PTG6559-5, include the Ad5 sequences from nt505 to nt4034. These cell lines are inferior to 293, in that they are about ten times less effective in supporting the growth of E1-deleted recombinant viruses. However, these cell lines were not tested for their ability to support homologous recombination in Ad vector production.

4. Gene Therapy

Somatic gene therapy involves the insertion of the correct configuration of a gene into an individual's somatic cells so that the normal gene product is produced to correct a metabolic disorder. Only a certain cell type is normally targeted by gene therapy. This can be done in two ways. The first method is done *ex vivo*, where cells from the affected tissue are removed and cultured *in vitro*; the gene is then introduced into these cells to correct the defect. Once the cells are grown to large enough quantities, they are transplanted back into the patient. The other method for gene therapy is done *in vivo*, where the gene is transferred to the target site without the need for tissue removal or culturing cells *in vitro* (Densick and Schuchman; 1998).

Human gene therapy, first approved in 1989, has relied mainly on virus-based delivery systems and has had a great influence on the generation of replication-deficient viral vectors (Wilkinson and Borysiewicz, 1995). The choice of the method used for gene delivery should address the risk involved, as well as efficiency and specificity of the virus (Table 5). Currently, there are over 140 human gene therapy protocols approved for clinical testing (reviewed in Hitt *et al.*, 1997). Most of these protocols use retroviral, adenoviral or adeno-associated viruses, while some use direct DNA injection or lipofection as a method of DNA delivery.

Retroviruses have been widely used in gene therapy. These vectors have been developed as replication-deficient viruses capable of stably integrating into the chromosome (reviewed in Wilkinson *et al.*, 1995). These viruses are uniquely suited for gene therapy since integration into the host cell genome is an obligatory part of their life cycle. However, they still have serious limitations: (1) they require dividing cells for stable integration, which limits their use *in vivo*, (2) insertional mutagenesis might arise from the random integration step, (3) low viral titers are produced from both the wild type and mutants grown in packaging cell lines, (4) a short shelf life makes it difficult to produce the virus in large quantities to be stored or shipped, and (5) recombination between the viral vectors and endogenous retroviruses might occur.

Adeno-associated viruses (AAV) are non-pathogenic parvoviruses that require co-infection with an Ad, or certain members of the herpes virus family, to complete their life cycle (reviewed in Fields *et al.*, 1996). In the absence of a helper virus, AAVs integrate at specific sites within the genome and exist in a latent state until the cell is infected with a helper virus. The virus then replicates and undergoes a lytic cycle. This family of viruses has several advantages (Kohn *et al.*, 1990): (1) AAVs do not cause any known diseases in humans, (2) they have a broad host range and do not require dividing cells in order to integrate, (3) the wild type virus integrates at a specific site (chromosome 19 in humans), and (4) more than 95% of the genome can be removed, allowing for foreign gene insertion. However, the use of AAVs as tools

for gene therapy also carries some disadvantages (Kohn *et al.,* 1990; Densick and Schuchman, 1998): (1) they cannot carry sequences larger than 5kb, (2) integrated AAVs can be activated by co-infection with the wild type AAV or a helper virus, causing the integrated provirus to be released, (3) permanent packaging cell lines have not been developed yet, and (4) site specific integration does not occur with the recombinant AAVs.

The herpes virus is also an attractive tool in gene therapy, firstly, due to the fact that it can be used to target the central nervous system, as well as its ability to infect non-dividing cells, the production of high titer viral stocks and its ability to carry large size inserts of foreign DNA (up to 30kb) (Breakefield, 1993). Despite the advantages, the herpes virus protocol for gene delivery is still under development, since the vectors produced have been shown to be cytotoxic, *in vivo* infection efficiency is low, and there is limited spread of the virus from the site of injection (Desnick and Shuchman, 1998).

Table 5: List of gene delivery methods.

Table shows advantages and disadvantages of the different techniques used to deliver genes in gene therapy studies. Compiled from Kessler *et al.*(1996), Desnick and Schuchman (1998).

	Retrovirus	Adenovirus	Adeno-Associated viruses	Herpes Virus
Titer	Low	High	Moderate	Moderate
Stable vs. Transient expression	Stable	Transient	Stable	Transient
Site-Specific Integration	No	Episomal	W/T: Yes Rec: No	Episomal
Requires Dividing Cells	Yes	No	No	No
Insert Size	Moderate	Moderate	Small	Large
Packaging Cells Available	No	Yes	No	No
Recombinant Vectors Cytotoxic	No	Yes	Yes	Yes

Problems associated with viruses, their side effects and possible risks, has led to the development of non-viral based gene delivery systems. Systems employing liposomes and DNA-protein conjugates have been investigated as alternatives to the use of viral vectors.

Liposomes are lipid bilayer vesicles that, depending on the method of preparation, may consist of a single bilayer or multiple bilayers surrounding an aqueous center (Felgner and Rhodes, 1991). The vesicles can range in size from a few nanometers to micrometers. Positively charged liposomes have been used to carry over DNA. Upon injection into a patient, these vesicles pose very little risk of an immune reaction or cause of a disease (Smith *et al.*, 1993). The inherent disadvantage of liposomes is the lack of specificity or specific cell-type targeting. The other problem associated with these vesicles is the lack of stability upon injection into animals, where they either break down or form aggregates. Moreover, liposomes are not as efficient as viruses in delivering genes to the target cell nucleus (Smith *et al.*, 1993).

5. Viral Vaccines And Genetic Immunisation

Recombinant vaccines are very similar to viral vectors used in gene therapy in that they both have been altered to carry and deliver foreign genes. However, the immune response, which is a major issue in gene therapy, is actually the target for recombinant vaccines. For vaccination, it is necessary to express the foreign gene long enough to stimulate an effective and long lasting immune response (reviewed in Hitt *et al.*, 1997).

Viruses are obligate intracellular parasites. Consequently, any interference with their entrance into the host's cell would prevent them from causing a disease. Blocking the initial viral-host cell interaction is the most convenient point of protection. Therefore, antibodies that recognise viral surface proteins would confer immunity against the virus. The antibodies of the host do not need to recognise all the proteins on the viral capsid; in fact, one subunit of the viral capsid protein is all that is needed to provide immunity (reviewed in Descamps *et al.*, 1996).

Immunisation against foot and mouth disease was one of the first methods to employ single synthetic peptide technology to provide protective immunity (Babiuk and Phillips, 1989). However, this technique carries a disadvantage due to the fact that viruses can easily mutate,

rendering the subunit vaccine useless in terms of providing protective immunity against the infectious agent.

The other option for protection is to use viral proteins that are targeted by cytotoxic T cells (CTLs), which lyse infected cells before the virus has completed its replication cycle. This will in turn reduce the amount of virus present and the subsequent chance of a clinical disease. Such vaccines have been shown to provide an enhanced protective immunity in the case of orthomyxovirus, where the nucleocapsid protein , conserved among different strains of influenza viruses, was shown to be a major target of CTLs (Babiuk *et al.*, 1996). Consequently, even if the virus experiences mutations or an antigenic shift in regions targeted by monoclonal antibodies, the CTLs would still recognise infected cells and cause their lysis.

Once the desired proteins have been identified, they can then be produced in large scale in either a prokaryotic or a eukaryotic system. Prokaryotic systems have been shown to produce viral proteins that can confer protective immunity *in vivo*; however, due to post-translational modifications, eukaryotic systems are more suitable for producing viral glycoproteins. Eukaryotic systems such as yeast, mammalian cell lines, insect cell lines and, recently, plants have been used for such purposes. All these systems have been shown to be very efficient at producing viral glycoproteins at high levels as well as maintaining the conformation of the proteins, which is critical for an effective immunisation. Hepatitis B was the first licensed recombinant vaccine that was produced in yeast (Babuik *et al.*, 1996).

The proteins could be produced in a cell line either through constitutive expression, where the gene that codes for the protein is integrated in the cellular genome, or through the use of a recombinant virus. In the first case, there may be problems associated with constitutive expression, such as toxicity to the cell expressing the protein; furthermore, unless the protein is secreted, the purification procedure might be very costly. The second option, which is to use a recombinant virus to carry the foreign gene, eliminates the toxicity aspect of the previous

method, but faces the problem of sample purification to produce a 100% virus-free vaccine (Kowalski *et al.*, 1993).

A novel vaccine production strategy was developed by using recombinant baculoviruses that carry foreign genes (Kang, 1988). They can be used to infect silk worms, resulting in the secretion of 1 mg of the desired protein from a single larvae. Further studies on the secreted proteins are being done to assess whether they had the appropriate post-translational modifications.

The debate regarding the use of live vaccines versus attenuated vaccines continues, where live incapacitated viruses, missing crucial genes, are thought to be safer options than attenuated vaccines. This is due to the fact that the genetic basis of attenuation in live attenuated vaccines is not very well understood and the risk of developing the disease is still possible. One of the first live vaccines to be available commercially was the pseudorabies virus, which contained a specific deletion in the thymidine kinase (TK) gene and/or viral glycoprotein (gC or gE gene) (Kit *et al.*, 1987). The virus was able to replicate *in vitro*, but was not able to complete its life cycle *in vivo*, thereby, greatly enhancing the safety aspect of the vaccination procedure.

Vaccinia virus is a member of the poxiviridae family, which, unlike most DNA viruses, replicates in the cytoplasm of infected cells by encoding their transcription and replication machinery (reviewed in Fields *et al.*, 1996). These viruses have a large genome of 185 kbp, allowing for large inserts of foreign DNA. Among their other advantages as recombinant vaccines are the following: they are readily administered, they confer long term protection, they are inexpensive, and they provide long term stability of the virus (Wilkinson and Borysiewicz, 1995). Although side effects have been well characterised, serious complications do occur. These complications include secondary spread of the lesion, rash, progressive vaccinia, aczema vaccinatum and encephalitic disease. Vaccinia mutants, carrying deletions in genes that are not essential for *in vitro* replication or expressing interleukin type 2 (IL-2) to stimulate the immune

system, are potential solutions to side effects which are currently being explored. Such modifications could improve the benefit-to-risk ratio, especially in high-risk groups including those with immune system disorders (Wilkinson and Borysiewicz, 1995).

Retroviruses, on the other hand, are not suited to be used as recombinant vaccine delivery systems, since they produce low levels of expression and they are potentially oncogenic due to their random integration. Moreover, they can be rescued by endogenous retroviruses. However, the main disadvantage of retroviruses is their requirement for dividing cells (reviewed in Fields *et al.*, 1996).

Ideal viral vaccines should provide a 100% success rate in preventing subsequent viral infection; however, complete protection is not realistic. The realistic ideal vaccine should have a success rate of over 90%. The vaccine should provide long-term protection within a few weeks of a single administration, thereby reducing the cost of re-immunisation. Furthermore, the vaccine needs to be safe, in that it will not cause adverse reactions, disease, or interference when simultaneously administered with other vaccines. The vaccine should also be genetically and thermally stable (reviewed in Hitt *et al.*, 1997).

In veterinary medicine, most of the pathogens enter via the mucosal surfaces; therefore, ideal vaccines should stimulate mucosal immunity, which would be best induced by intranasal or oral delivery. Thus, the vaccine should be designed to be delivered by mucosal routes (Babiuk *et al.*, 1996). In the veterinary field, the cost of vaccination is a crucial consideration, where the cost of a single vaccination should not exceed a few cents per administration, especially in developing countries.

Genetic immunisation employs the use of naked plasmid DNA as the source of the coding region for the vaccine. This technology, described as the "revolution in vaccine biotechnology" (Ulmer *et al.*, 1996), has had a strong foundation, built on the observation that when plasmid DNA was introduced into an animal's tricep muscle (Wolff *et al.*, 1990), the genes were

expressed for an extended period of time. This would allow enough time for the immune system to recognise the foreign proteins and produce the appropriate immunity against it. This technique has been used to show that genes from different sources (bacterial, viral, reporter genes, etc.) can be expressed and used in different animals, ranging from fish, chickens, rabbits, cows and non-human primates (Babiuk *et al.*, 1996).

The major advantages of this technology are: (1) the possibility of including several genes from different sources on the same plasmid in order to induce protective immunity against several infectious agents in one immunisation procedure, (2) the absence of the risk of developing a disease following immunisation, (3) the achievement of the proper conformation of the protein following antigen post-translational modifications and folding of the protein, (4) the induction of both humoral and cellular immune responses, (5) the possibility of life-long immunity (still under investigation), and (6) in the case of multiple vaccines, the lack of vaccine incompatibilities (Babiuk *et al.*, 1996).

6. Establishing Cell Lines

Immortal transformed cells have been widely used in studies involving oncogenesis, differentiation and development, clinical neurology (Geller *et al.*, 1991) and virology, where they are employed as tools to generate viral vectors or to simply generate high titer viral stocks.

The first observation of the ability of Ad to transform cells was in 1962 when Trentin and co-workers, showed that Ad12 was able to cause oncogenic tumours in newborn rodents. That study generated a lot of interest and research into the possible link between adenoviruses and human cancers. All subsequent research failed to reveal adenoviral DNA presence in human tumours, but they did show that the E1 region of Ad12 had to be present in order for the virus to cause a tumour in newborn rodents. Ad12 integration in the cellular genome is thought to be a

non-sequence-specific insertional recombination, in which patch homologies are observed between sequences from both the virus and from those of the cellular genome (Doerfler, 1996).

Senescence is a phenomenon that characterises the limited proliferative capacity of normal mammalian cells. There are several models proposed for the causes of cellular senescence. One model suggests that senescence is a process characterised by the accumulation of abnormal macromolecules until cell division ceases (Bryan and Reddel, 1994). The accumulation of such abnormal products (deaminated proteins and oxidised amino acids) has been documented in senescent cells. Another view is that senescence is a programmed event, since senescent cells have been shown to express new genes as well as repress normally expressed genes (Goldstein, 1990). This theory was supported by a study where human fibroblasts were fused with immortal human cervical cancer cells exhibited a limited division potential in culture (Bryan and Reddel, 1994). The third theory explaining the case of senescence proposes a role for the conserved telomeric sequences. The repeated sequence TTAGGG is used by the DNA replication machinery during cell division. If this sequence is lost during successive cell cycles, eventually the cell will be prevented from dividing. This growth arrest was suggested to be linked to an activation of p53-dependent cell division arrest in response to DNA damage (Dulic *et al.*, 1994). While the phenomenon of senescence and the details of it have not yet been determined, this limited cellular passaging capacity can be overcome by immortalisation *in vitro*. Primary cells can be immortalised using a number of methods including the use of oncogenes, growth factors (Spandidos, 1985) , chemical carcinogens, and cellular genetic alterations (Gonos and Spandidos, 1993). The immortalised phenotype really represents more of a decreased differentiation potential than an increase in replication potential. Immortalisation is the first step in tumourogenesis, while the second step is transformation. Viral (Ad E1 region, SV40 and polyoma large T antigen as well as E6 and E7 of human papiloma virus (Bryan and Reddel, 1994) and cellular (*ras*, *myc*, *fos*, *jun* and some mutants of p53 protein (Spandidos (editor), 1992)

oncogenes have been shown to have the ability to immortalise cells. The immortalising oncogenes appear to eliminate the function of some cellular proteins, such as the retinoblastoma and the normal p53 protein, which seem to prevent cell proliferation.

Normal cells have a limited number of passages *in vitro* (approximately 12 generations), after which the cell culture appears to go into crisis resulting in cell death. Following immortalisation, the cells exhibit a life-span of over 100 generations in culture, as well as uninhibited growth, cloning in agar, growth on confluent monolayers and lower serum dependence.

The second step in tumourogenesis is transformation. The events leading to transformation are not very well understood; however, transformed cells have distinct properties such as anchorage independence, the ability to grow in suspension, the loss of contact inhibition, the ability to form foci in culture, high saturation density, a low serum requirement, over-expressed oncogenes or deleted suppressor genes, a modified extracellular matrix and the ability to cause invasive tumours *in vivo*.

Oncogenes involved in immortalisation and transformation have different properties: those capable of immortalising primary cells have the common feature of nuclear localisation, while those capable of transforming cells have a mainly cytoplasmic localisation (Gonos and Spandidos, 1993)

I. Prospects of BAVs for gene therapy and vaccine application

Bovine adenoviruses (BAVs) can be a successful mean of gene therapy, and viral vaccine. They can be used in veterinary medicine as well as in human medicine. In a study by Mittal *et al.* (1995), BAV3 carrying the firefly luciferase gene in place of the E3 region was constructed. Although BAV3 is non-permissive in humans, upon its infection in human (293) and bovine (MDBK) cell lines, luciferase gene expression was detected in both cell lines. The application

of non-permissive viruses in viral vaccine studies was shown to be successful in vaccinating dogs (Prevec *et al.*, 1990), skunks and foxes (Charlton *et al.*, 1992) with a recombinant human Ad5 viral vector carrying the rabies glycoprotein gene. A very low percentage of the population has been exposed to BAVs. Therefore, the virus would have a better chance of delivering the gene of interest without getting degraded by a pre-existing immune response.

VII. Objectives of the Current Study

The primary goal of this research project was to establish a packaging cell line capable of complementing the E1 functions *in trans*. To achieve such a goal, the following number of objectives ought to be undertaken:

1) Construction of pCMV-E1 and establishing a cell line from embryonic primary cells
2) Construction of pCMV-E1-*neo* and establishing a cell line from established cell lines
3) Characterization of the constructed cell line in terms of the following:
    a) Expression of E1 products
    b) Expression of trasnfected DNA
    c) Complemenation of E1-deletion mutant of bovine adenovirus type 2
4) Construction of recombinant BAV2 plasmids carrying the *lac*Z gene in place of the deleted E1 and/or E3 region

VIII. Materials and Methods

A. Bacterial strains

The bacterial strains used in cloning and propagation of plasmid DNA were *Escherichia coli* (*E. coli*) DH5 α (Genotype: F$^-$ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 λ$^-$ *thi*-1 *gyr*A96 *rel*A1; Life Technologies^{TM}) and XL1-Blue MR (Genotype: *Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*; Stratagene®). These *E. coli* strains were grown in Luria-Bertani medium (LB: 1% Bacto-Tryptone, 0.5% Bacto Yeast extract, 1 % Sodium Chloride, pH 7.0; autoclaved at liquid cycle for 20 minutes at 15 lb/sq. in.), supplemented with 100 μg/ml ampicillin or 20 μg/ml kanamycin, depending on the type of antibiotic resistance selection needed at the time. Cells were stored in Luria-Bertani medium (LB) with 15% glycerol at -20°C.

1. Preparation of Competent Cells

Competent cells were prepared according to Sambrook *et al.* (1989). A frozen stock of *E.coli* cells was streaked on an LB-agar (Luria Bertani medium supplemented with 2% agar ; BioShop) plate, which was inverted and allowed to grow overnight in an oven incubator at 37°C. A single colony was picked and grown in 5 ml LB for about 5 hours; this was subsequently inoculate a 500 ml flask of LB. The cells were allowed to grow until reached an OD_{600} of 0.45 to 0.55 and then harvested by centrifugation (Beckman Avanti^{TM} J-25) at 3,500 rpm, 4°C, for 10 minutes. The cell pellet was gently resuspended in 50 ml of transformation buffer (75mM $CaCl_2$, 5 mM Tris Base, pH 7.6; sterilised by filtration through 0.45 micron Gelman Sciences Acrodisc® filter), left on ice for 16 hours and then collected again by spinning at 3,500 rpm for 10 minutes. The cells were then resuspended in 3 ml of transformation buffer with 15% glycerol

and allowed to sit on ice for 30 to 45 minutes, 150 µl aliquots were dispensed into Eppendorf tubes and stored at -80°C until needed.

B. DNA Ligation

T4 recombinant DNA ligase (NEB) was used to perform all DNA ligations. Ligations were completed in a total volume of 20 µl at 16°C for 4-16 hours in Ligase buffer (50mM Tris-HCl, 10mM $MgCl_2$, 10mM DTT, 1mM ATP, 50 µg/ml BSA; pH 7.8), supplied by NEB. A typical ligation contained 50-300 ng of digested DNA insert and 50-150 ng of linearized plasmid DNA, 2 µl of ligase buffer, 0.5 µl of ligase (diluted to 10 units/µl) and sterile distilled water up to a final volume of 20 µl.

C. Transformation

Competent *E. coli* were transformed according to Sambrook *et al.* (1989). A tube containing frozen competent cells was placed on ice, after which 10 µl of the ligation mixture was added and then allowed to sit on ice for 30 minutes with occasional shaking. The cells were shocked by heating at 42 °C for 45 seconds and then put back on ice for 2 minutes. Nine hundred µl of SOC media (2% Bacto-Tryptone (BioShop), 0.5% Yeast extract (BioShop), 0.05 % Sodium Chloride, 2.5mM KCl, pH 7.0; autoclaved for 20 minutes at 15 lb/sq. in., liquid cycle; supplemented with sterile $MgCl_2$ to a final concentration of 10mM and glucose to a final concentration of 20mM) were added to the cells, which were subsequently incubated at 37°C with gentle shaking in a bench top shaker (New Brunswick Scientific Gyrotary water bath shaker G76) for 45 minutes. Following incubation, the cells were spread (100 µl per plate) on LB-agar plates supplemented with either ampicillin or kanamycin. The cells were allowed to grow for 16 hours at 37°C into individual colonies, which were picked for plasmid extraction and analysis.

D. Plasmid DNA Isolation

1. Small Scale DNA Isolation

The small-scale purification of plasmid DNA was carried out according to Sambrook *et al.* (1989). Single colonies were picked, using autoclaved wooden applicator sticks (Fisher*brand*®), from the LB-agar plates and transferred to a glass test tube containing 2 ml LB (supplemented with the appropirate antibiotic for selection). The tubes were then transferred to the bench top shaker and incubated at 37°C with shaking for 16 hours. The cells were harvested by transferring the culture to autoclaved Eppendorf tubes which were then centrifuged at 12,000 rpm for 30 seconds (Sorval® MC12V). The supernatant was decanted from the tubes and 100 µl of lysozyme solution (10mM ethylenediamine tetraacetic acid [EDTA], 50mM glucose, 125 mM Tris-HCl, pH 8.0; sterilised by filtration) was added to the cell pellet. The tubes were vortexed (American Hospital Supply Corporation Deluxe Mixer) until the cell pellet was completely resuspended. The solution was then allowed to incubate at room temperature for 10 minutes before 200 µl of alkaline SDS (1% SDS, 200mM NaOH) was added. The solution was mixed gently, allowed to sit at room temperature until the solution cleared and then 150 µl of sodium acetate (3M; pH 4.8) was added. The solution was mixed, incubated on ice for 20 minutes and then centrifuged for 5 minutes at 12,000 rpm. The supernatant was transferred to new autoclaved Eppendorf tubes and 900 µl of cold 95% ethanol was added to each. The solution was mixed and then centrifuged for 5 minutes at 12,000 rpm. The supernatant was decanted and the DNA pellet that precipitated was washed with 200 µl of 70% ethanol, air dried for 10 minutes and then resuspended in either 100 µl of TE buffer (100 mM Tris, 1mM EDTA; pH 7.5; Sterilised by autoclaving) or sterile distilled water.

2. Large Scale DNA Isolation

The appropriate transformed *E. coli* culture was used to inoculate a 500 ml LB flask, supplemented with antibiotics. The culture was allowed to shake overnight at 37°C in the bench top incubator.

a) Cesium Chloride Gradient

The cesium chloride gradient method of plasmid DNA purification was performed according to Sambrook *et al.* (1989). Initially, the cells were harvested by transferring the culture to autoclaved bottles (250 ml Sepcor® Polypropylene centrifuge bottles) and then centrifuging (Beckman Avanti® J-25 centrifuge) at 8,000 rpm for 8 minutes. The supernatant was decanted from the bottles and the cell pellet was completely resuspended by vortexing in 10 ml of lysozyme solution. The solution was then allowed to incubate on ice for 15 minutes before 20 ml of alkaline SDS was added. The solution was mixed gently, allowed to incubate at room temperature for 10 minutes and then 15 ml of sodium acetate (3M; pH 4.8) was added. The solution was mixed gently, incubated on ice for 30 minutes and then centrifuged for 15 minutes at 10,000 rpm. The supernatant was then transferred to a new autoclaved centrifuge bottle and 90 ml of cold 95% ethanol were added to it. The solution was mixed, allowed to incubate at -20°C for 20 minutes and then centrifuged for 15 minutes at 10,000 rpm. The DNA pellet was resuspended in 5 ml of autoclaved water before 10 ml of cold 95% ethanol was added. The solution was then incubated at -20°C for 10 minutes before it was centrifuged again at 10,000 rpm for 15 minutes. The final pellet was air dried for 10 minutes before being resuspended in 2 ml of sterile distilled water.

Subsequently, 150 µl of ethidium bromide (10 mg/ml) was added and the density of the solution was brought up to 1.55 to 1.59 g/ml by adding the appropriate amount of cesium chloride (CALEDON). The solution was transferred to Beckman Quick-Seal® centrifuge tubes

and sealed by heating. The samples were centrifuged (Beckman TL-100 ultracentrifuge) for 20 hours at 60,000 rpm and 22°C. Following centrifugation, two prominent red bands could be visualised. The lower band was extracted using a hypodermic syringe attached to a needle and transferred to a sterile Eppendorf tube. The DNA was washed several times with water-saturated isoamyl alcohol until the solution was no longer pink in colour. Subsequently, two volumes of sterile water were added and the DNA was ethanol-precipitated, centrifuged at 10,000 rpm for 10 minutes, air dried, and resuspended in a suitable amount of TE buffer or sterile water.

(1) DNA Dialysis

DNA samples purified using the cesium chloride gradient technique were dialysed before they were used for mammalian cell culture transfection. A 5-centimetre piece of Spectra/Por® 3 (Spectrum Medical Industries, Inc.) membrane tubing was first autoclaved, at 20 lb./sp.in. for 10 minutes on liquid cycle, immersed in water, and allowed to cool before it was used. The DNA solution was transferred to the tubing which was then placed in two litres of TE buffer. The solution was dialysed for several hours, before fresh TE buffer was added and dialysis continued overnight.

b) Maxi-prep Wizard Kit

DNA was purified using the Wizard™ *plus* Maxipreps DNA Purification System (Promega®) according to the manufacturer's instructions. A previously grown 500 ml cell culture was transferred to sterile 250 ml centrifuge bottles and centrifuged for 10 minutes at 8,000 rpm and 4°C. The cell pellet was resuspended in Cell Resuspension Solution (50 mM Tris-HCl, 10mM EDTA, 100 µg/ml RNase A; pH 7.5). The cell pellet was completely resuspended by vortexing. Afterward, 15 ml of cell lysis solution (0.2M NaOH, 1% SDS) was added and mixed by inversion until the solution turned clear (20 minutes). Subsequently, 15 ml of neutralisation solution (1.32M potassium acetate, pH 4.8) was added and the solution was

again mixed by inversion. The solution was then centrifuged for 15 minutes at 10,000 rpm and 22°C. The clear supernatant was passed through sterile filter paper (Whatmans® #1). Once the supernatant was filtered, 20 ml isopropanol was added and the solution was mixed well before centrifugation for 15 minutes at 10,000 rpm and 22°C. Following centrifugation, the supernatant was discarded, after which the DNA pellet was dried at room temperature and resuspended in 2 ml of TE buffer. The resuspended DNA was then mixed with 10 ml of Wizard® maxipreps DNA purification resin and transferred to a Wizard® Maxicolumn, which was attached to a vacuum manifold port. Once the excess liquid was removed from the column, 25 ml of Column Wash Solution (80 mM potassium acetate, 8.3 mM Tris-HCl, 40 μM EDTA, pH7.5, 55% ethanol) was added. A subsequent wash was done with 5 ml of 80% ethanol. The column was allowed to dry for 5 minutes before the DNA was eluted in 2 ml of preheated (65°C to 70°C) water. This was accomplished by spinning the column in a 50cc screw-cap centrifuge tube at 2,500 rpm for 5 minutes. The eluted DNA was passed through a 0.2 micron Luer-Lok® filter before storage at 4°C.

E. DNA and RNA Concentration Determination

The purity of DNA or RNA was determined by measuring the absorbance of the sample at 260 nm and 280 nm (Hitachi U-2000 Spectrophotometer). An OD_{260}/OD_{280} of 1.8 to 2.0 indicated a pure preparation.

The DNA concentration was determined by measuring the absorbance at 260nm and applying the following formula: $[DNA]=(A_{260} \times \text{Dilution factor} \times 50)/1000$ μg/μl. For the determination of RNA concentration, the following equation was applied: $[RNA]=(A_{260} \times \text{Dilution factor} \times 40)/1000$ μg/μl.

F. *Restriction Enzyme Digestion*

Restriction enzymes were used according to the manufacturer's (NEB) recommendations. A typical digestion contained about 2μg of DNA, 2μl of 10x NEBuffer (supplied by the manufacturer) supplemented with 100μg/ml BSA (if specified by the manufacturer, depending on the restriction enzyme used), 2 to 5 units of restriction enzyme, and sterile distilled water to a final volume of 20 μl. The digestion was allowed to incubate in an oven incubator at 37°C for 4 to 8 hours before the samples were analysed by gel electrophoresis.

G. *CIP/SAP Treatment of DNA*

1. Calf Intestinal Phosphotase (CIP)

CIP was used according to the manufacturer's (NEB) recommendations. A dephosphorylation reaction with CIP typically contained 1 to 5μg of vector DNA, 0.2μl (1 unit) of CIP, 2 μl of supplied CIP buffer (50mM NaCl, 10mM Tris-HCl, 10mM $MgCl_2$, 1mM Dithiothreitol, pH 7.9) and sterile distilled water to a final volume of 20 μl. The reaction was incubated at 37°C for one hour before the DNA was phenol extracted and ethanol precipitated.

2. Shrimp Alkaline Phosphotase (SAP)

The shrimp alkaline phosphatase was used according to the manufacturer's recommendations (Boehringer Mannheim®). About 50 ng of linearized vector DNA were mixed with 2 μl of the SAP buffer provided (500mM Tris-HCl, 50mM $MgCl_2$, pH 8.5), 1 μl of SAP (1unit/μl) and sterile distilled water to a final volume of 20 μl. The reaction was incubated at 37°C for 10 minutes, after which the enzyme was inactivated at 65°C for 15 minutes.

H. Klenow Treatment of DNA

Klenow Fragment (NEB) is a proteolytic product of DNA Polymerase I. Klenow retains the polymerase activity, but it has lost 3'→5' and 5'→3' exonuclease activity through mutation and proteolytic cleavage respectively. Klenow was used according to the manufacturer's recommendations. In fill-in experiments, 1 μg of DNA was mixed with 2 μl of the supplied Klenow (10 units) buffer (100mM Tris-HCl (pH 7.5), 50mM MgCl2, 75 mM dithiothreitol), 33 μM of deoxynucleotide triphosphate (dNTP) and 1 unit of Klenow (5 units/μl). The reaction mixture was incubated at 25°C for 15 minutes, phenol extracted, and ethanol precipitated.

I. Polymerase Chain Reaction (PCR)

Primers used in PCR (Saiki *et al.* 1985; Mullis *et al.* 1986) experiments were designed using VECTOR NTI 4.0 software (InforMax) and synthesised by Procyon Biopharma Inc. (London, ON.). The primers were designed to be 20 to 26 nucleotides in length, possess a G/C content of more than 50%, and a have a melting temperature of 58°C to 65°C. PCR reactions typically contained 10 to 50ng of template DNA, 50 to 250 μM of dNTP mix (equal amounts of dATP, dTTP, dGTP and dCTP), 10 to 100 μM of forward and reverse primer, 0.25 to 2.5 mM $MgCl_2$, 0.2 μl of *Taq* Polymerase (MBI Fermentas; 5 units/μl), 5 μl of supplied Taq buffer (100mM Tris-HCl (pH 8.8), 500 mM KCl and 0.8% nonidet P40) and sterile, distilled water up to a final volume of 50 μl. The reactions were run in a SingleBlock™ System (EasyCycler™ Series, ERICOMP INC.). The samples were initially deantured for 3 minutes at 94°C. The template was amplified in 32 PCR cycles consisting of the following: denaturation (1 minute at 94°C), primer annealing (2 minutes at 55 °C) and primer extension (3 minutes at 70°C). Afterward, a final primer extension was done at 70°C for 10 minutes.

J. Purification of DNA Fragments

1. GeneClean®

GeneClean® (BIO 101 Inc.) was used to purify DNA fragments from agarose gels, according to the manufacturer's recommendations. Generally, the desired DNA band from a previously run gel was observed under a UV transilluminator, excised, transferred to an Eppendorf tube, and weighed. A volume of 3 M NaI equal to three times the weight of the agarose band was added to the tube. The agarose was then allowed to dissolve at 55°C before 5 µl of GlassMilk® was added, mixed and allowed to incubate at room temperature for 5 minutes. The sample was then centrifuged at 12,000 rpm for 5 seconds and the supernatant was decanted. The GlassMilk® pellet was washed three times with New Wash (provided by the manufacturer, stored at -20°C). The pellet was allowed to dry before its resuspension in 30µl of sterile distilled water. The sample was incubated at 55°C for 3 minutes in order to elute the DNA, and then centrifuged at 12,000 rpm. The supernatant was collected and stored at 4°C for future work.

2. Phenol:chloroform Extraction

Phenol:chloroform extraction was used to remove proteins from DNA samples. The phenol:chloroform mixture contained 25:24:1 of phenol:chloroform:isoamyl alcohol respectively (stored at 4°C). The mixture was equilibrated to a pH of 7.8 to 8.0 using Tris HCl (pH 8.0). An equal volume of phenol:chloroform mixture was added to the sample, mixed by inversion several times, and centrifuged at 13,000 rpm for 5 minutes. The upper layer was transferred to a fresh tube and 3M sodium acetate, pH 7.5, (10% of the sample volume) was added. The DNA was then ethanol precipitated, air dried and resuspended in a suitable volume of sterile water or TE buffer. The DNA was stored at 4°C.

K. Gel Electrophoresis and Documentation

Agarose gels were made using 0.6 to 1% w/v agarose in TAE buffer (40 mM Tris base, 10mM sodium acetate, 1.8 mM EDTA; pH adjusted to 7.8 using glacial acetic acid). The mixture was heated to boiling before it was allowed to cool to 50°C. Ethidium bromide was added to a concentration of 0.25μg/ml. The mixture was then poured into a polycarbonate cast to solidify. The gel was then transferred to an electrophoresis box and TAE buffer was used as the running buffer.

Typically, 18 μl of digested DNA was mixed with 3 μl of loading buffer (50% sucrose, 10mM EDTA, 1% SDS, 0.1% bromophenol blue; filtered through #1 Whatman® paper) prior to loading into the pre-cast gel. A lambda DNA marker (lambda HindIII digest; NEB) was used to estimate the size of the sample DNA bands. The samples were electrophoresed at 5 to10 volts/cm using a BIORAD™ 200/2.0 power supply. Subsequently, the bands were observed under a UV transilluminator box (Fisher Scientific® 312 nm variable intensity transilluminator FBTIV-614) and photographed using the Snappy® program (Play Incorporated™) via a video camera.

L. Mammalian Cell Lines

MDBK cells (Bovine Kidney, S. Madin and N.B. Darby; ATCC Number: CCL-22) and their derivative M5 cells were the main cell lines used for viral growth, plaque assays, and transfections. The following cell lines were also used and tested for permissivity to BAV2 and efficiency of DNA uptake: Bu (IMR-31 Buffalo lung, S.B. Dwight; ATCC Number: CCL 40), EBTr (Embryonic bovine trachea, A.J. Kniazeff, W.A. Nelson-Rees and N.B. Darby; ATCC Number: CCL 44), BT (Bovine turbinate, A.W. McClurkin; ATCC Number: CRL 1390), Bln (Bovine lymph node, ATCC Number: CRL 6017), FB2.K (Bovine Kidney, ATCC Number: CRL 6033), FB5.Bm (Fetal bovine bone marrow, ATCC Number: CRL 6043), FB5.Ln (Fetal

bovine lymph node, ATCC Number: CRL 6044), LB9.K (Bovine Kidney, ATCC Number: CRL 6055), LB10.K (Bovine Kidney, ATCC Number: CRL 6061) and SJMRF (Buffalo, ATCC Number: CRL 6072).

Cells were grown as described in Burleson *et al.* (1992), in 2 to 20ml Minimum Essential Medium (MEM) supplemented with 2mM L-Alynyl-L-Glutamine (Gibco BRL supplied in 0.85%NaCl), 0.225% w/v sodium bicarbonate, 10% donor or horse serum, and 1% antibiotic (contains 10,000 units/ml of penicillin, 10,000 µg/ml of streptomycin, 25 µg/ml of amphotericin B in 0.85% NaCl).

Cells were grown to 80% to 90% confluency before they were passaged at a ratio of 1:4. The cell monolayer was initially washed with 1 to 5 ml of versene (0.53mM EDTA, 0.137M NaCl, 2.7mM KCl, 3.2mM Na_2HPO_4 , 1.1mM glucose, 1.47mM KH_2PO_4; sterilised by autoclaving for 15 minutes at 15 lb/sq.in. on liquid cycle) prior to incubation in 1 to 10 ml versene and 0.25 to 2 ml trypsin (2.5g/L trypsin and 0.85 g/L NaCl; Gibco BRL) at 37°C. The cells were allowed to lift off the plate for 5 minutes before they were transferred to a 50cc tube and centrifuged at 1,500 rpm for 10 minutes. The cells were then resuspended in growth medium, and transferred to appropriately sized plates before they were returned to the CO_2 incubator (Fisher Scientific IsoTemp® incubator; Model 546). The cell culture plates (35mm plate surface area: 962mm^2; 100mm plate surface area: 7,854mm^2; 150mm pate surface area: 17,671mm^2) were normally seeded at a density of 300 cells/mm^2. The incubator was kept humid by placing a bowl of sterile water inside. The CO_2 level was kept at 5% and the temperature was maintained at 37°C.

M. Fetal Calf Primary Cell Culture

Fetal bovine lung and kidney tissues were obtained from a deceased pregnant cow (Kingma Meat Products Ltd., Wellandport, Ontario). The tissues were rinsed with PBS buffer,

dissected into 1 cm^3 fragments and treated with trypsin for one hour at room temperature. Afterwards, the resulting mixture was filtered through sterile cheese cloth (Sigma), centrifuged for 10 minutes at 1000 rpm and 22°C. The cell pellet was resuspended in growth medium, as described in the previous section, and cultured in 60 mm cell culture plates. The plates were placed in a CO_2 incubator at 37°C and the media was replaced every three days.

N. Mammalian Cell Lines Frozen Stocks

Cells from confluent plates were lifted off the plate using versene and trypsin, centrifuged for 10 minutes at 1,500 rpm and 4°C, and resuspended in donor serum that was supplemented with 10% dimethyl sulfoxide (DMSO). Afterwards, cells were transferred to 2 ml tubes and kept at -80°C for 16 hours before they were transferred to a liquid nitrogen cryogenic tank for storage.

O. Cell Line Transfections

In this study, two different transfection techniques, calcium phosphate and lipofectamine® transfection, were used to assess DNA uptake, attempt to rescue a mutant or establish a cell line.

1. Calcium Phosphate

The calcium phosphate transfection prodedure was performed according to Graham et al. (1973). Preceding transfection, cells were subcultured the night before and plated at 300 cell/mm^2 (300,000 cells in a 35 mm plate); fresh media was added the following morning. To each 35 mm plate, 5 µg of plasmid DNA was mixed in TE buffer (pH 8.0, filter sterilised) to a final volume of 75 µl with 25 µl of 2.5 M $CaCl_2$ (filter sterilised). The DNA-$CaCl_2$ mixture was slowly added to 100 µl of 2x Hepes-buffered saline solution (280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO_4.2H_2O$, 12 mM dextrose, 50 mM HEPES; filter sterilised). The solution was allowed to incubate at room temperature for 30 minutes to allow for the formation of calcium phosphate-DNA precipitate. Subsequently, the mixture was added to the cells and allowed to

incubate in a CO_2 incubator at 37°C. The media was replaced after 3 to 5 hours. The cells were permitted to grow for 24 to 48 hours post-transfection, and depending on the experiment, were subjected to selection for antibiotic resistance, stained for *lac*Z activity, or tested for viral growth.

2. LIPOFECTAMINE™

LIPOFECTAMINE™ (Gibco BRL®) reagent is a 3:1 (w/w) liposome mixture of 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) (Chemical Abstracts Registry name: N- [2- (2,5- bis[(3- aminopropyl)amino]- 1-oxpentyl}amino)ethyl] -N,N- dimethyl- 2,3- bis (9- octadecenyloxy)- 1- propanaminium trifluoroacetate), the polycationic lipid, and dioleoyl phosphatidylethanolamine (DOPE), the neutral lipid, in water. It is suitable for the transfection of DNA into cultured eukaryotic cells.

a) Transfection Optimisation

The optimal transfection protocol was determined by interpretation of the results in this thesis using the plasmid pCMVβ, a plasmid containing the *E.coli lacZ* gene under the control of the human cytomegalo virus (CMV) promoter. In a 35mm tissue culture plate, approximately 3 x 10^5 cells were seeded in 2ml of growth medium supplemented with serum and antibiotics. The cells were then incubated in a CO_2 incubator at 37°C until the cells were 50% to 60% confluent

For each transfection, the DNA (0.1, 0.2, 0.4, 0.6, 0.8, 1.6, 3.2 μg) were diluted into 100μl serum-free MEM medium. In another tube, 2.5μl of Lipofectamine™ reagent was diluted into 100μl serum-free MEM medium. The two solutions were combined, mixed gently, and incubated at room temperature for 30 minutes. Meanwhile, the cells were washed with 1 ml of serum-free medium and then covered with 2 ml of serum-free medium. The lipid-DNA complexes were added onto the washed cell monolayer.

The cells were incubated for 4 hours in a CO_2 incubator at 37°C; following the removal of the transfection mixture, 2 ml of growth medium containing 12% serum and 1% antibiotic was

added. Cells were stained for *lac*Z activity 24 to 48 hours post-transfection. The optimum conditions for transfection were observed when the following procedure was used: 0.4 μg of plasmid DNA and 2.5 μl of lipofectamine® with a cell incubation time of 4 to 6 hours.

This procedure was also used to transfect DNA for stable expression and establishment of cell lines. When the plasmid pCMV-E1-neo was used, the cultures were not stained post-transfection. Althernatively, cells were passaged at a ratio of 1:10 into growth medium containing Geneticin (GibcoBRL®), selective for the reporter *neo* gene.

3. LIPOFECTAMINE PLUS ™

The Lipofectamine Plus™ tranfections were performed according to the manufacturer's recommendations and carried out in the same manner as the normal Lipofectamine™ transfection, described in the previous section. The only modification was the addition of 3 μl of PLUS reagent (for 35 mm cell culture plates) to the plasmid DNA prior to mixing the DNA with the Lipofectamine™.

4. FuGENE ™

FuGene ™ (Beohringer Mannheim®) reagent is an exclusive blend of non-liposomal lipids and other compounds in 80% ethanol. The manufacturer promises minimal cytotoxicity for eukaryotic cells and that transfections can be performed in the presence of a normal serum concentration.

Prior to transfection, 3 x 10^5 cells were seeded in a 35mm plate with 2 ml of growth medium. The following day, 4 μl of FuGENE ™ reagent were added to 100μl of serum-free medium. The mixture was allowed to sit at room temperature for 5 minutes, after which the diluted FuGENE ™ reagent was added drop-wise to another tube containing 1 μg of plasmid DNA. The FuGENE ™ -DNA complex was allowed to form by incubating at room temperature

for 15 minutes. Following incubation, the transfection mixture was added drop-wise to the cells, which were already covered with 2 ml of growth medium, ensuring even dispersal. The cells were incubated in a CO_2 incubator at 37°C for 24 hours before they were stained for *lacZ* activity.

5. Electroporation

Electroporation (Newmann et al., 1982) is the application of brief, high-voltage electrical pulses which cause the formation of small (nanometers in diameter) pores in the cell membrane. Subsequently, the DNA can be directly picked up through these pores into the cytoplasm and then into the nucleus for expression.

A confluent 100 mm plate (8.5×10^6 cells) was used per transfection. The cells were lifted off the plate with trypsin and verscene, washed with phosphate-buffered saline solution (PBS; 140mM NaCl, 2.6mM KCl, 4mM Na_2HPO_4, and 1.5mM KH_2PO_4, pH 7.4 , sterilised by autoclaving), resuspended in 0.5 ml of PBS and then kept on ice until they were ready to be electroporated. Cells were then mixed with a final DNA concentration of 1 to 40 μg/ml and electroporated at 700 v/cm, 1000Ω and 25μFD using the Gene Pulser® II (BIO-RAD) and the Gene Pulser® cuvettes (0.4 cm electrode). Typically, 50% to 80% of the electroporated cells are destroyed in the process. Therefore, the cells were diluted 1:10 with growth medium and plated in two 24-well plates. The cells were allowed to recover for 24 hours before antibiotic selection was applied.

P. Selection with Geneticin®

Cultured mammalian cell lines differ widely in their sensitivity to Geneticin (G418; Gibco BRL). The appropriate concentration used for selecting stably, transfected cells had to be determined experimentally. Twenty four to 48 hours following transfection, new medium containing G418 was added to the cells to select for those expressing the neomycin resistance.

Non-transfected cells from different cell lines were tested for resistance to the antibiotic. The concentration of Geneticin® (50 to 100 μg/ml) that was shown to have an effect on the non-transfected cells was sustained in further experiments to select for stably transfected cells. Fresh growth medium containing the antibiotic was added to the plates every 2 to 3 days until most of the cells had been killed, after which time the concentration of Geneticin® was decreased to 25 to 75 μg/ml. The cells were surveyed under antibiotic selection until they were confirmed to express the E1 region of BAV2.

Q. Isolation of Single Focus

During selection with the antibiotic G418, several foci were observed to be continuously growing. A single focus were isolated in one of two ways: (1) by lifting up the cells, diluting them 1:10 and plating them in a 24-well plate at a concentration of 1 cell/plate or (2) by using autoclaved aluminium rings to isolate the foci from the rest of the plate, lifting up the cells from inside the ring and then transferring these cells to another plate.

R. Mammalian Cell Counter Staining

Mammalian cells were grown on glass slides for 24 hours and then fixed using formalin fixation buffer (150 mM NaCl, 5% formaldehyde; pH 7.4) at 4°C for 24 hours. The cells were then rinsed with distilled water and dehydraded through graded ethanol solutions (50%, 70%, 95% and 100%) for 1 minute each. Slides were then washed in xylene for 1 minute, and air dried for 15 minutes.

Afterwards, the cells were stained with hematoxylin (Sigma) for 30 minutes, washed in 0.1X SSC for 1 minute, and then in 2X SSC (0.3 M NaCl and 0.03 M $Na_3C_6H_5O_7.2H_2O$) for 15 minutes. The slides were then treated with another cycle of successive ethanol washes, as before. Subsequently, the cells were stained with eosin (Sigma) for 30 minutes and then washed in 95%

and 100% ethanol for 3 minutes each. The slides were later soaked in xylene for 20 minutes and mounted in 50% glycerol.

S. *Histochemical Assay for lacZ Activity*

Cells previously transfected with the plasmid carrying the *lacZ* reporter gene were stained for *lacZ* activity. The cells were washed with PBS and then fixed with 1 ml of 4% paraformaldehyde (4% parafomaldehyde in PBS; the solution was heated and the pH was adjusted to 10 to allow the paraformaldehyde to dissolve; the solution was cooled to room temperature and the pH was adjusted to 7.2). The cells were incubated at room temperature for 5 minutes, after which the solution was aspirated and the cells were washed three times with PBS. Afterward, the staining solution, 1mg/ml x-gal (5-bromo-4-chloro-3-indolyl-β-$_D$-galactopyranoside; stock of 20 mg/ml in dimethylformamide, stored at -20°C; Sigma) diluted in the mixer (100mM sodium phosphate (pH 7.3), 3mM $K_3Fe(CN)_6$, 3mM $K_4Fe(CN)_6$, 1.3mM $MgCl_2$; pH7.4), was added. The cells were incubated in a CO_2 incubator at 37°C for 16 hours before blue cells were counted for statistical analysis.

T. *Viral Strain Propagation*

BAV2, strain 19 (ATCC VR-314), was propagated in MDBK cells according to the procedure described in Burlesson *et al.* (1992). Sixteen hours after the cells were passaged, they were washed with PBS^{2+} (PBS supplemented with 1mM $CaCl_2$ and 0.5mM $MgCl_2$) and the virus was added at a concentration of 1 moi (multiplicity of infection). The cells were incubated in a CO_2 incubator at 37°C for one hour, the unattached virus was aspirated, and growth medium containing 6% serum and antibiotics was added to the plates. CPE (cytopathic effect) normally appeared in four days, after which the cells and the media were harvested and transferred to a 50 cc tube. Subsequently, the media were centrifuged for 10 minutes at 4,500 rpm and 4°C. The supernatant was transferred to fresh tubes and stored at 4°C. The cells were resuspended in PBS

and put through three freeze-thaw cycles, using liquid nitrogen and a 37°C water bath. Afterwards, DMSO (10% final concentration) was added before the samples were stored at -20°C.

U. Hirts Extraction

The procedure of Hirts extraction was performed as modified by Haj-Ahmad and Graham (1986). Typically, the Ad particles were first resuspended in TE buffer and mixed in equal volume with 2x lysing buffer (20mM EDTA, 20 mM Tris HCl, 1% SDS; pH 8.0), supplemented with 1mg/ml pronase (20mg/ml stock in 10mM Tris HCl, 10mM NaCl, pH 7.5; stored at -20°C; Boehringer Mannheim). The mixture was incubated in an oven incubator at 37°C for 16 hours, after which it was extracted with phenol/chloroform/isoamyl alcohol and then centrifuged at 8,000 rpm for 10 minutes. The upper aqueous layer was measured using a graduated cylinder and transferred to a fresh tube. A volume of 3 M sodium acetate, equal to one tenth the volume of the aqueous layer, was added. After mixing, 95% cold ethanol, equal to twice the total volume of the solution, was added to the tube. The solution was mixed and centrifuged for 15 minutes at 10,000 rpm and 4°C. The DNA pellet was washed with cold 70% ethanol and allowed to dry at room temperature. The DNA was resuspended in an appropriate volume of sterile water or TE buffer depending on the amount of viral DNA recovered.

V. Plaque Assay

The plaque assay technique (described in Burlesson *et al.*,1992) was used to determine the number of infectious particles in a purified stock of Ad. Generally, MDBK cells were plated in 35 mm 6-well plates at a concentration of 300 cell/mm^2 (300,000 cells in a 35 mm plate). The cells were allowed to grow for 16 hours in a CO_2 incubator at 37°C, after which the medium was vacuum aspirated and the cells were washed in PBS^{2+}. Meanwhile, ten fold dilutions (10^{-1} to 10^{-9}) of the virus stock being tested were made in PBS^{2+}. One hundred µl of each dilution was

added to duplicate plates after which the cells were incubated in a CO_2 incubator at 37°C for one hour to allow the virus to adsorb to the cell membrane. Subsequently, the unbound virus was vacuum aspirated and the cells were covered with 3 ml of the overlay mix (2 x MEM supplemented with 24 mM $MgCl_2$ and 4% serum, mixed with an equal volume of melted 1.2% noble agar). Following solidification of the overlay mix, the plates were transferred and maintained in a CO_2 incubator at 37°C until plaques could be observed. The cells were then fixed with 10% formalin for 30 minutes prior to the removal of the overlay agar layer. The cells were washed with PBS and stained with 2 ml of 1% crystal violet by incubating at room temperature for 30 minutes. Afterwards, once the staining solution was removed, the plaques were counted. The virus dilution that resulted in observable and distinct plaques was used to estimate the number of pfus (plaque forming units). To estimate this value, the number of plaques counted was multiplied by the dilution factor and the reciprocal value of the volume used. The number of pfu was reported as a concentration of pfu/ml.

W. Southern Hybridization

Localising specific sequences of DNA was accomplished using a modification of the Southern technique (Southern, 1975). About 10μg of genomic DNA was digested with the desired restriction enzyme and the bands were separated by 0.7% agarose gel electrophoresis.

1. Transfer of DNA

After electrophoresis, the unused parts of the gel were trimmed and the DNA was denatured by soaking the gel fragment in 200 ml of denaturation solution (1.5 M NaCl and 0.5 M NaOH) for 45 minutes. The gel was then rinsed with distilled water and soaked in neutralisation solution (1 M Tris, 1.5 M NaCl; pH 7.4). The neutralisation solution was replaced with fresh solution and the gel fragment soaked again for 15 minutes.

A pyrex tray with a sponge block was filled with 10 x SSC (1.5 M NaCl and 0.15 M $Na_3C_6H_5O_7.2H_2O$) until the sponge appeared completely wet and saturated with the solution. The sponge was then covered with two pieces of 3MM® Whatman paper, eliminating any air bubbles trapped between the sponge and the Whatman paper. The gel was placed on top of the Whatman paper. Cling Wrap was used to cover the exposed parts of the sponge in order to direct the flow of the solution through the gel.

A piece of nylon membrane (GeneScreen Plus®, DuPont™), the size of the gel, was wetted with sterile distilled water and then soaked in 10 x SSC for 5 minutes. Once completely soaked, the membrane was placed on top of the gel and a glass rod was used to eliminate any trapped air bubbles. The membrane was then covered with a piece of Whatman paper and topped with a 10-cm thick stack of paper towelling. The paper towelling was weighed down with a 500 g weight. Transfer of DNA was allowed to proceed for 16 hours after which the membrane was soaked in 6x SSC for 5 minutes, dried at room temperature, and then cross linked using UV Stratalinker™ 1800 (Stratagene®) for 1 minute. The membrane was then transferred to a hybridization cylinder for labelling.

2. Radiolabelling of DNA Probe

DNA probes were labelled using the Nick Translation System (Promega®). The kit is supplied with: (1) DNase I, which creates nicks within the DNA double strand and (2) DNA Polymerase I, which adds a new residue while the 5′→3′ exonuclease activity of this enzyme removes the old residue at the 5′ end of the nick.

A typical nick translation reaction contained 10 µl of nucleotide mix (100µM GTP, TTP, CTP), 5 µl of 10 x Nick Translation buffer (500mM Tris-HCl, 100 mM $MgSO_4$, 1mM DTT; pH 7.2), 1 µg of DNA, 7 µl [α-^{32}P]dATP (70µ Ci at 400 Ci/mmole and 10mCi/ml), and 5 µl of DNA Polymerase/DNase I mix (1µg/µl DNA Polymerase, 0.2ng/µl DNase I, 50% glycerol, 50mM

Tris-HCl, 10mM $MgSO_4$, 0.1mM DTT and 0.5 µg/µl BSA). The reaction was incubated for 1 hour at 15°C, after which 5 µl of stop solution (0.25M EDTA; pH 8.0) was added.

3. Prehybridization, Hybridization and Washing

Prehybridization and hybridization were carried out at 65°C in a Hybaid hybridisation oven (Hybaid® micro-4). The membrane was prehybridized at 65°C for 30 minutes in 10 ml of hybridization solution (1% SDS, 1M NaCl, 10% dextran sulfate supplemented with 100 µg of salmon sperm DNA). Hybridization was done in 30 ml of hybridization solution (supplemented with 100 µg of salmon sperm DNA and 50 µl of the radiolabelled probe; boiled for 5 minutes and cooled to 65°C before use) at 65°C for 16 hours. Subsequently the membrane was washed in 100 ml of 2 x SSC for 10 minutes at room temperature, then in 2 x SSC (supplemented with 0.1% SDS) for 1 hour at 65°C, and finally in 0.1 x SSC for 10 minutes at room temperature.

4. Film exposure and developing

Following hybridization, the membrane was completely dried at room temperature, placed between Cling Wrap and a sheet of Whatman paper (underneath) and placed in an X-ray cassette containing a BioMax film (Kodak®, Scientific Imaging Systems). The film was exposed for 1 to 16 hours at -80°C or room temperature, depending on the amount of radioactivity present on the membrane; it was then developed and fixed using a Kodak GBX developer and Kodak GBX fixer. The film was finally rinsed with distilled water and dried at room temperature.

X. Trizol® Extraction of Total RNA

TRIZOL®LS Reagent (Life Technologies™), a mono-phasic solution of phenol and guanidine isothiocyanate, was used to isolate total cellular RNA. After removing the growth medium, the cells in a 150mm cell culture plate were covered with 5 ml of TRIZOL®LS reagent. The reagent was then used to wash the cells off of the plate. Following the complete mixing of

the cells with the reagent, the mixture was allowed to incubate at room temperature for 5 minutes. Subsequently, the mixture was extracted using 1 ml of chloroform for 2 to 3 minutes followed by a 15 minute-centrifugation at 10,000 rpm and 4°C. The resulting upper phase was transferred to a new tube and mixed with 2.5 ml of isopropanol and then centrifuged at 10,000 rpm and 4°C for 15 minutes. The RNA pellet was washed once with 5 ml of 75% ethanol and centrifuged for 5 minutes at 10,000 rpm and 4°C. The RNA pellet was air dried and resuspended in 1ml of RNase-free water (0.1% diethyl pyrocarbonate, incubated overnight in an oven incubator at 37°C then autoclaved for 15 minutes in liquid cycle) by incubating in a water bath at 55°C to 60°C for 10 to 15 minutes. The integrity of the purified RNA was checked by analysis using 0.9% agarose gel electrophoresis.

Y. Northern Hybridization

Thirty μg of isolated RNA were mixed with 5 μl of RNA loading buffer and heated to 65°C for 20 mins prior to separating the sample by agarose gel electrophoresis (1% agarose in 0.1 M phosphate buffer, pH 6.8). The RNA was separated by electrophoresis for 4 to 6 hours at 1 v/cm using a phosphate running buffer. Following electrophoresis, the integrity of the RNA was determined under UV light. The RNA was then transferred to a nylon membrane (as described for the Southern hybridisation) without pre-treatment of the gel. Prehybridisation, hybridisation and film exposure were carried out as previously described.

IX. Results

The strategy used for developing a packaging cell line for recombinant BAV2 was patterned, with modifications, after the packaging cells used for human Ad vectors. In that system, the 293 packaging cell line, which consitutively expresses Ad5 E1 products, not only complemented the missing E1 functions in the vector intentionally removed to accommodate the size of the foreign gene insert, but also served as the standard cells for contransfection to generate other recombinant Ad regardless of the presence of E1 in the vector (Haj-Ahmad and Graham, 1986). However, since recombinant Ad5 generated through the 293 packaging cell line often co-purified with wild-type revertants, the subsequent strategy for a BAV2 packaging cell line was modified to avoid generating unwanted wild-type revertants.

The desired BAV2 packaging cell line was designed to incorporate the following properties: 1) The cell line must minimize or prevent the generation of revertants; 2) the cell line must be readily transfected; 3) the gene or genes for complementing missing viral functions must be stably integrated in the cellular genome and constitutively expressed at levels appropriate for packaging viral particles; and 4) the packaging cell line must support routine propagation of the recombinants in expanded scales.

To achieve these objectives, we focused on developing a cell line from primary bovine cells by immortalisation or complete transformation with E1 of BAV2, the rationale being that primary cells transformed by the E1 oncogenes will result in a stable cell line as far as the retention and expression of the E1 genes are concerned. Such cells will continue to grow in culture as long as E1 expression is maintained since the transformed phenotype is dependent on E1 expression (Graham *et al.*, 1977). The type of packaging cell line we hoped to isolate by this method was one capable of generating recombinants with missing E1, with or without

replacement by a foreign gene insert, and one able to support subsequent propagation of the recombinant virus.

In order to establish the desired BAV2 packaging cell line, a transforming plasmid containing BAV2 E1 coding sequences with their appropriate transcriptional control elements was constructed. Several considerations must be taken into account in the design of such a plasmid. First, it must readily transform primary cells by virtue of E1A and E1B expression. This required precise selection of BAV2 E1 sequences to be included in the transforming plasmid. Previous studies have demonstrated that BAV2 genetic organization is similar to those of the widely studied human Ads and that both E1A and E1B regions have high percentage of identity with those of human Ad E1A and E1B (Salmon and Haj-Ahmad, 1994 ; Ojkic, 1997). By simple sequence alignment and comparison, delineating those sequences comprising the complete E1A and E1B genes can be done with a high degree of accuracy. Second, the resulting cell line must avoid one of the deficiencies associated with the 293 packaging cell line, which is its propensity to produce wild-type revertants from E1-deletion/replacement mutants (reviewed in Hitt *et al.*, 1997). Studies have shown that revertants generated by 293 cells occurred by genetic recombination between homologous sequences present in the viral genome and those in 293 cells (reviewed in Hitt *et al.*, 1997). The mechanisms involved in homologous recombination are well known, hence it was possible to incorporate features into the transforming plasmid that prevents genetic recombination leading to wild-type revertants. These features are elaborated in the next section.

A. Construction of a transforming plasmid containing BAV2 E1

The strategy for constructing a plasmid intended for immortalization and transformation of primary cells with eventual use as packaging cells for BAV2 E1-deletion vectors is shown in Figure 6. The basic design includes placing the complete BAV2 E1 coding sequence under

control by a cytomegalovirus (CMV) transcriptional element (enhancer/promoter). In this design, the CMV promoter replaces the E1A promoter and all sequences from leftward on. By doing this, viral sequences at the extreme left end that can serve as an initiation site for homologous crossover are replaced by foreign sequences consisting of the CMV control elements. Elimination of crossovers near the left end effectively destroys chances for a double-crossover that is required for generating wild-type revertants (Figure 7 and Figure 8).

The overall strategy for the construction of the transforming plasmid, pCMV-E1, as shown in Figure 9, describes the series of cloning steps required to arrive at the desired plasmid. The details of each cloning step and the methods used to verify the resulting intermediate plasmids are described in the legends to the subsequent figures. Briefly, the first cloning step involved pBB12 , a plasmid containing the left 17.5% of the BAV2 genome (Salmon, 1993) as the source of E1A and E1B coding sequences. This plasmid was modified by inserting a linker into the 1.1% position to convert the *EagI* site into *BamHI* resulting in the plasmid pBB12-B (Figure 10). This step was necessary to allow fusion of the CMV promoter with the E1 sequences (Figure 11) at the 1.1% position that contained the converted *BamHI* site. The CMV promoter was obtained from the plasmid pCMV53, which is a derivative of the commercially supplied pCMVβ. The plasmid pCMV53 (Figure 12 and Figure 13) contained a convenient *BglII* site at the 3'-end of the CMV promoter to serve as the site for fusing with the E1 sequences. The resulting plasmid was called pCMV-E1 (Figure 11).

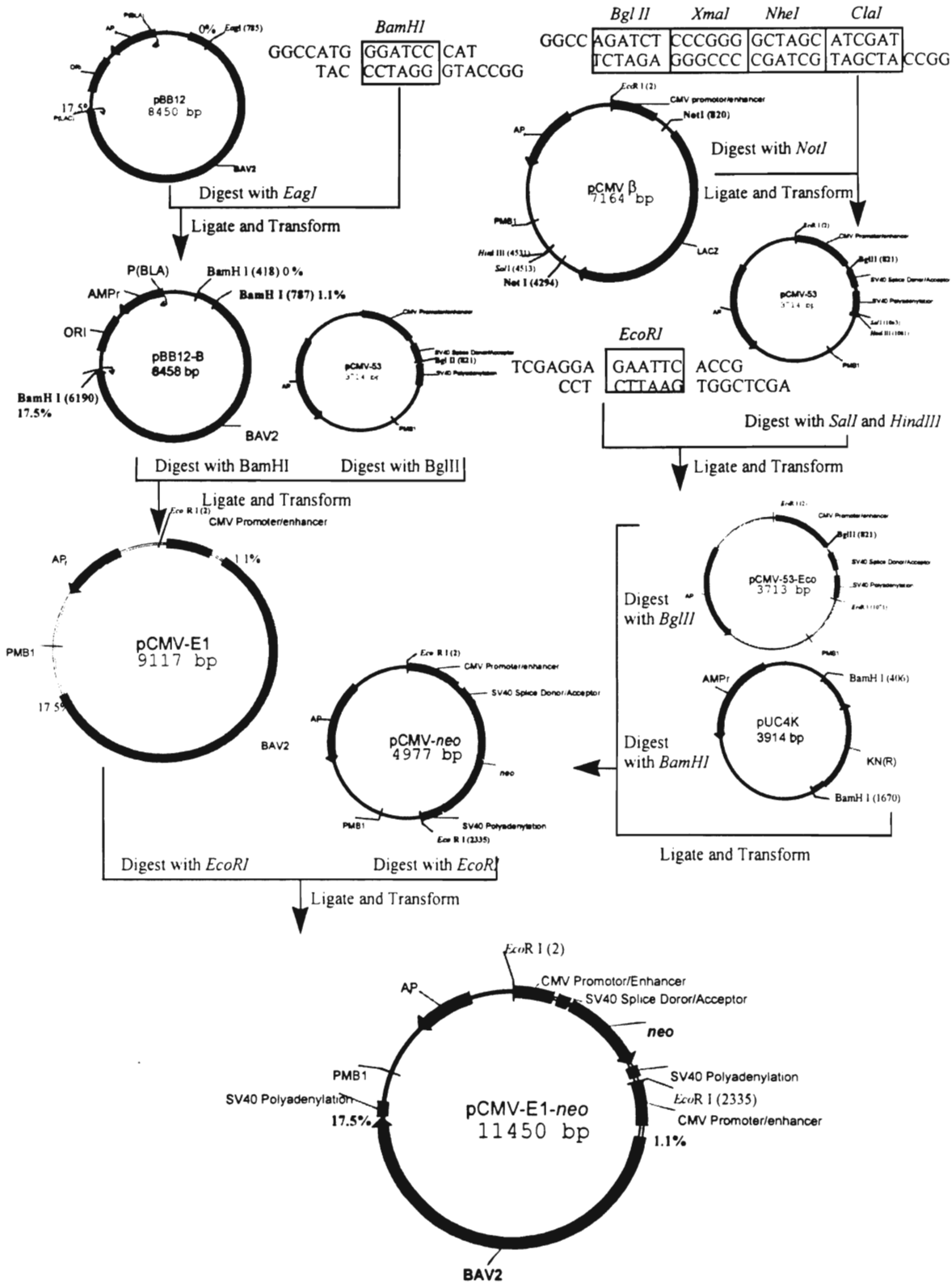


Figure 6: Overall construction strategy for the plasmid pCMV-E1-*neo*

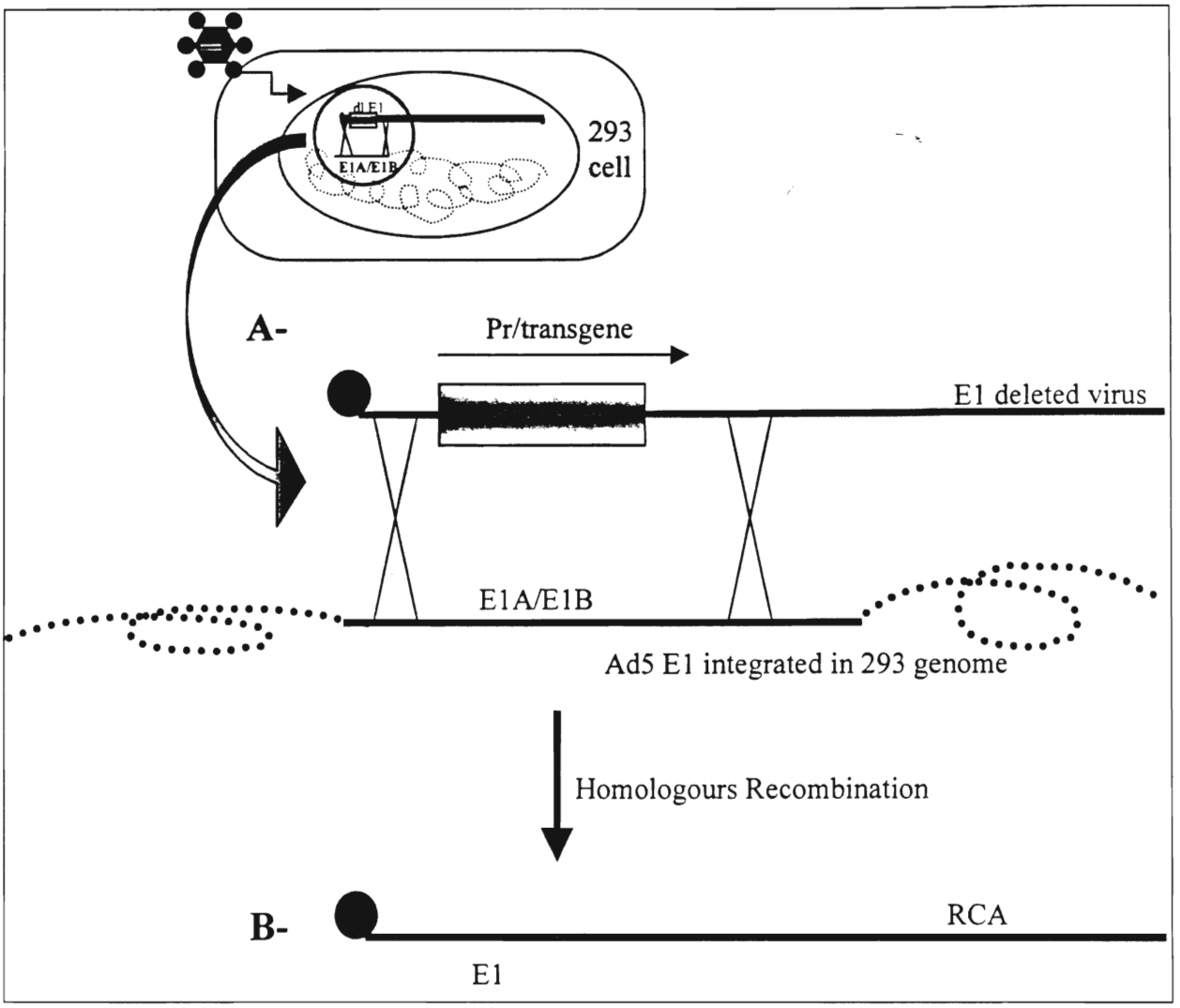


Figure 7: Ad reversion to wild-type through homologous recombination in 293 cells.

Diagrammatic representation of homologous recombinations that result in the emergence of replication competent Ad (RCA) during propagation of E1 deleted Ads in 293 cells.

A - Homologous recombination occurs between homologous sequences in the 293 chromosome and the viral genome.

B - RCA emergence as a result of a double cross over between the integrated sequences and the E1-attenuated viral genome resulting in restoration of missing E1 sequences.

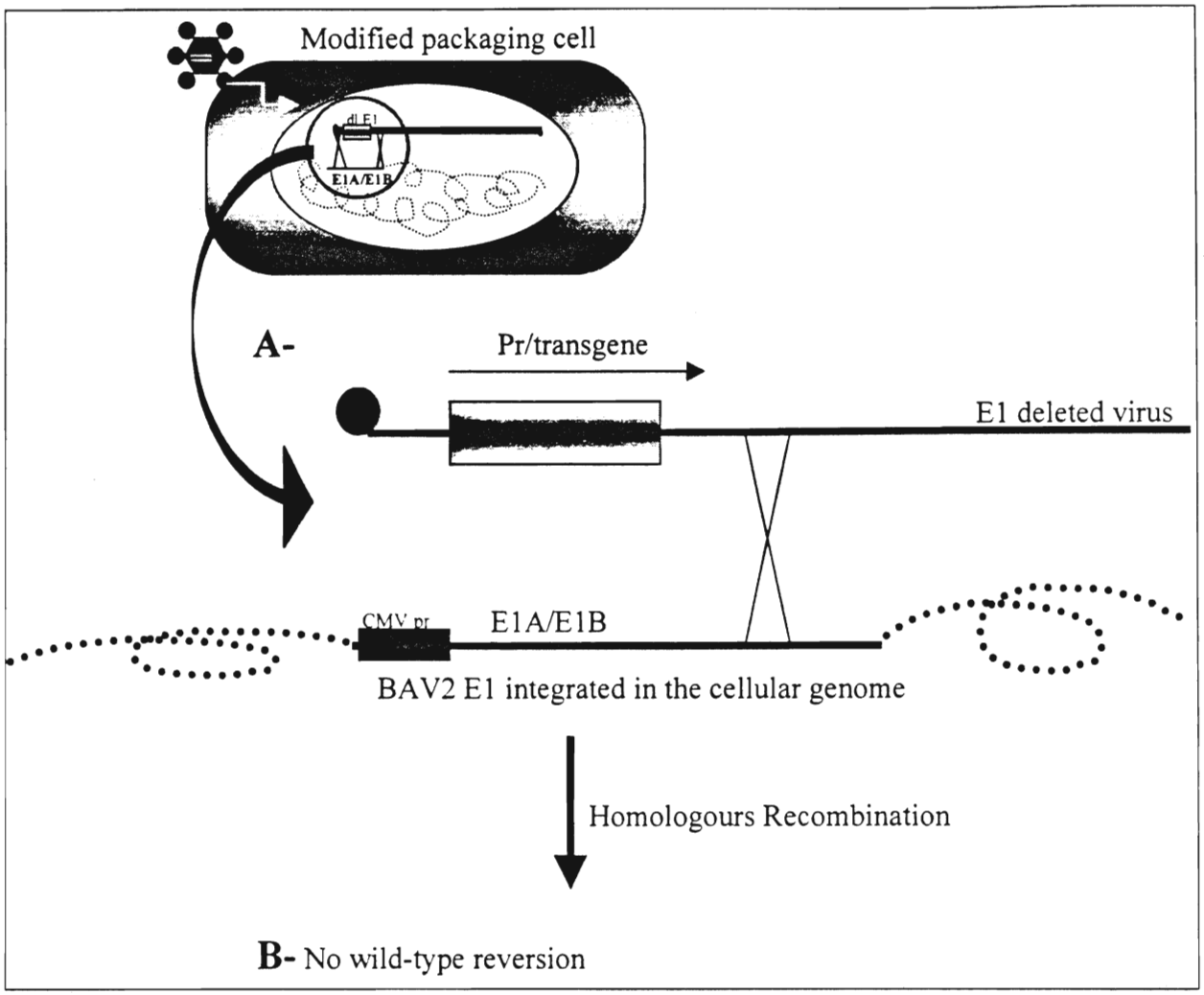


Figure 8: Prevention of generation of Ad wild-type revertants.

Diagrammatic representation of cell line engineering to minimize reversions to wild-type. **A** - E1A promoter was replaced with the CMV promoter in order to eliminate the possibility of a homologous recombination at the left end of the genome.
B – Wild-type revertants are not generated since one cross over does not restore the missing E1 coding sequences.

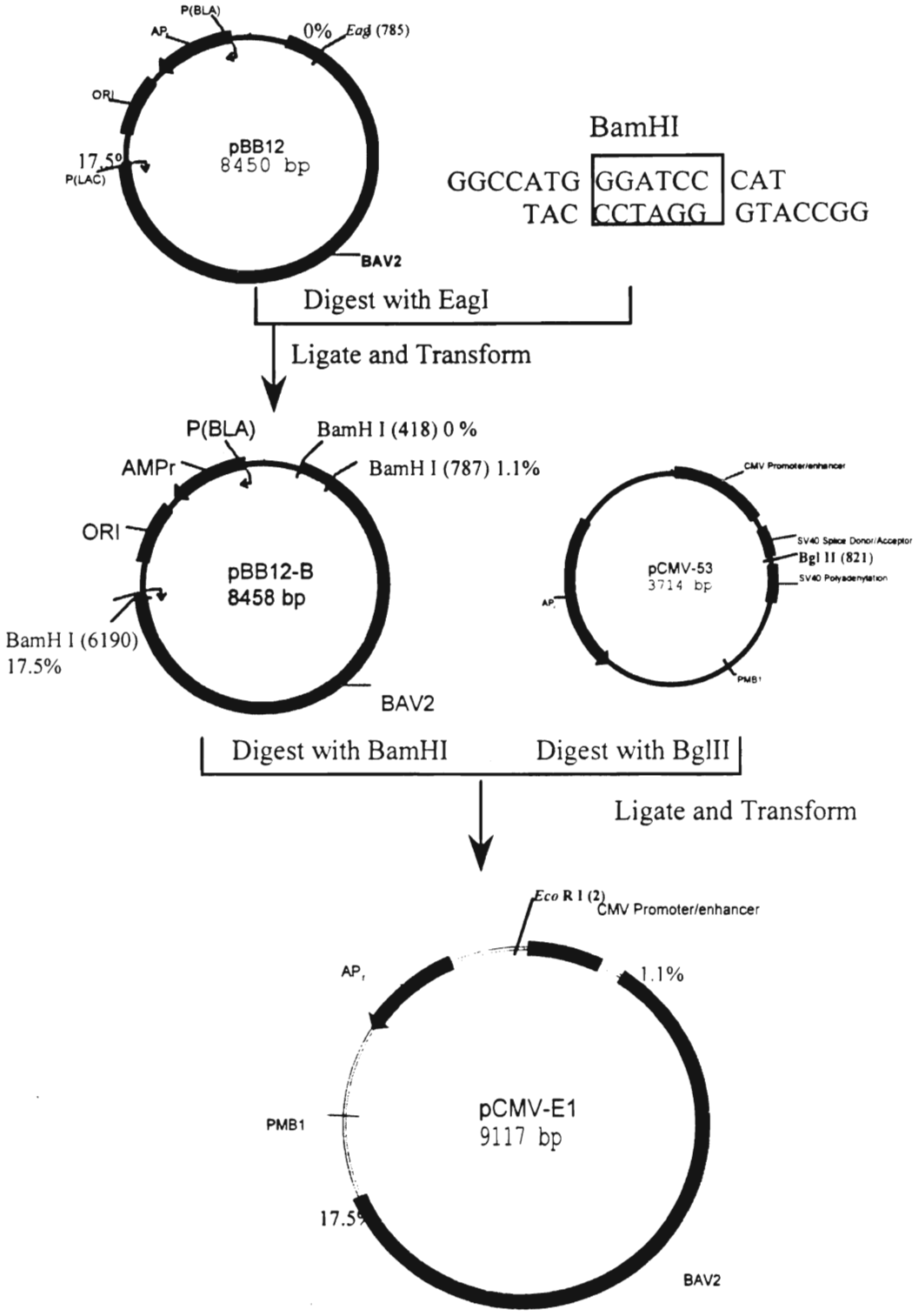
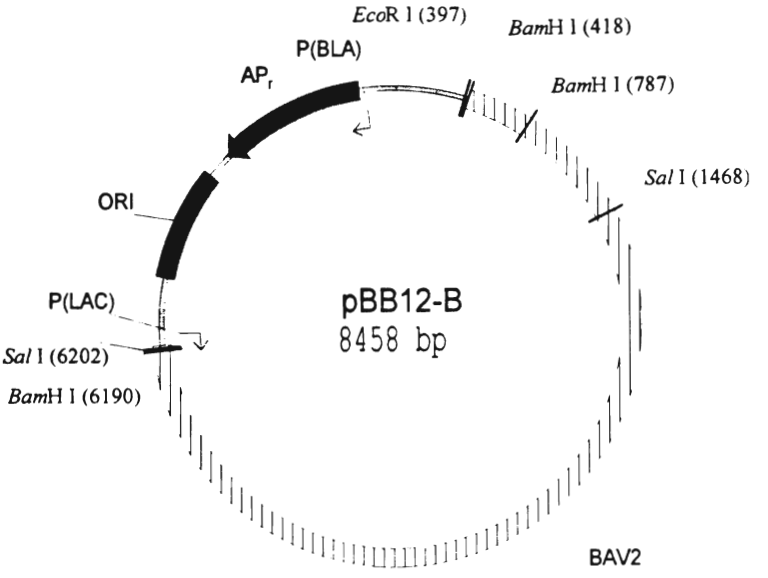


Figure 9: Strategy for constructing the plasmid pCMV-E1.

I.



II.

Enzymes	BamHI	EcoRI	SalI
Fragment Sizes (bp)	5,403	8,458	4,734
	2,686		3,724
	369		

III.

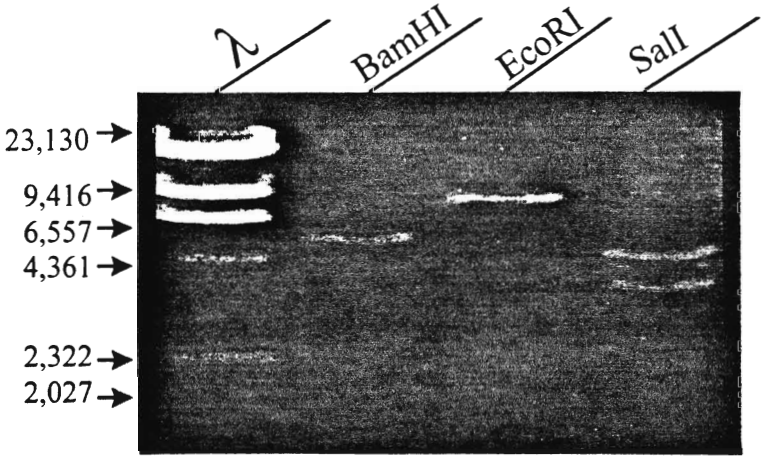
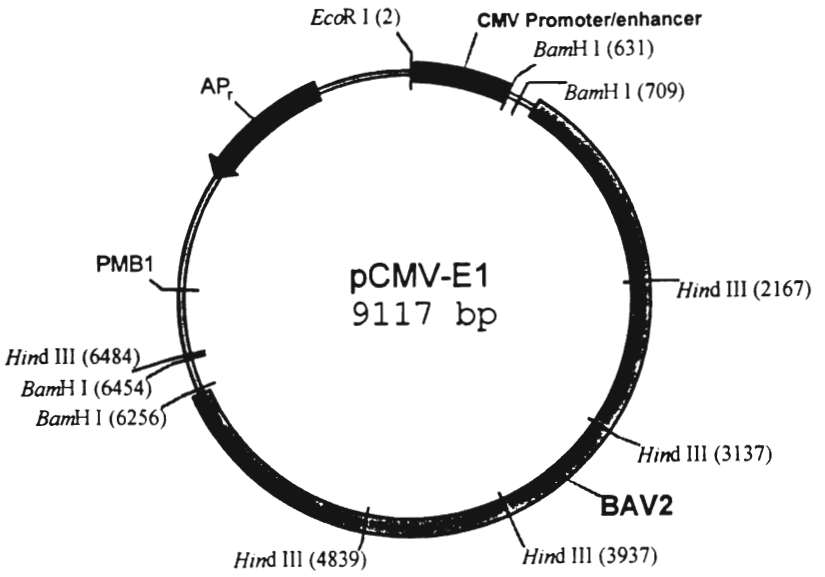


Figure 10: Restriction analysis of pBB12-B.

PBB12-B was constructed by replacing the unique EagI restriction site with a *BamHI* restriction site using a DNA linker as shown in Figure 9. (I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, shows the plasmid pBB12-B digested with *BamHI*, *EcoRI*, and *SalI* respectively.

I.



II.

Enzymes	EcoRI	BamHI	HindIII
Fragment Sizes (bp)	9,117	5,547	4,800
		3,294	1,645
		198	970
		78	902
			800

III.

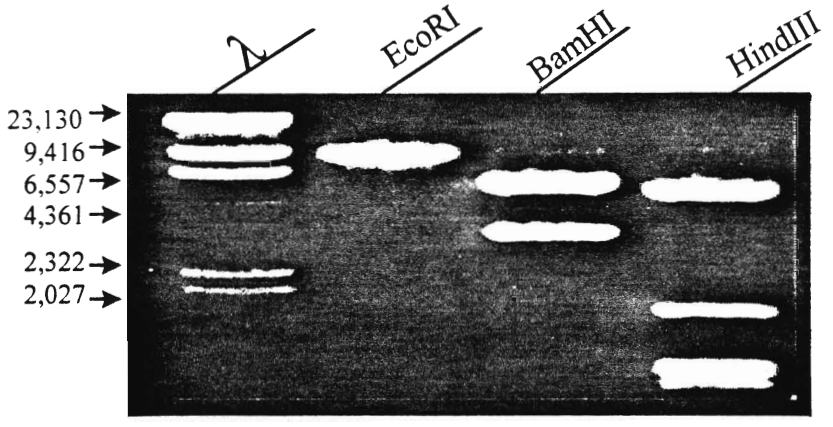


Figure 11: Restriction analysis of pCMV-E1.

The plasmid pCMV-E1 was constructed by cutting the plasmid pBB12-B with the restriction enzyme *BamHI* and the resulting 5.4kb DNA fragment was inserted into the restriction site *BglII* in the plasmid pCMV-53. The resulting plasmid, pCMV-E1, carries E1 region of BAV2 (1.1-17.5%) under the control of the CMV promoter instead of the BAV2 E1 promoter (Figure 9). (I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, shows the plasmid pCMV-E1 digested with *EcoRI*, *BamHI* and *HindIII* respectively.

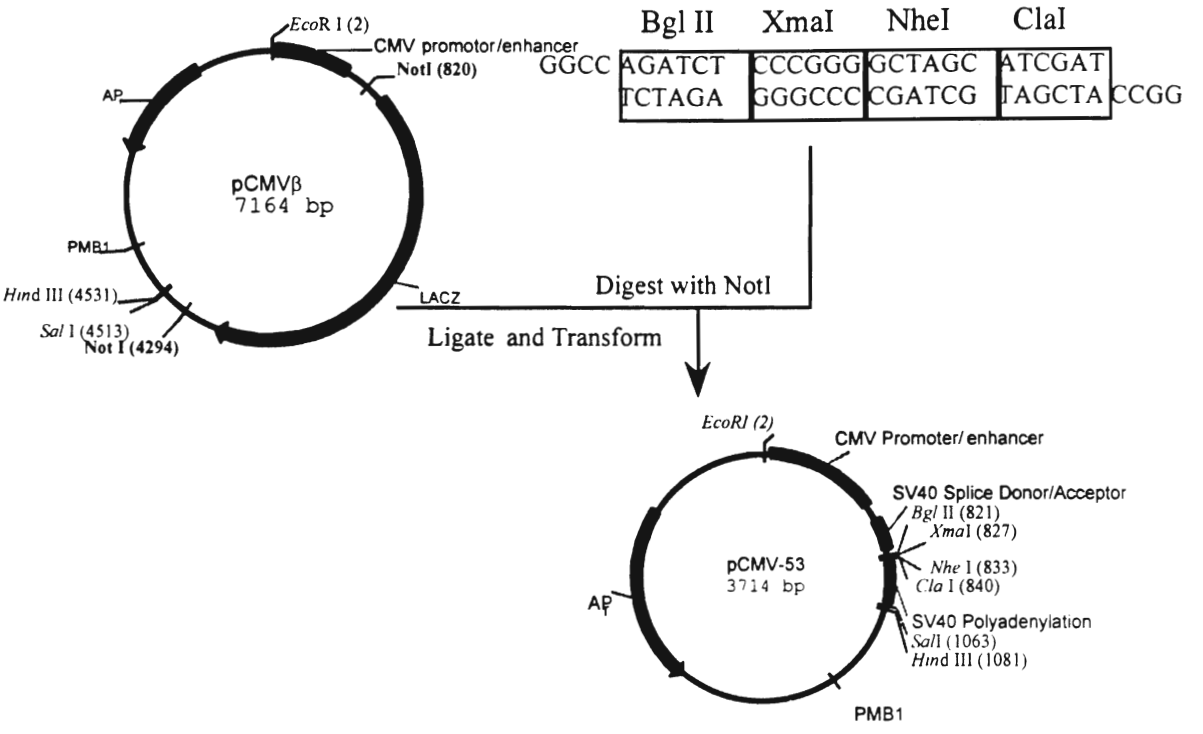
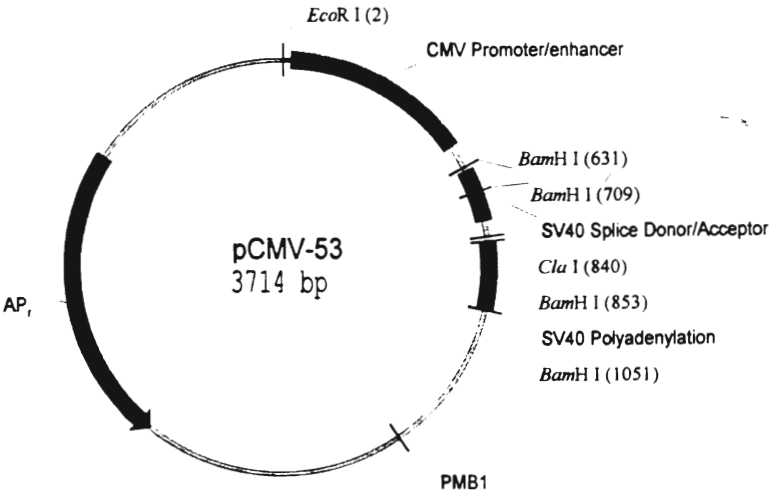


Figure 12: Construction strategy of the plasmid pCVM-53.

I.



II.

Enzymes	BglII	BamHI	HindIII	XhoI
Fragment Sizes (bp)	3,714	3,294	3,714	3714
		198		
		144		
		78		

III.

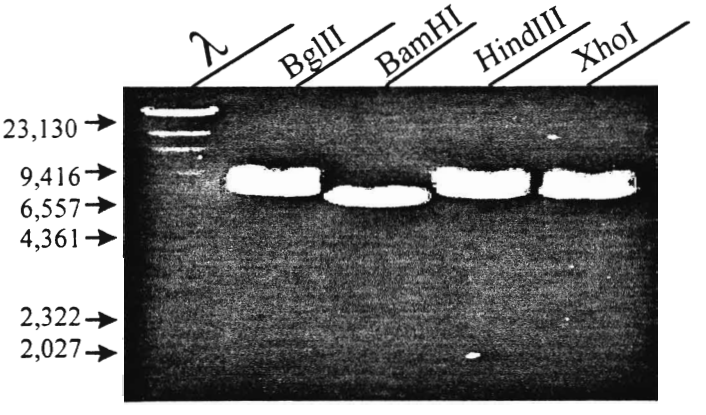


Figure 13: Restriction analysis of pCMV53.

This plasmid was constructed by cutting the plasmid pCMVβ with the restriction enzyme NotI to release the *lac*Z gene and inserting a linker at that site which would introduce a polylinker containing the restriction sites of *BglII*, XamI, NheI and *ClaI* restriction enzymes as shown in Figure 12. The orientation or the polylinker was confirmed as shown in Figure 14. (I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, and 5 show the plasmid pCMV 53 digested with *BglII*, *BamHI*, *HindIII* and *XhoI* respectively.

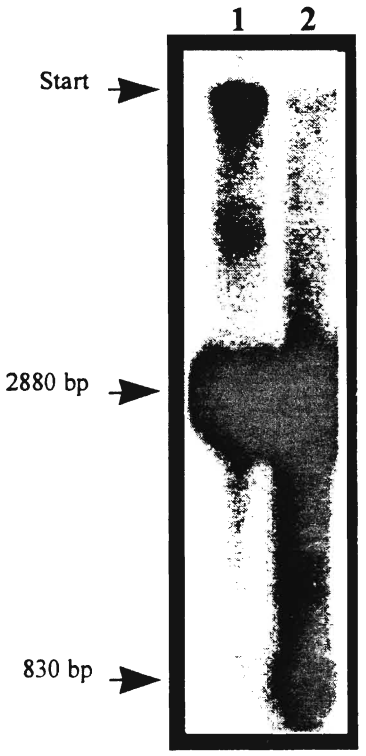


Figure 14: Orientation of the linker in the plasmid pCMV-53.

Autoradiograph of an agarose gel used to identify the orientation of the linker in the plasmid pCMV-53. Since the linker was a *NotI* insert, it could assume two orientations in the plasmid. To determine the orientation, the following experiment was done: First, the plasmid pCMV-53 was digested with the restriction enzyme *BglII*, then used klenow to fill the sticky ends in a reaction mixture that contained α-^{32}P-dATP. Then the plasmid was digested with *ClaI* and *EcoRI*. The 820 bp framgment resulting from the digestion with *EcoRI* and *ClaI* would be labelled with ^{32}P ATP only if the linker went in the following direction: *BglII*, *XmaI, NheI*, *ClaI* at 821, 827, 833, and 840 bp respectively on the plasmid. Lane 1 shows a radioactive band with an approximate size of 2880bp, but it does not show a radioactive band at 820bp which means that the orientation of the linker is the following: *ClaI*, *NheI, XmaI*, *BglII* at 821, 827, 833, and 840 bp respectively on the plasmid (referred to as the "minus" orientation). In Sample 2, another clone shows two radioactive bands, one at 2880bp and the other at approximately 820bp. The radioactive band at 820bp indicates that the linker was inserted in the opposite direction to sample 1 (referred to as the "plus" orientation). The plasmid vector from sample 2 (clone #16) was used in subsequent experiments.

B. Transfection of Embryonic Kidney and Lung Cells

Transformation of primary cells obtained from organs of a bovine fetus was carried out to explore the possibility of establishing cell lines that can serve as packaging cells for BAV2 recombinants, specifically those that are replication-deficient due to deletion of E1 functions. As previously mentioned, this approach to establishing a bovine cell line is inherently advantageous since such cells are only able to survive in cell culture if they acquired the immortalized phenotype due to E1A effects alone, or the fully transformed phenotype due to cooperative effects of E1A and E1B.

Thus, primary cells from bovine kidney and lung organs from an unborn fetus were prepared as described in Materials and Methods. The source fetus was obtained from a local slaughterhouse and appeared to be in its third trimester of gestation, judging from its size. Thus, monolayer preparations of primary cells were transfected with the plasmid pCMV-E1 using the calcium phosphate technique. Selection for outgrowers that form foci was based on the method used in transformation assays as previously described (Graham *et al.*, 1977).

Transfected cells were observed carefully for signs of foci formation for several weeks in pCMV-E1-transfected kidney or lung cell preparations and comparing them with mock-transfected controls. Monolayers of primary cells first appeared as mixture of fibroblastic and epithelial type. With or without pCMV-E1, the transfected cells continued to grow, requiring passaging at times to prevent overcrowding in the plates. Over time, there was a gradual appearance of predominantly fibroblastic cells and disappearance of those with epithelial morphology. No foci were ever observed. Eventually, the fibroblastic cells became quiescent and appeared to have stopped growing. At this stage, the cultures were discarded.

The attempt to establish immortalized cells from primary cultures of bovine kidney and lung cells was unsuccessful and it may have been due to couple reasons. First, BAV2 E1 might

have failed to transform primary bovine cells since BAV2 E1 might have an inherently poor transforming activity hence it cannot readily immortalize primary cells. The second possibility is that the bovine cells used for transfection might have already reached terminal differentiation, adding to the difficulty in isolating de-differentiated, transformed phenotype. These possibilities are further explored in the Discussion.

C. *Biochemical Transformation of Established Bovine Cell Lines*

In this effort to isolate a suitable packaging cell for BAV2, the focus was shifted to using available established cell lines from which derivatives can be developed by transfection with BAV2 E1 linked to a selectable marker. In this approach, transfected cells will be biochemically transformed by virtue of a *neo* selectable marker to be linked with pCMV-E1. The strategy was to isolate cells that constitutively express E1 from the population of G418-resistant cells (*neo*$^+$).

To link the *neo* gene with the E1-containing plasmid, pCMV-E1, the *neo* coding sequence was obtained from a commercial plasmid, pUC4K (Clonetech^{TM}). Since the *neo* gene in this plasmid did not contain a mammalian transcriptional control element, a derivative was constructed so that the selectable marker was now under the control of the CMV promoter (Figure 15). The cloning of this plasmid required an intermediate called pCMV-Eco (Figure 15 and Figure 16) that was used to obtain the plasmid pCMV-*neo* (Figure 17). The final step for linking the *neo* gene with the E1-containing plasmid involved the transfer of the *neo*-containing fragment from pCMV-*neo* into pCMV-E1, to produce the pCMV-E1-*neo* (Figure 18 and Figure 19).

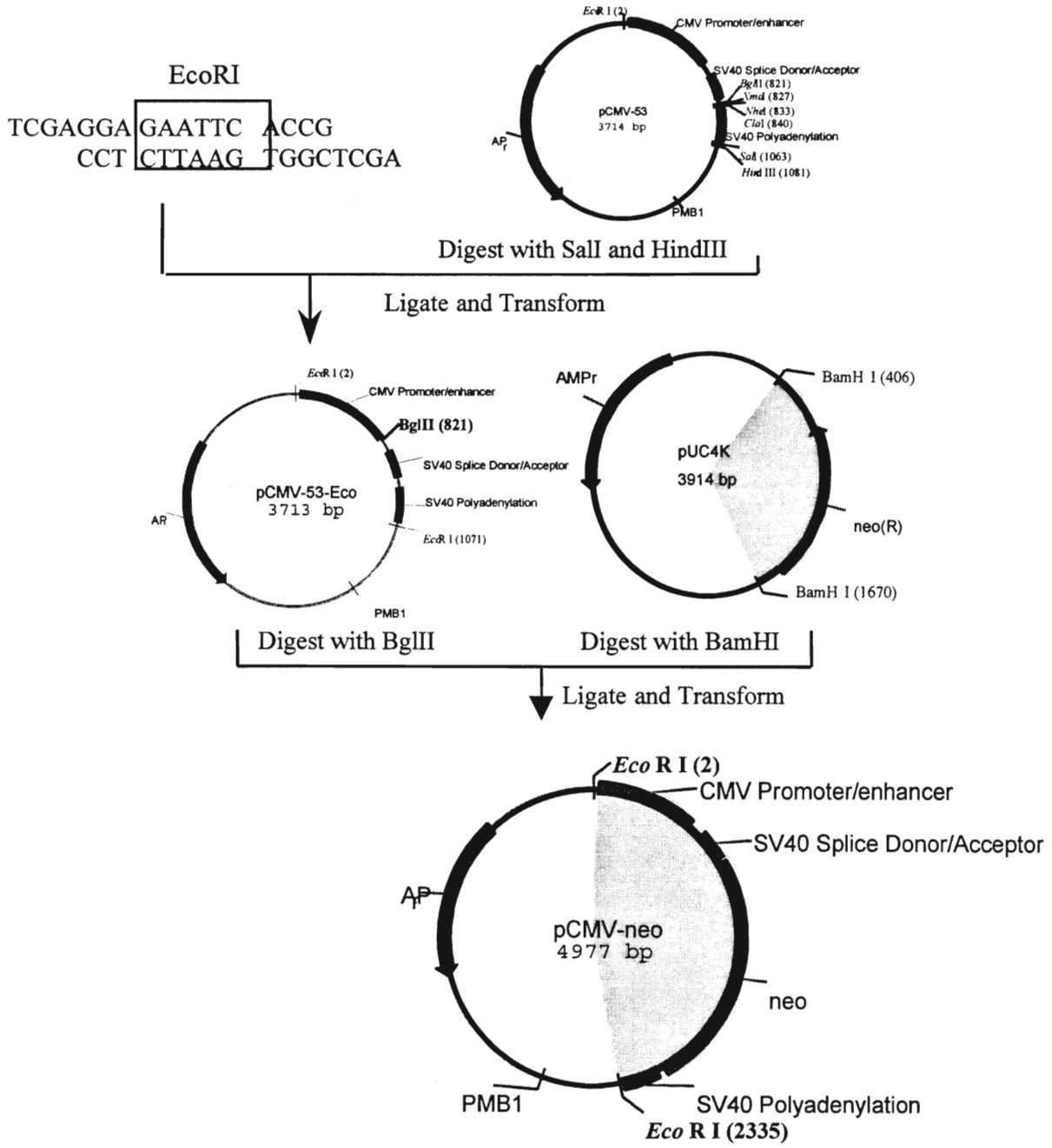
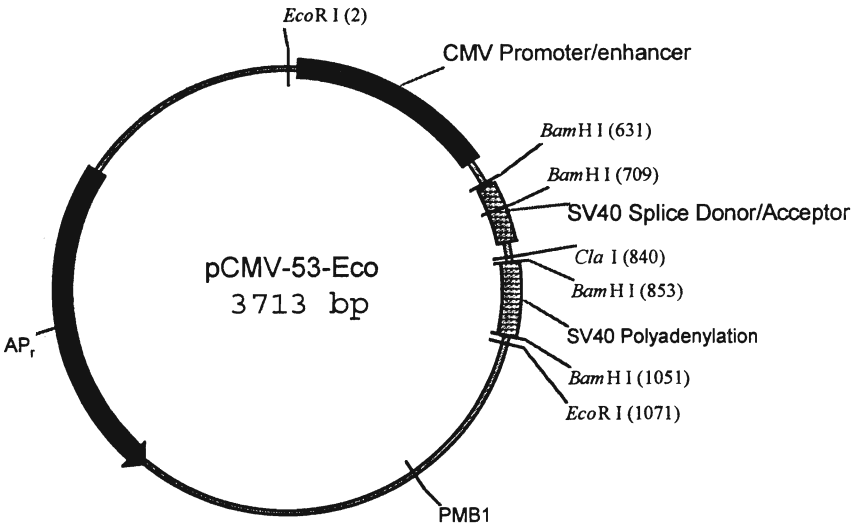


Figure 15: Construction strategy of the plasmid pCMV-*neo*.

I.



II.

Enzymes	ClaI	EcoRI	BamHI
Fragment Sizes (bp)	3,713	2,644	3,293
		1,069	198
			144
			78

III.

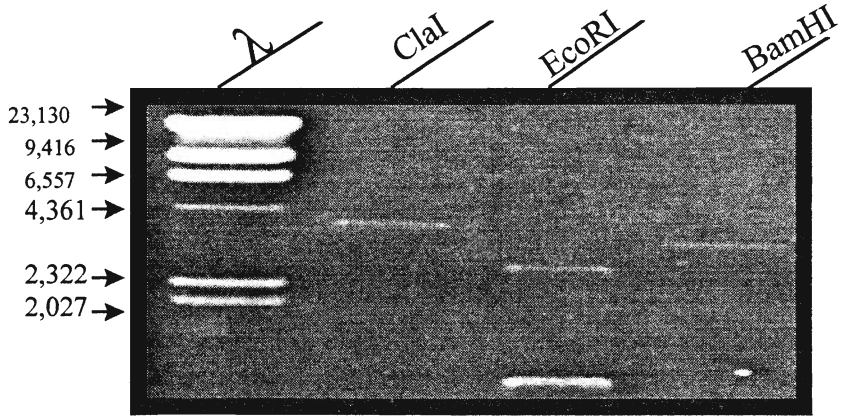
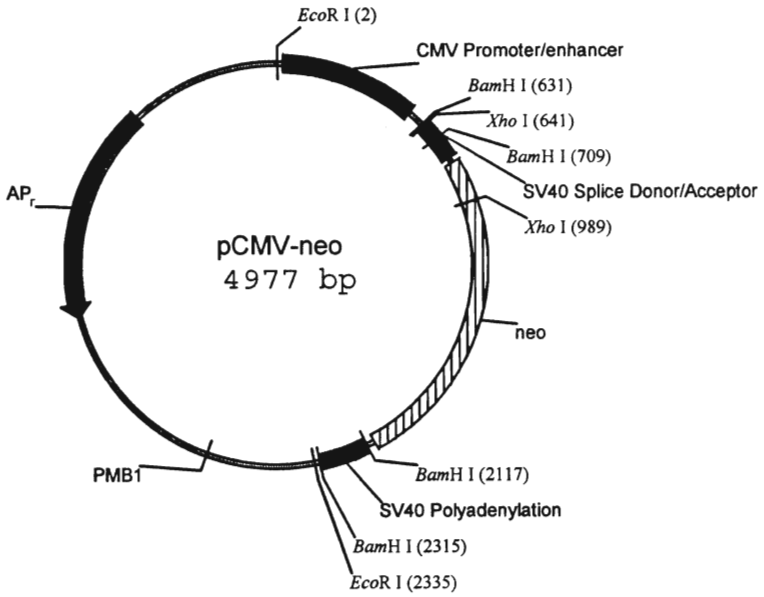


Figure 16: Restriction analysis of pCMV 53-Eco.

This plasmid was constructed by cutting the plasmid pCMV-53 with the restriction enzymes *SalI* and *HindIII* and ligating it to a linker that is compatible to the *SalI* and *HindIII* sticky ends without restoring the restriction sites but instead it introduces a new *EcoRI* restriction sequence at that site. (I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, shows the plasmid pCMV 53-Eco digested with *ClaI*, *EcoRI* and *BamHI* respectively.

I.



II.

Enzymes	BamHI	EcoRI	XhoI
Fragment Sizes (bp)	4,977	3,293	4,629
		1,408	348
		198	
		78	

III.

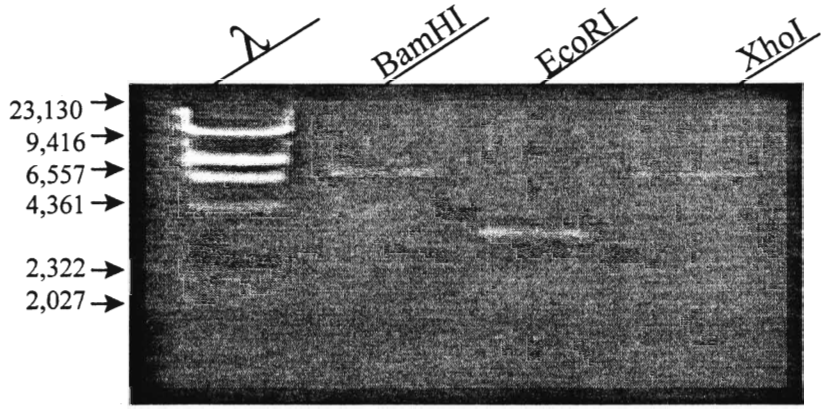


Figure 17: Restriction analysis of pCMV-*neo*.

The *neo* gene was released from pUC4K using the restriction enzyme *BamHI* and inserted into the *BglII* site, which has compatible restriction sticky ends to the sticky ends generated by the enzyme *BamHI*, in the plasmid pCMV-53-Eco. (I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, shows the plasmid pCMV-neo digested with *BamHI*, *EcoRI* and *XhoI* respectively.

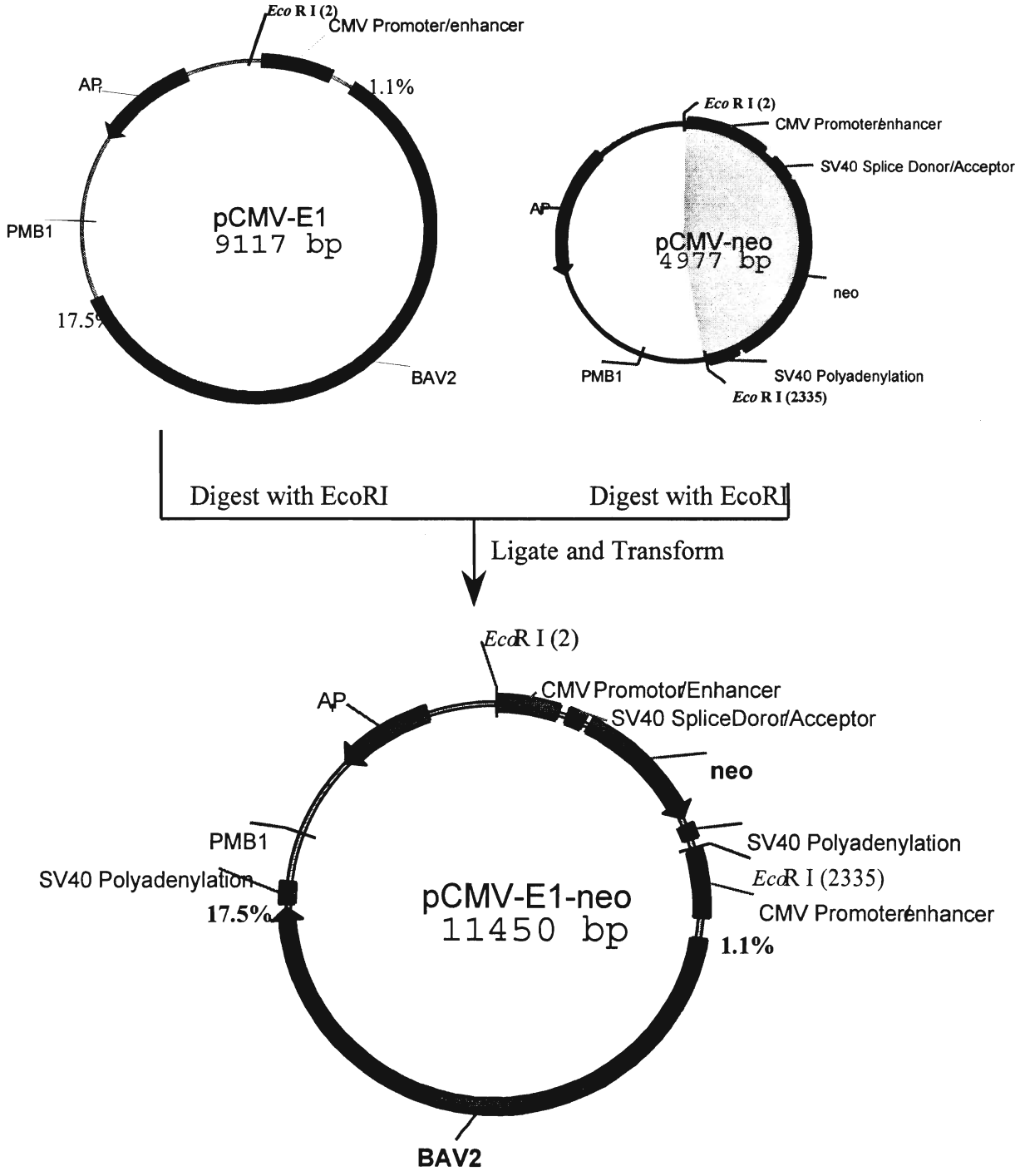
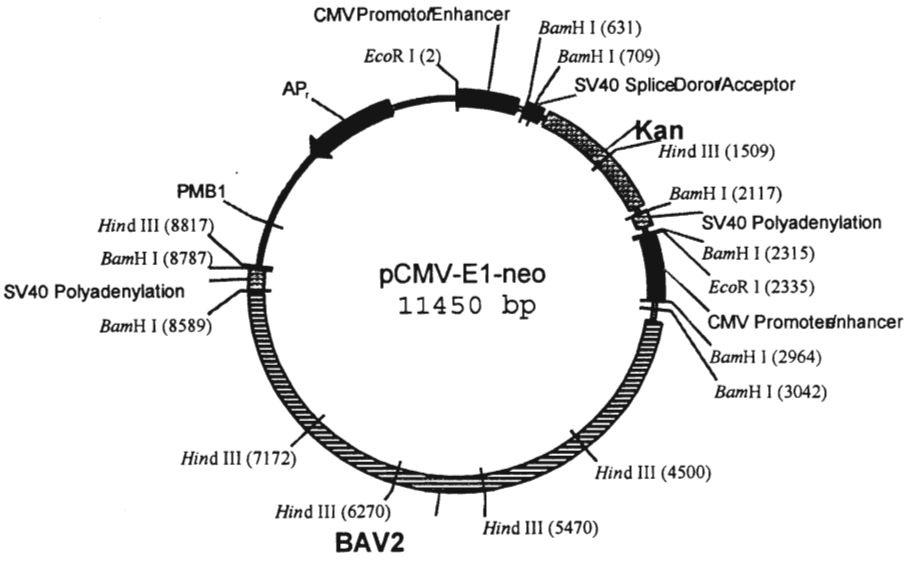


Figure 18: Construction strategy of the plasmid pCMV-E1-*neo*.

I.



II.

Enzymes	EcoRI	BamHI	SalI
Fragment Sizes (bp)	9,117	5,547	4,964
	2,333	3,294	3,478
		1,408	1,756
		649	1,252
		198	
		78	

III.

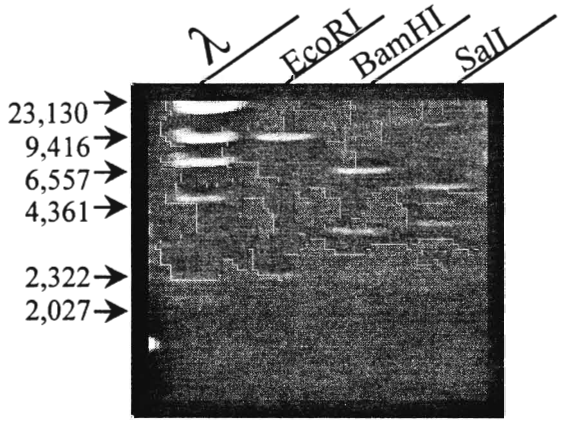


Figure 19: Restriction analysis of pCMV-E1-*neo*.

This plasmid was constructed by combining the plasmids pCMV-E1 and pCMV-neo. The *neo* gene under the control of the CMV promoter was released from pCMV-neo with the restriction enzyme *EcoRI* and cloned into the *EcoRI* site in pCMV-E1. (I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, shows the plasmid pCMV-E1-neo digested with *EcoRI*, *BamHI* and *SalI* respectively.

D. Transformation of MDBK Cells

MDBK cells were the first established cells to be transfected with pCMV-E1-*neo*. To obtain high efficiency of gene transfer, electroporation was used as described in Materials and Methods. Briefly, the cells were allowed to recover for 24 hours in MEM media supplemented with 10% FBS serum before selection with the drug G418 was initiated. When foci were visible, under light microscopy, cells were lifted by trysinization and plated at 1 cell/well to isolate single clones. Independent foci were isolated to a total of 48 for further characterization. Each clonal transformant was expanded for analysis of E1 expression.

E. Analysis of G418-Resistant MDBK Cells

1. Northern Blot

Quantitative assessment for E1 expression in the 48 independent foci was carried out by Northern blotting analysis. Figure 20 shows the result of probing for E1-specific transcripts from extracts of the 48 colonies. The result showed that 2 isolates, labeled L-23 and L-24 respectively, in a total of 48 expressed high levels of E1 transcripts. These cell lines were then subjected to further investigations in order to study their DNA uptake efficiency.

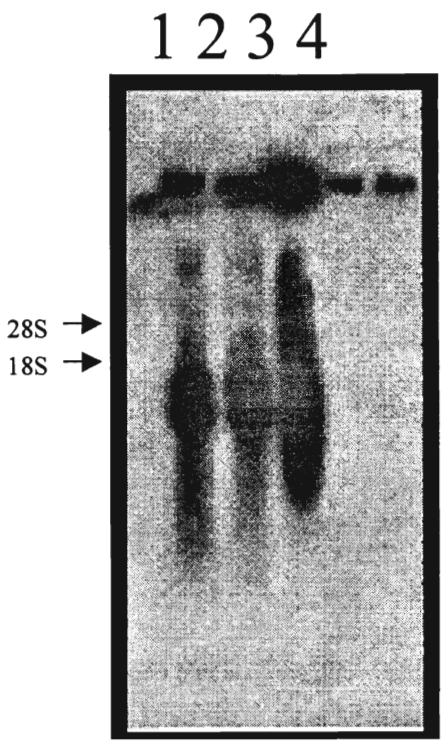


Figure 20: Northern blot analysis.

Total RNA was isolated from the cell lines, L-23 (lane 1), L-24 (lane 2), MDBK infected with BAV2 (lane 3) and MDBK control RNA (lane 4). The RNA isolated was subjected to agarose gel eclectrophoresis and then transferred to a nylon membrane. Positions of 18S and 28S bands are indicated on the left end of the gel. The membrane was probed with E1 of BAV2 (0 to 0.6%).

2. DNA uptake analysis

The efficiency of the MDBK-derived cells that express high levels of BAV2 E1 was determined in a transient expression assay using the *lacZ* reporter gene in the plasmid pCMVβ using the calcium phosphate and lipofectamine methods for transfering DNA. These methods revealed that both transformed cell lines L-23 and L-24 were, like the original cell line, MDBK, very poor at transient DNA uptake and expression (Figure 21) compared to other cell lines, such as Hela and 293. These results led to the idea of acquiring other established cell lines, testing them for DNA uptake and then choosing the efficient cell lines at picking up DNA to be transfected with pCMV-E1-n*eo*.

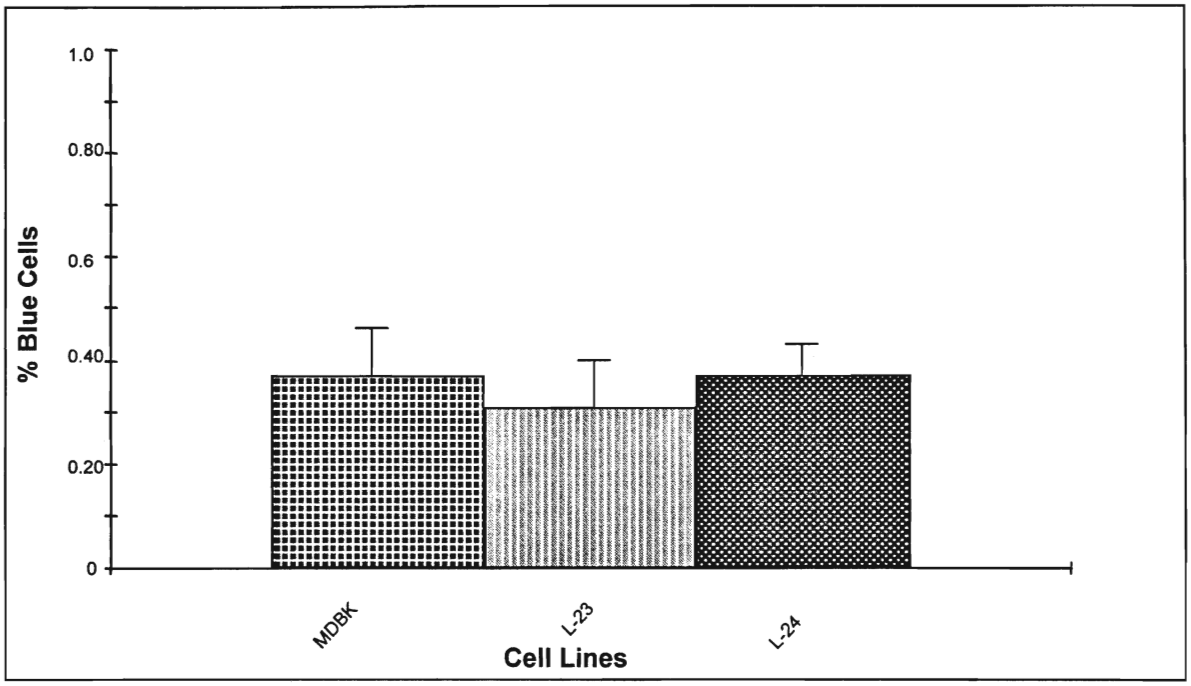


Figure 21: Transient DNA uptake and expression studies.

DNA uptake studies on MDBK, and transformants L-23 and L-24. Cell transfections, with the plasmid pCMVβ, was done in 6-well plates when cell confluency reached 50 percent. The cells were fixed and stained for *lac*Z activity 24 hours post-transfection. Blue cells were counted in ten randomly selected fields per plate and numbers are expressed as a percentage. Error bars show standard deviation value for three sets of data from independent sets of experiments.

F. Characterisation of Other Bovine Cell Lines

The low efficiency in DNA uptake of the transformed cell lines, L-23 and L-24, persuaded us to test other pre-established bovine cell lines. A total of nine cell lines (CCL-40, CCL-44, CRL-1390, CRL-6017, CRL-6033, CRL-6043, CRL-6055, CRL-6061, CRL-6072) were initially tested for permissivity to BAV2 and then for DNA uptake efficiency. The goal of this study was to find a cell line that is permissive to BAV2 and is efficient at DNA uptake. The cell line with these properties could then be used to be transfected with pCMV-E1-neo in order to establish a packaging cell line.

1. Permissivity to BAV2

All cell lines were tested for permissivity to BAV2 viral infection. The cells were grown to about 80 % confluency after which they were infected with BAV2 at 10 moi (multiplicity of

infection). All lines showed CPE (cytopathic effect) four to six days post infection. DNA was isolated from the cells following complete CPE. Restriction analysis of the isolated DNA revealed the presence of BAV2 DNA. Therefore, all cells were permissive to BAV2 infection.

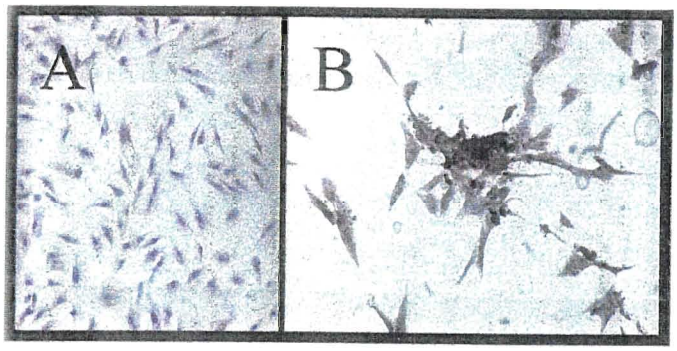


Figure 22: A photograph of counter stained CCL 40 cells

A - Normal CCL 40 cell morphology (40 x magnification)
B - Morphology of CCL 40 cells exhibiting cytopathic effect 48 hours post infection with BAV2 at 10 multiplicity of infection (moi) (40 x magnification)

2. DNA uptake efficiency

The nine other bovine established bovine cell lines were then tested for reporter DNA uptake in a transient expression assay. The plasmid pCMVβ , which carries the bacterial *lacZ* gene, was used again in these studies. Following transfection, the cells were stained with X-gal for *lacZ* activity. The blue cells were counted under light microscopy and an average of 10 fields per plate was calculated and presented with a standard deviation (Figure 23).

The results showed that CCL 40 appeared to have a transfection efficiency of about 30%, compared to 60% for 293 cells used as a control. Another cell line, CRL 6055, showed a comparably high efficiency. All other cell seven cell lines were poor in transfection (below 10%) (Figure 24).

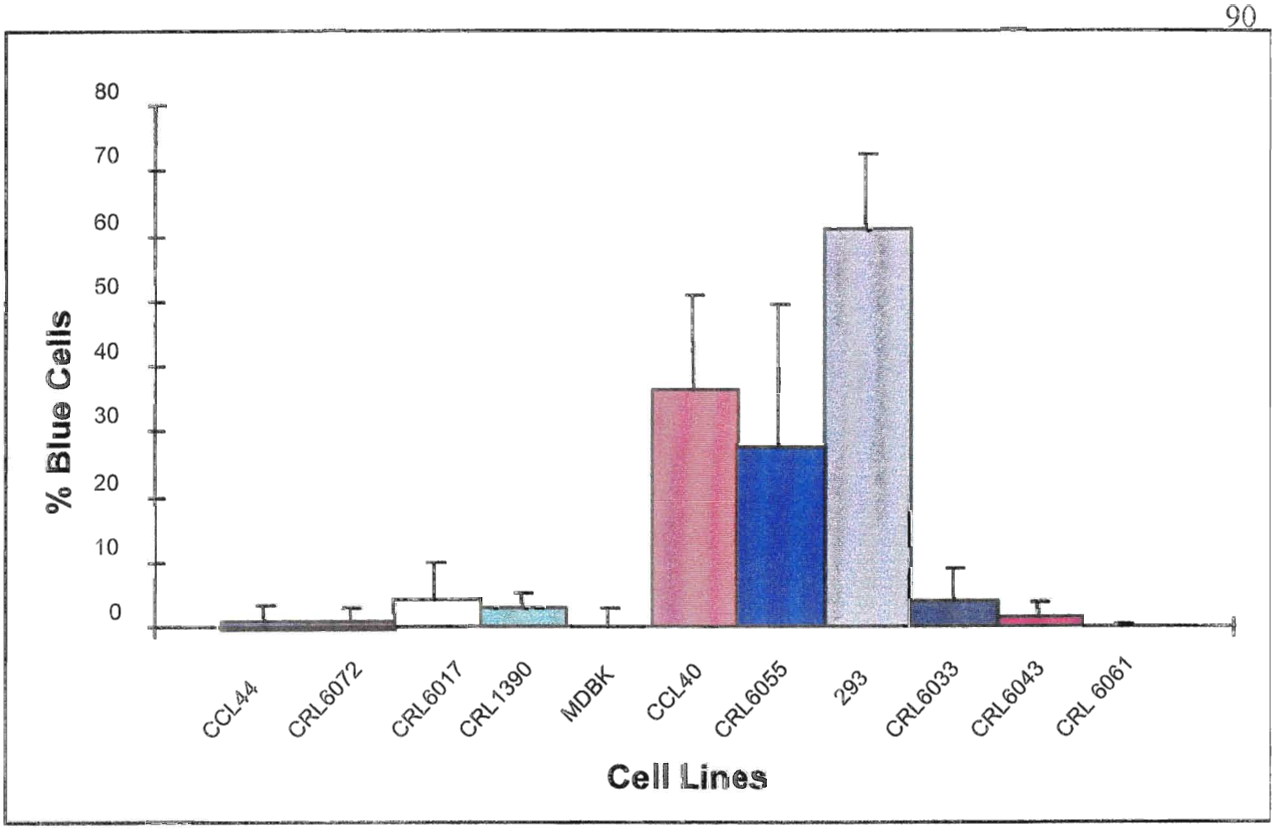


Figure 23: Transient DNA uptake efficiency and expression in various bovine cell lines.

Determination of transfection efficiency of various established bovine cell lines. Cell transfections, with the plasmid pCMVβ, in 6-well plates when cell confluency reached 50 percent. The cells were fixed and stained for *lac*Z activity 24 hours post-transfection. Blue cells were counted in ten randomly selected fields per plate and numbers are expressed as a percentage. Error bars show standard deviation value for three sets of data from independent sets of experiments.

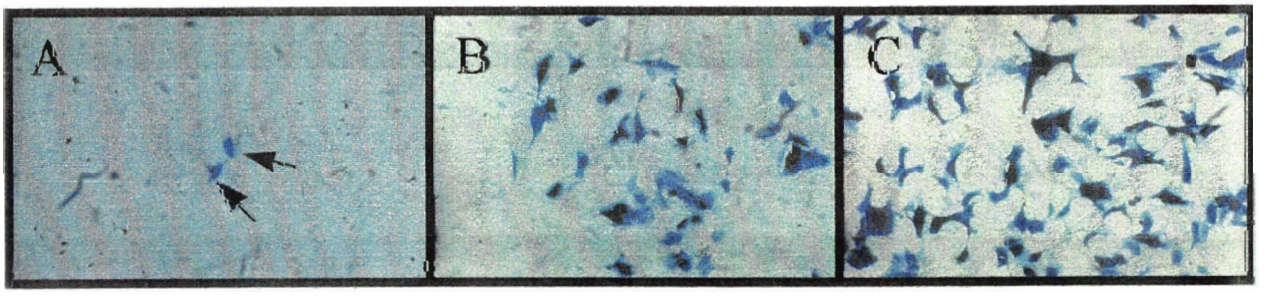


Figure 24: Photographs of mammalian cells lines stained for *lac*Z activity.

Cells were stained following transfection with the plasmid pCMVβ which carries the bacterial *lac*Z gene under the control of the CMV promoter.
A - MDBK cells, arrows indicate blue cells (40 x magnification)
B - CCL 40 cell line (40 x magnification)
C - 293 cells (40 x magnification)

3. Transfection and Selection

Due to its high transfection efficiency, the cell line CCL-40 was chosen for its high DNA uptake efficiency to be used in establishing a packaging cell line. The cells were transfected with the plasmid vector pCMV-E1-*neo*. The transfection and selection procedures were done in the same manner as in the case of MDBK cell lines. However, in a titration experiment to determine the level of sensitivity of these cells to the drug G418 revealed that CCL-40 cell line is more sensitive than MDBK cell line. MDBK cells can tolerate a level up to 75 μg/ml of G418 where CCL-40 cells can tolerate a maximum concentration of 25 μg/ml of G418 before cell death occurs. Therefore, a selection concentration of 50 μg/ml G418 was used.

Following selection, the resistant cells were expanded as a heterogeneous mixture without subcloning into single foci. Two cell lines were grown separately, namely, Pro-cell 1 and Pro-cell 2. These two cell lines were then subjected to northern blot analysis to determine the presence of E1 mRNA trasncripts, which would be an indication of E1 expression.

4. Northern Blot

In order to determine the level of E1 expression, total RNA extracted from the cell lines ProCell 1 and ProCell 2 was subjected agarose gel ecletrophoresis and followed by northern blot analysis. Using an E1 probe (0 to 6% of BAV2 genome) both cell lines revealed the presence of BAV2 E1 region (Figure 25).

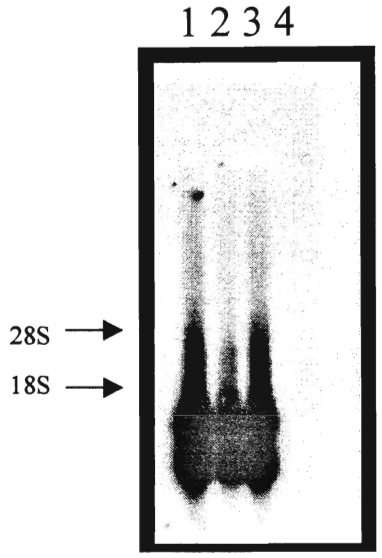


Figure 25: Northern blot analysis.

Total RNA was isolated from the cell lines, ProCell 1 (lane 1), ProCell 2 (lane 2), CCL-40 infected with BAV2 (lane 3) and CCL 40 control RNA (lane 4) . The membrane was probed with E1 of BAV2 (0 to 6% of the BAV2 genome).

G. Development of shuttle vectors for rescuing BAV2 recombinants With the lacZ gene in place of E1 Region

In the following study, a plasmid vector carrying part of BAV2 DNA was used to insert the *lac*Z gene into previously established deletions in E1 region of BAV2. The plasmid was co-transfected with wild type viral DNA in order to allow for homologous recombination which would result in a mutant virus carrying the *lac*Z gene in place of E1 region.

In order to rescue a mutant using homologous recombination, the plasmid DNA and the viral DNA have to have an area of overlap which in case of contact, a cross over would occur resulting in a viral genome that carries the foreign gene. Therefore the plasmid DNA was linearized with *BglII* restriction enzyme and the viral genomic DNA was digested with *BamHI* resulting in approximately 4 kbp of region of overlap (Figure 26). Both plasmid and genomic DNA were co-transfected into L-23 and L-24 cell lines at different concentrations using the calcium phosphate method. Although, viral mutants were not generated during co-transfection

experiments, wild-type BAV2 virus was generated by transfecting cells with non-digested viral DNA.

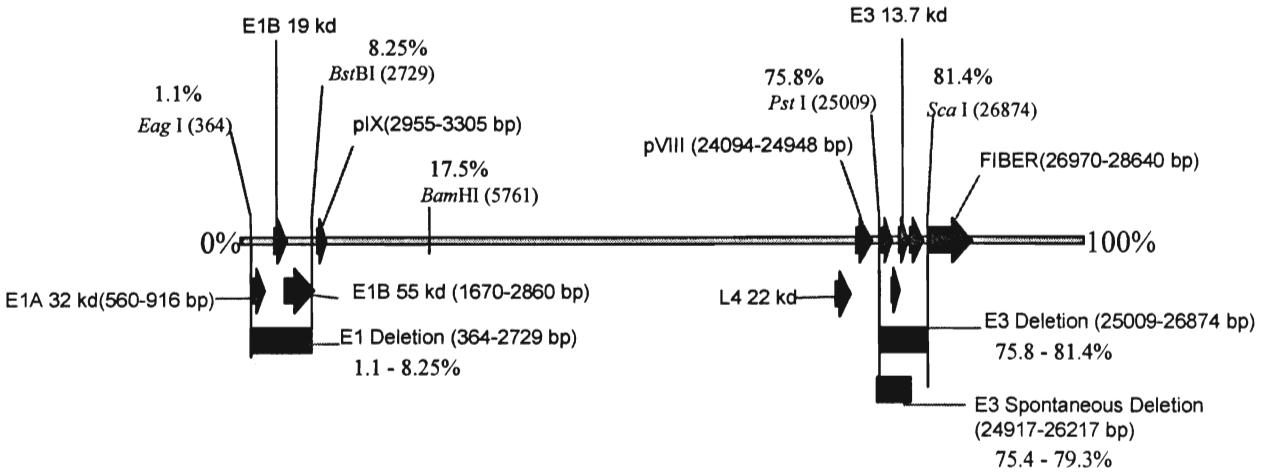


Figure 26: A schematic representation of the BAV2 genome.

The genome (33034 bp) is divided by convention into percentile units going from 0% to 100%. The E1 deletion was done using the restriction enzymes EagI and BstBI shown on the map at 1.1% and 8.25% respectively. The E3 deletion was made by using the restriction enzymes *PstI* and *ScaI* shown at 75.8% and 81.4% respectively. The spontaneous E3 deletion occurred, as indicated on the map, between 75.4% and 79.3 %.

1. Construction of pdlE1E-Z

The plasmid pdlE1E-Z was constructed to be used as a tool in BAV2 mutant rescue experiments. The *lacZ* gene was inserted in the forward orientation in this scheme. The cloning strategy involved first the modification of the plasmid pCMVβ in order to introduce a unique *KpnI* restriction site, resulting in the construction of the plasmid pCVMβ-K (Figure 27). This plasmid was confirmed by restriction enzyme analysis as shown in Figure 28. The expected restriction DNA fragments were observed on the gel (Figure 28). The plasmid pCMVβ-K was then combined with the plasmid pdlE1-E to give the plasmid pdlE1-E-Z. The plasmid pdlE1E-Z contains BAV 2 sequence from 0% to 40.4% with the E1 region deleted using the restriction enzymes EagI at 1.1% and BstBI at 8.25% (E1A and E1B regions extend from 1.7% to 2.8% and

5.1% to 8.66% respectively). The *lacZ* gene under the control of the CMV promoter was inserted in the deletion in the forward direction.

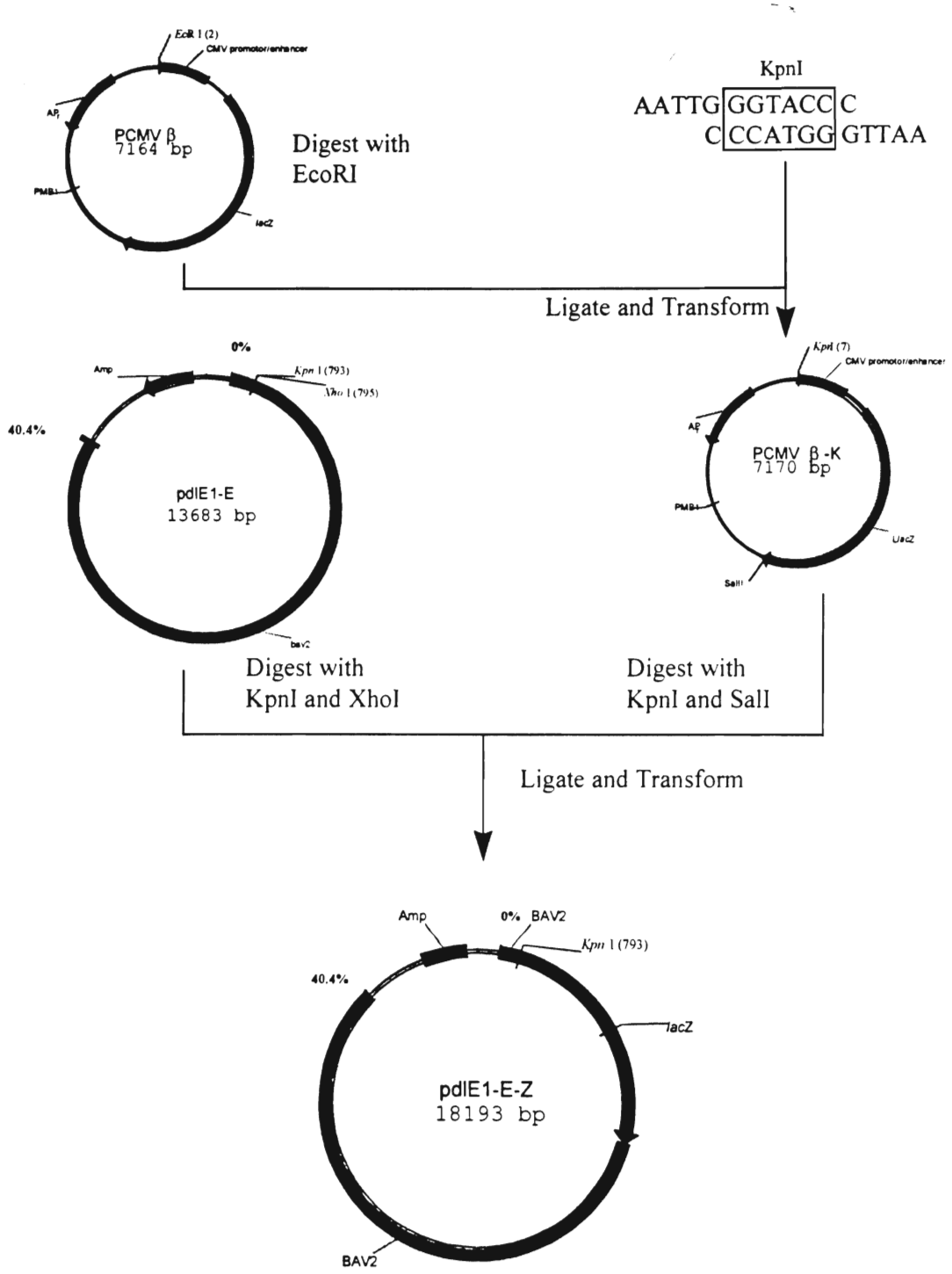
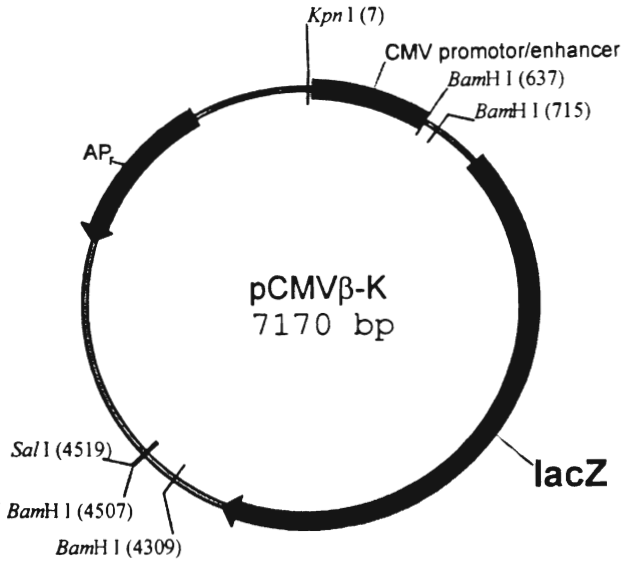


Figure 27: Cloning strategy for pdlE1E-Z.
The commercially available plasmid, pCMVβ, was modified using a linker to introduce a new *KpnI* restriction site instead of the unique *EcoRI* site. The resulting plasmid, pCMVβ-K was combined with the plasmid pdlE1-E, which carries up to 40.4% of the left end of BAV2 genome. The final plasmid, pdlE1-E-Z contained the *lacZ* gene under the control of the CMV promoter being expressed in the forward orientation.

I.



II.

Enzymes	KpnI	BamHI	SalI
Fragment Sizes (bp)	7,170	3,594	7,170
		3,300	
		198	
		78	

III.

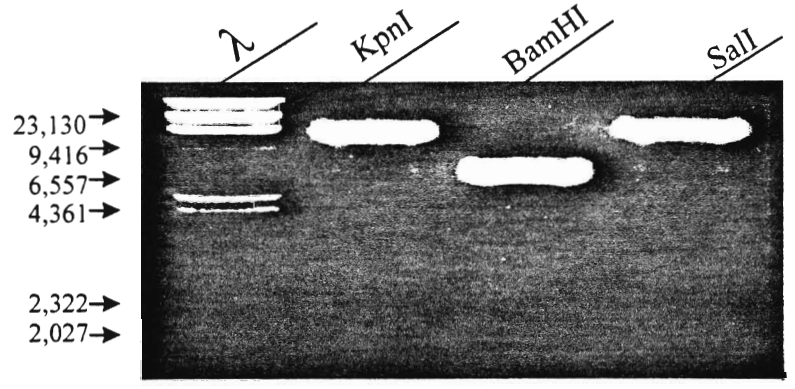
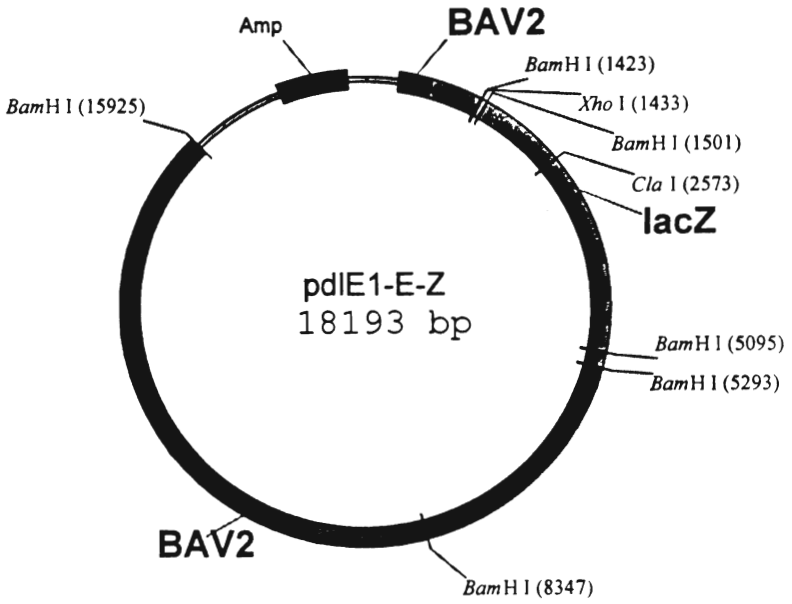


Figure 28: Restriction analysis for pCMVβ-K.

This plasmid was constructed by cutting the plasmid with the restriction enzyme *EcoRI* and ligating in the presence of a linker that carries compatible sticky ends to the *EcoRI* restriction site but carries the *KpnI* recognition sequence. (I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, shows the plasmid pCMVβ-K digested with *KpnI*, *BamHI* and *SalI* respectively.

I.



II.

Enzymes	ClaI	BamHI	XhoI
Fragment Sizes (bp)	18,193	7,578	18,193
		3,691	
		3,594	
		3,054	
		198	
		78	

III.

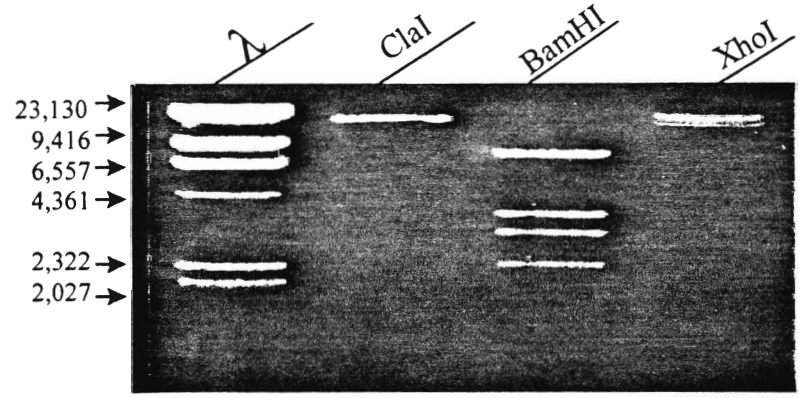


Figure 29: Restriction analysis of pdlE1E-Z.

The plasmid pdlE1E-Z was constructed by digesting the plasmid vector, pCMVβ-K with the restriction enzymes *KpnI* and *SalI* . The resulting fragment was cloned into the *KpnI* and *XhoI* restriction sites in the plasmid pdlE1-E. (I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, shows the plasmid pCMVβ-K digested with *ClaI*, *BamHI* and *XhoI* respectively.

The use of the plasmid pdlE1E-Z in BAV2 mutant rescue experiments requires the use of packaging cell lines that express the E1 BAV2 region. Therefore, transfections were done using the cell lines L-23, L24, ProCell 1 and ProCell 2. The plasmid DNA was co-transfected with wild-type viral DNA at a one to one ratio using both lipofectamine® and calcium phosphate method. However, these attempts did not result in the generation of a BAV2 mutant.

2. Construction of pdlE1E-Z-R

In a study by Bramson and co-workers (1996), the expression of foreign DNA inserted in place of deleted E3 region was influenced by the presence or the absence of intact E1 region in the viral vector. When E1 region was kept intact, the foreing gene was only expressed in the rightward orientation, however when the viral vector contained an E1-deletion, the foreign gene was observed to be expressed when present in both orientation. Consequently, we decided to insert the *lac*Z gene in both forward and reverse orientations, in case one of the orientations does not result in active transcription of the gene.

The plasmid pdlE1E-Z-R therefore, contained the same E1 deletion as pdlE1E-Z, however the *lac*Z gene was inserted in the reverse direction. This plasmid was also used to attempt to rescue a BAV2 mutant with an E1 deletion. The *lac*Z gene was inserted in the reverse direction in case the forward orientation had a negative influence on the expression of the other viral genes resulting in a non-viable virus. Therefore, the reverse orientation should have opposite or no effects in terms of modulating other viral gene expression that would inhibit viral growth.

The cloning strategy involved the modification of the plasmid pCVMβ in order to introduce a unique *SalI* site in place of the unique *EcoRI* site (Figure 30). That resulted in the construction of the plasmid pCMVβ-S. The plasmid pCMVβ-S was confirmed by restriction

enzyme analysis and the expected fragments were observed on the agarose gel electrophoresis as shown in Figure 31. The following step was to combine the plasmid pCMVβ-S with the plasmid pdlE1E resulting in the construction of the plasmid pdlE1E-Z-R, which was confirmed by restriction enzyme analysis, as shown in Figure 32. The expected DNA fragment size was observed on the agarose gel electrophoresis.

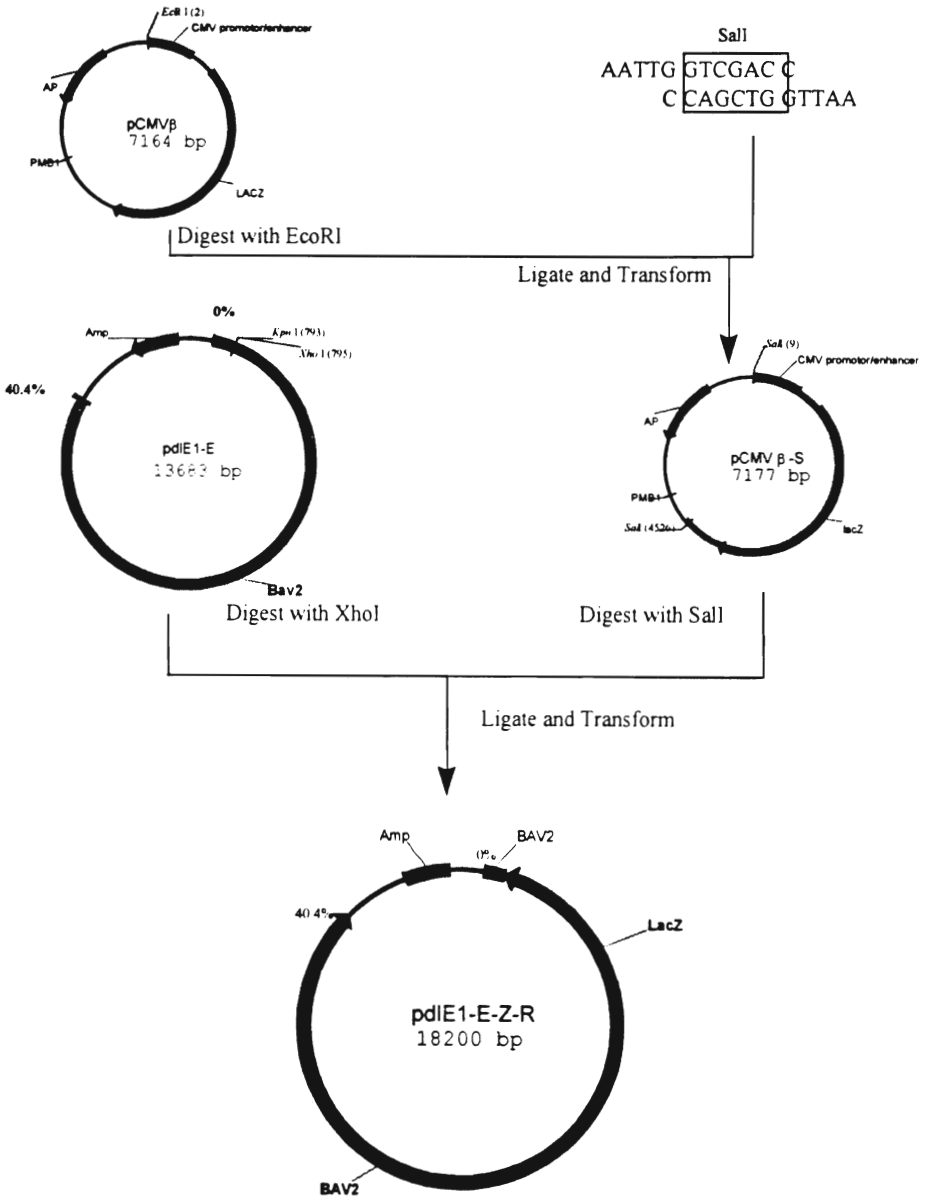
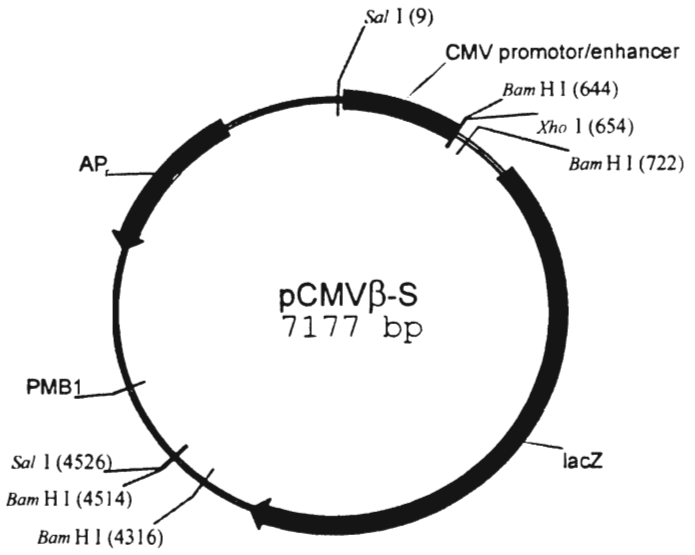


Figure 30: Cloning strategy for pdlE1E-Z-R.

The commercially available plasmid, pCMVβ, was modified using a linker to introduce a new *SalI* restriction site instead of the unique *EcoRI* site. The resulting plasmid, pCMVβ-S was combined with the plasmid pdlE1-E, which carries up to 40.4% of the left end of BAV2 genome. The final plasmid, pdlE1-E-Z contained the *lacZ* gene under the control of the CMV promoter in place of the E1 region and being expressed in the reverse orientation.

I.



II.

Enzymes	XhoI	BamHI	SalI
Fragment Sizes (bp)	7,177	3,594	4,517
		3,307	2,660
		198	
		78	

III.

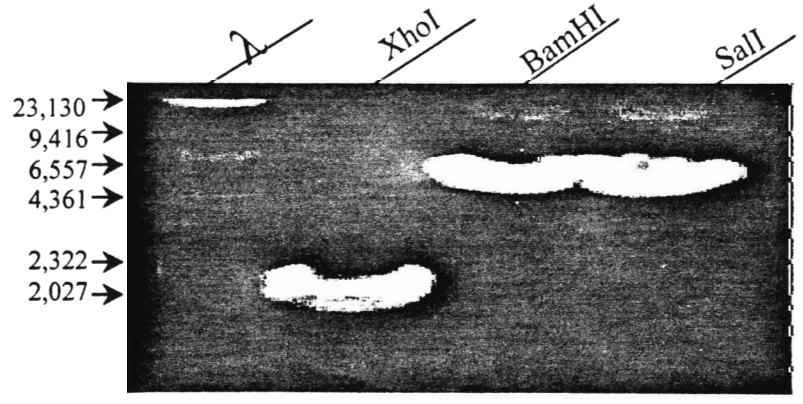
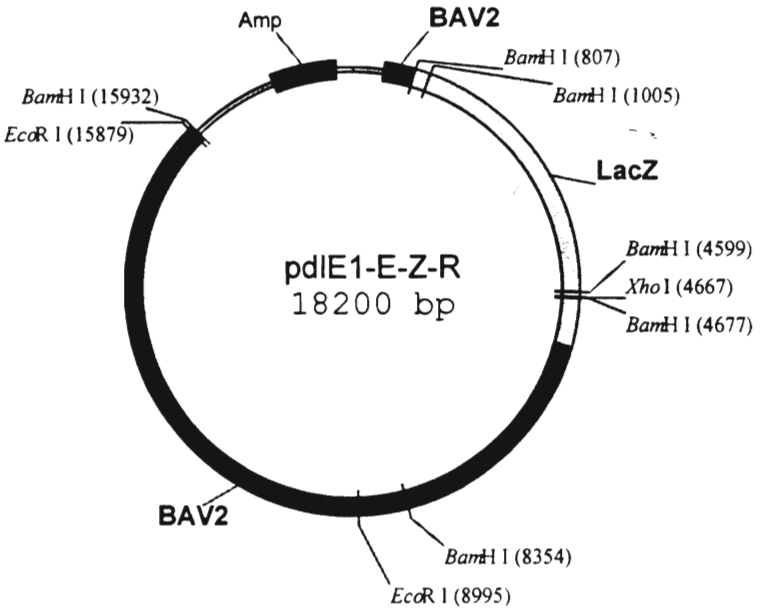


Figure 31: Restriction analysis of pCMVβ-S.

This plasmid was constructed by cutting the plasmid pCMVβ with the enzyme *EcoRI* and ligating in the presence of a linker that contains compatible sticky ends to the *EcoRI* restriction site but carries the recognition sequence for the enzyme *SalI*. (I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, shows the plasmid pCMVβ-K digested with *BamHI*, *HindIII* and *XhoI* respectively.

I.



II.

Enzymes	EcoRI	BamHI	XhoI
Fragment Sizes (bp)	11,316	7,578	18,200
	6,884	3,677	
		3,594	
		3,057	
		198	
		78	

III.

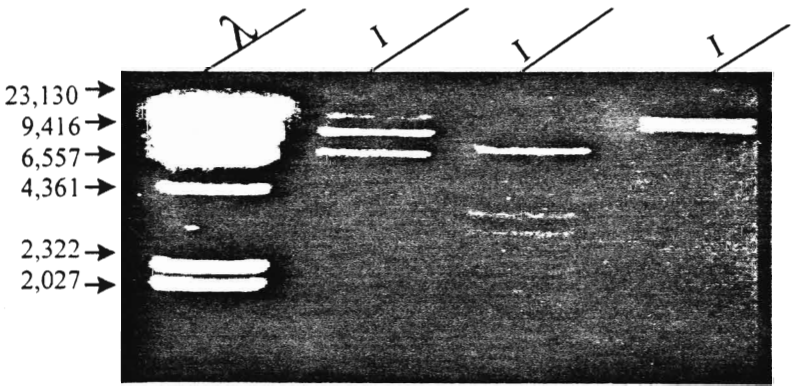


Figure 32: Restriction analysis of pdlE1E-Z-R.

This plasmid was constructed by digesting the plasmid pCMVβ-S with the restriction enzyme *Sall* , the resulting fragment was ligated to the plasmid pdlE1E-Z-R after being digested with the enzyme XhoI. (I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, shows the plasmid pdlE1E-Z-R digested with *EcoRI*, *BamHI* and *XhoI* respectively.

The plasmid pdlE1E-Z-R was transfected into the E1 expression cell lines, namely L-23, L-24, ProCell 1 and ProCell 2. The lipofectamine® and the calcium phosphate methods were used in these transfections. However, that did not result in rescuing a BAV2 mutant.

3. Construction of pdlE3-5-Z

The plasmid pdlE3-5-Z contains BAV2 sequences from 64.8% to 100%. The E3 region occurs on the BAV2 genome between protein VIII (72.9-75.5%) and the fibre protein (81.6% to 86.7%) at 75.7% to 81.36%. The E3 deletion was done using the restriction enzymes *PstI* at 75.8% and *ScaI* 81.4%. The insertion of the *lacZ* gene in place of the E3 region would allow for rescuing a BAV2 viral mutant. The E3 deletion mutants do not require the E1 expression packaging cell lines. Therefore, normal CCL40 and MDBK cell lines were used in these rescue attempts.

The cloning strategy of the plasmid pdlE3-5-Z included a single cloning step on combining the plasmid pdlE3-5 with the plasmid pCMVβ-K as shown in Figure 33. The plasmid was confirmed using restriction enzyme electrophoresis and the expected fragment sizes were observed on the agarose gel electrophoresis (Figure 34).

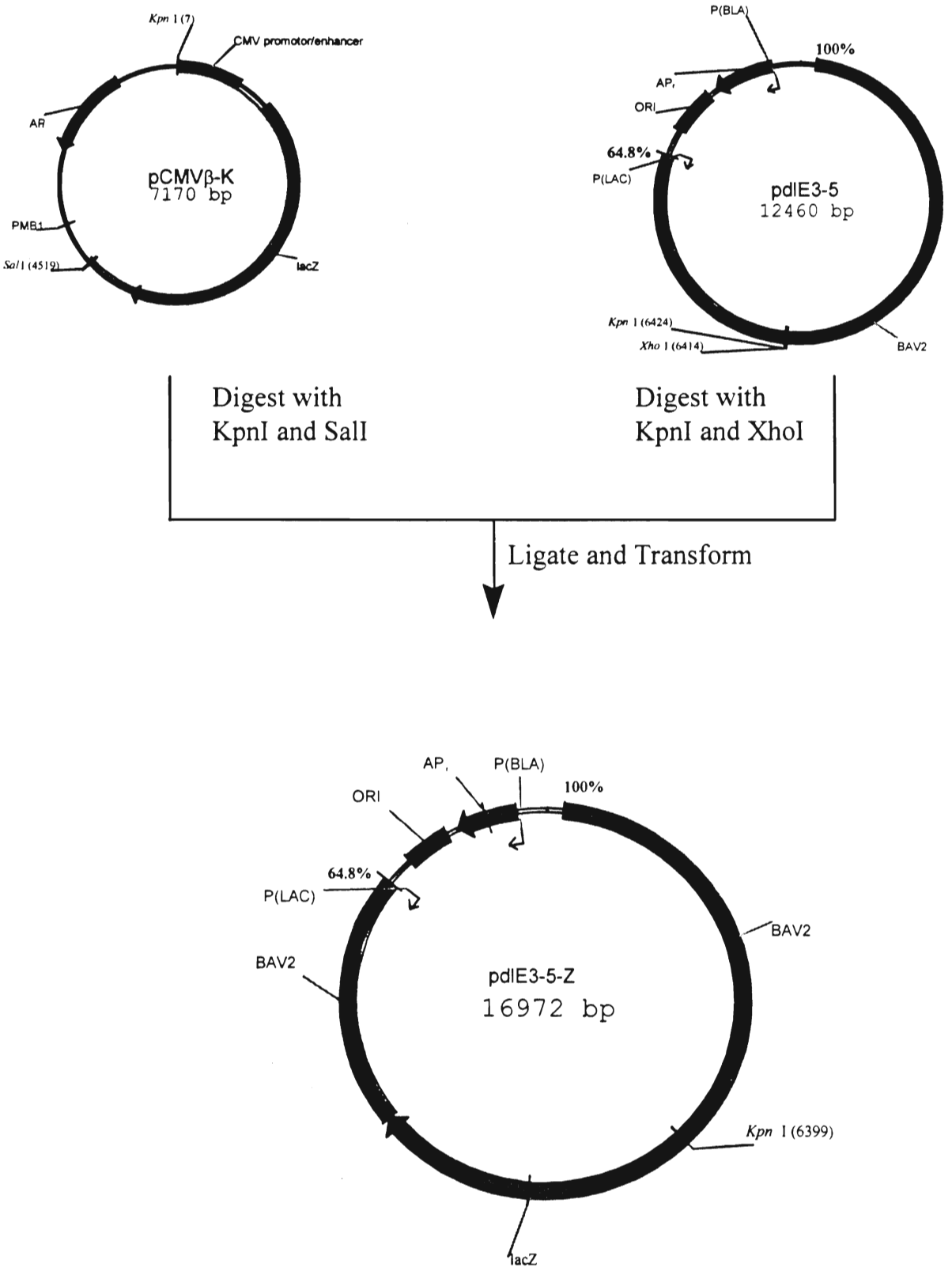
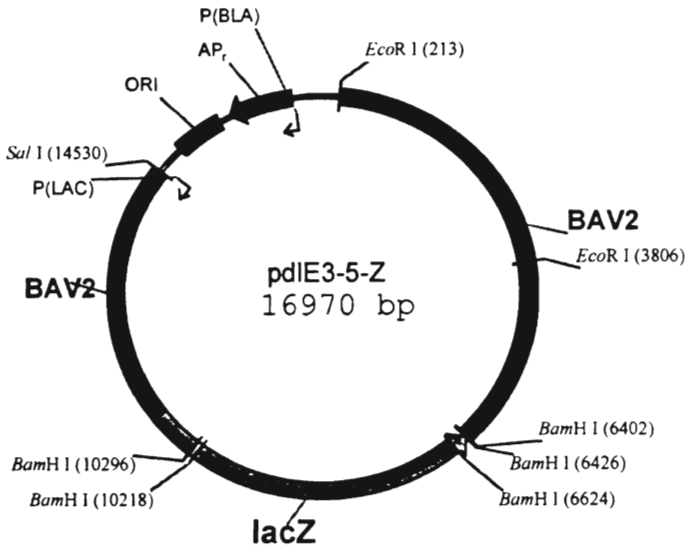


Figure 33: Cloning strategy for pdlE3-5-Z.

The plasmid pCMVβ-K was combined with the plasmid pdlE3-5, which carries the right end of BAV2 genome from 64.8% to 100%. The final plasmid, pdlE3-5-Z contained the *lac*Z gene under the control of the CMV promoter in place of the E3 region and being expressed in the reverse orientation.

I.



II.

Enzymes	EcoRI	BamHI	SalI
Fragment Sizes (bp)	13,377	13,3594	16,970
	3,593	3,594	
		198	
		78	
		24	

III.

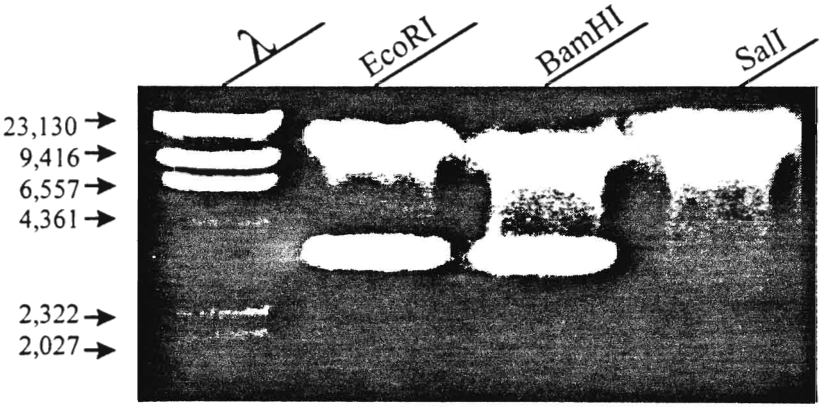


Figure 34: Restriction analysis of pdlE3-5-Z.

This plasmid was constructed by digesting the plasmid pCMVβ-k with the restriction enzymes *KpnI* and *SalI* . The resulting fragment was inserted in the *KpnI* and *XhoI* restriction sites in the plasmid pdlE3-5-Z. (I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, shows the plasmid pdlE3-5-Z digested with *EcoRI*, *BamHI* and *SalI* respectively.

The final construct, pdlE3-5-Z was co-transfected with wild type BAV2 viral DNA that was previously digested with the restriction enzyme *XmaI* to eliminate the right end of the

genome. Therefore allowing an overlap region of approximately 6.1 kb. These transfections employed both the lipofectamine® and the calcium phosphate methods. However, these transfections did not result in a BAV2 viral mutant.

H. Rescuing a BAV2 mutant with an E3 deletion

The full BAV2 genome was cloned into a cosmid vector, cosBAV2, and upon its transfection into M5 cell line, it was shown to result in BAV2 viral particles (Ojkic, 1998). The advantage of having the whole genome cloned into a cosmid vector is that it eliminates the dependence on homologous recombination for the generation of viral vectors during viral mutant rescue attempts. Therefore, the objective of this study was to modify the cosBAV2 by replacing E3 viral genes with the *neo* gene and use the new construct to attempt to rescue a viral vector. Consequently, cosBAV2 was modified through a series of cloning to suit our strategy (Figure 35). The final construct, cosBAV2-*neo* , was then used to attempt to rescue a mutant by transfecting the linearized cosmid vector into MDBK, M5 and CCL 40 cell lines.

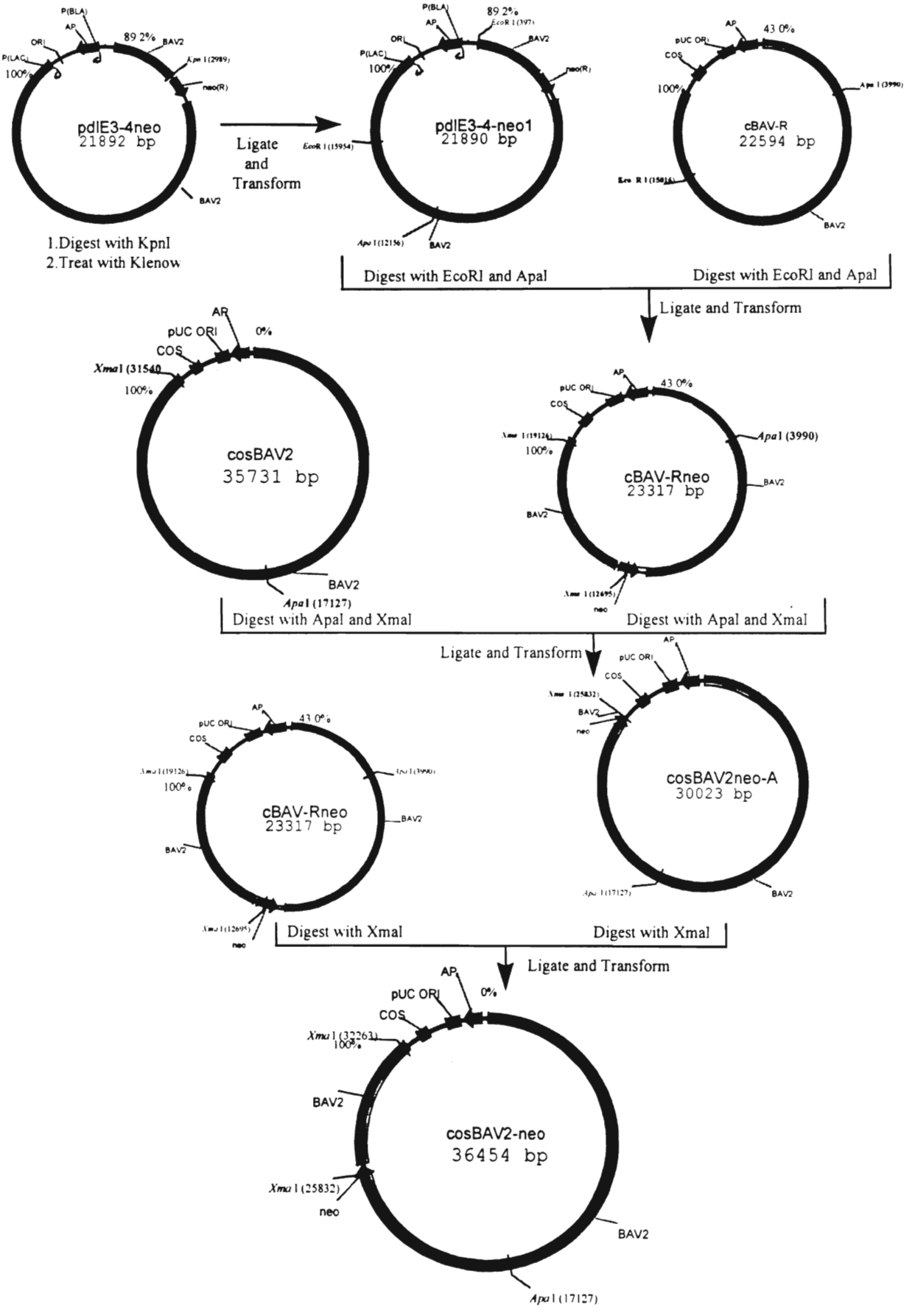
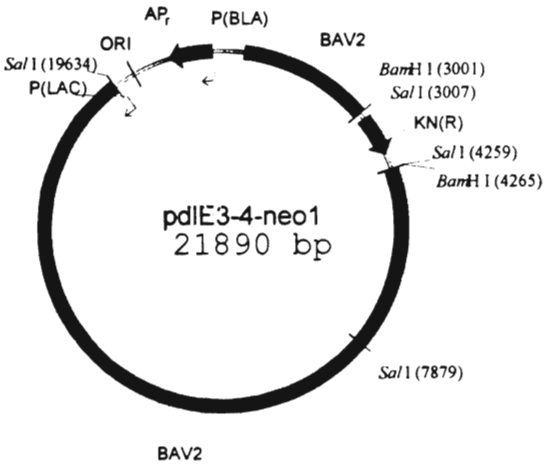


Figure 35: Cloning strategy for cosBAV2-*neo*.

1. Construction of cosBAV2-*neo*

The first step in constructing cosBAV2-neo was the elimination of a unique *KpnI* restriction site from pdlE3-4Kan plasmid vector, which contained BAV2 sequence from 89.2% to 100% with the *neo* gene cloned in place of deleted the E3 coding region (75.8 to 81.4%). That resulted in the construction of the plasmid pdlE3-4-neo 1 which was confirmed by restriction enzyme digestions followed by gel electrophoresis analysis, shown in Figure 36. The plasmid pdlE3-4-neo1 was combined with the plasmid cBAV-R to construct the plasmid cBAV-R-*neo* which was confirmed as shown in Figure 37. Once the integrity of cBAV-R-*neo* was confirmed, it was combined with cosBAV2 to construct cosBAV2-*neo*-A. This plasmid contained partial sequence of the *neo* gene, therefore, kanamycin resistance selection was not applied during screening for this plasmid. The cosmid construct, cosBAV2-*neo*-A, was confirmed through restriction analysis as shown in Figure 38. In order to add the missing sequences of the *neo* gene, cosBAV2-neo-A was combined with cBAV-R *neo* to construct cosBAV2-neo. This cosmid vector contained the full BAV2 genome with a *neo* gene replacing the E3 region from 75.8 to 81.4%. This final construct was confirmed through restriction analysis and gel electrophoresis as shown in Figure 39.

I.



II.

Enzymes	BamHI	KpnI	SalI
Sizes	20,626	No restriction	11,755
	1,264	sites present	5,263
			3,620
			1.252

III.

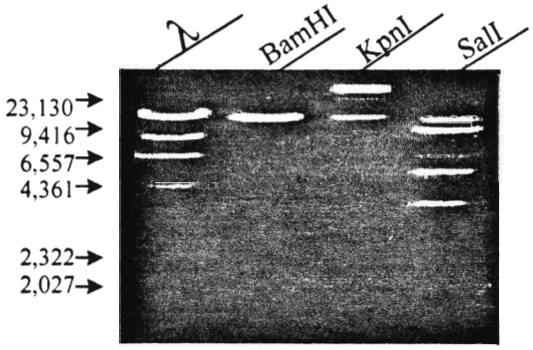
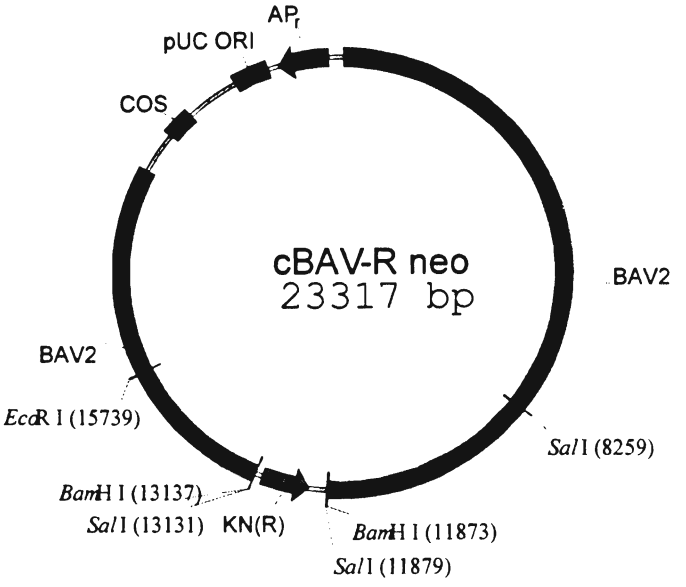


Figure 36: Restriction analysis of pdlE3-4-*neo*1.

This plasmid was constructed by cutting pdlE3-4-neo plasmid with the *KpnI* restriction enzyme followed by a treatment with the klenow enzyme to fill the sticky ends (Figure 35). The resulting plasmid pdlE3-4-Kan-1 was confirmed on agarose gel electrophoresis (I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, shows the plasmid pdlE3-4-neo1 digested with *BamHI*, *KpnI* and *SalI* respectively.

I.



II.

Enzymes	EcoRI	BamHI	SalI
Fragment Sizes (bp)	23,317	22,053	18,445
		1,264	3,620
			1,252

III.

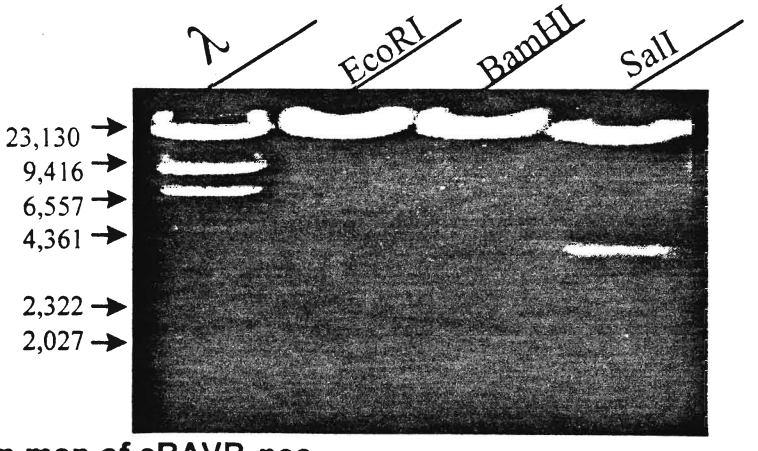
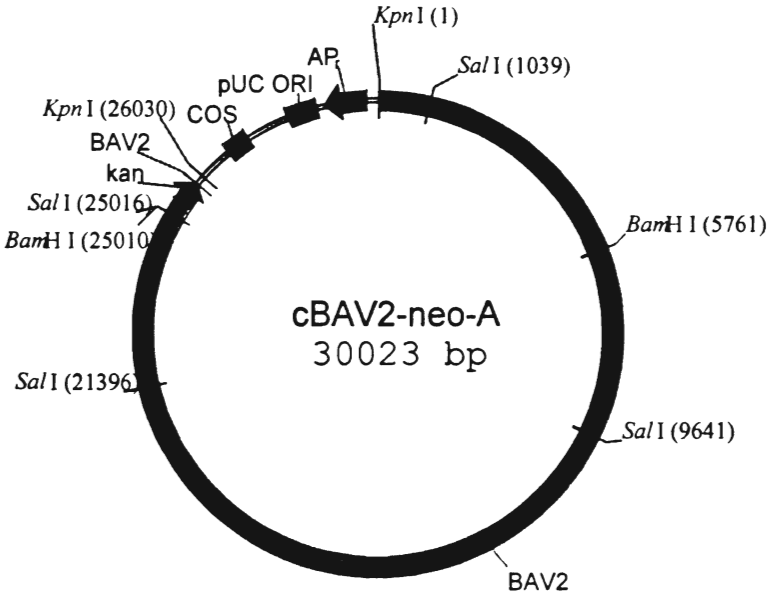


Figure 37: Restriction map of cBAVR-*neo*.
The plasmid pdlE3-4kan was digested with the restriction enzymes *EcoRI* and *ApaI*. The resulting fragment was inserted into the same restriction sites in the cosmid vector cBAV-R resulting in the construction of the cosmid vector cBAV-R*neo* (Figure 35). (I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, shows the plasmid cBAVR-neo digested with *EcoRI*, *BamHI* and *SalI* respectively.

I.



II.

Enzymes	BamHI	KpnI	SalI
Sizes	19,249	26,029	11,755
	10,774	3,994	8,602
			6,046
			3,620

III.

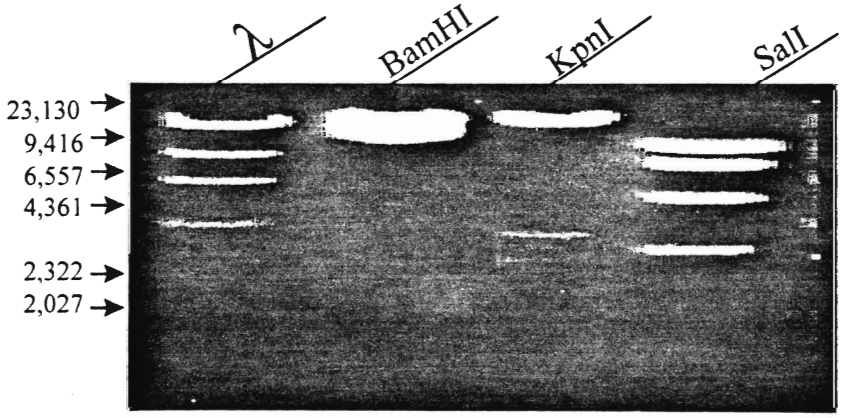
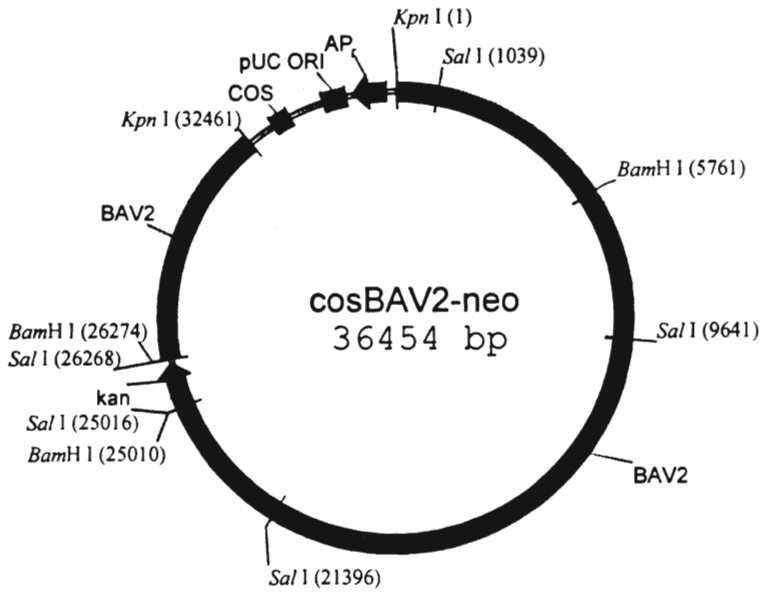


Figure 38: Restriction analysis of cBAV2-*neo*-A.

This plasmid was constructed by transferring the *ApaI* to *XmaI* fragment from the cosmid vector cBAV-R*neo* the same restriction sites in cosBAV2 (Figure 35). (I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, shows the plasmid cBAV2-neo-A digested with *BamHI*, *KpnI* and *SalI* respectively.

I.



II.

Enzymes	BamHI	KpnI	SalI
Sizes	19,249	32,460	11,755
	15,941	3,994	11,225
	1,264		8,602
			3,620
			1,252

III.

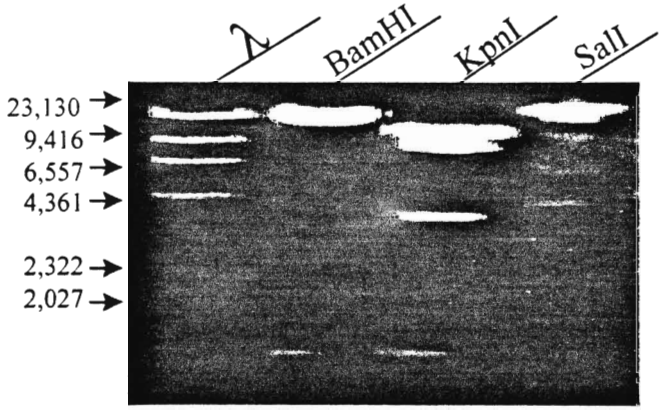


Figure 39: Restriction analysis of cosBAV2-neo.

The cosBAV2-neo cosmid vector was constructed by combining cBAV-R*neo* with cosBAV2*neo*-A. CosBAVR*neo* was digested with the restriction enzyme *XmaI* and the released fragment was inserted into the *XmaI* site in cosBAV2-neo-A to result in a complete BAV2 clone that contains the *neo* gene in place of the E3 deleted region from 75.8 to 81.4% (Figure 35). (I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, shows the plasmid cosBAV2-neo digested with *BamHI*, *KpnI* and *SalI* respectively.

2. Transfection of cosBAV2-neo into bovine cell lines

The cosBAV2-neo clone was used to attempt to rescue a mutant by transfecting the closed circular cosmid or the linearized form, by digesting it with the restriction enzyme *KpnI*, into MDBK, M5 and CCL40 cell lines. However, these transfections did not result in the generation of a mutant virus. The possible reasons for are discussed further in the discussion section.

X. Discussion

Ad vectors are being rapidly developed for use in gene therapy and viral vaccine technologies for applications in humans and other animals. Numerous human clinical protocols using adenoviruses have already entered phase I clinical trials (Bilbao *et al.*, 1998). Although there are over 40 serotypes of human Ads, only subgroup C serotypes 2 and 5 are predominantly used as vectors. Other serotypes and Ads from non-human sources are also being examined for their potential as vectors.

A property of Ad vectors is their ability to remain in episomal forms in the nucleus of the host cell and only rarely integrate within the cellular genome. Therefore, the use of Ad vectors carries an advantage since the risk of integration and the consequent hazard of insertional mutagenesis are minimized or even eliminated. However, this property of Ads also carries a disadvantage in gene therapy, since expression of foreign DNA tends to be only transient (Verma and Somia, 1997).

The wild-type Ad genome is 30 kbp to 44 kbp. The so-called "First generation" Ad vectors, with deleted E1 and E3 regions, can accommodate an insert of up to 8 kbp of foreign DNA (Levrero *et al.*, 1991). Although these viruses are attenuated and require a packaging cell line to support viral DNA replication *in vitro*, viral proteins are still expressed at very low level *in vivo*, and activate the immune system (reviewed in Hitts *et al.*, 1996). To improve First generation vectors, Ad vectors were further attenuated by including more deletions to produce the so-called "Second generation" Ad vectors. The Second generation vectors include the so-called "gutless" vectors which contain only the ITRs and a packaging sequence around the transgene (Fisher *et al.*, 1996). Such vectors, allow for large insert size of foreign DNA but require helper virus for propagation. The development of vectors containing only few genes, as in the "gutless" vectors, has resulted in prolonged *in vivo* transgene expression in liver tissue

(Schieder *et al.*, 1998) and in the muscle (Chen *et al.*, 1997), therefore, improving the rate of success of transgene delivery through the use of Ad vectors.

Highly purified, attenuated Ad vectors are required for *in vivo* expression studies. However, since these are almost invariably grown in 293 cells, they are likely non-homogenous. Several methods have been developed to detect wild-type Ad contaminants, which arise during virus growth in the 293 cell line. In a study by Wei and co-workers (1995), one plaque-forming unit (pfu) of wild-type virus can be detected, using a PCR-based method, in 10^9 pfu of recombinant viruses. Therefore, this highly sensitive detection system enhances quality control in the production of adenoviral vectors that are free of wild-type virus and suitable for gene therapy applications. Another area of advancement in Ad vectors is in the preparation and storage of stock virus. In a recent study by Croyle and co-workers (1998), a highly efficient purification process for recombinant adenoviral vectors was developed. This protocol, based on the cesium chloride purification method that require as long as 45 hours (Green and Pina, 1963), employs the use of sucrose concentration gradient instead of cesium chloride, therefore eliminating desalting step and consequently decreasing total preparation time by 15 hours. The same study showed enhanced storage half-life of viral stocks by using lyophilized preparations instead of the traditional glycerol storage method. A lyophilized viral stock of 1.4×10^{12} pfu/ml was reduced to only 0.6×10^{12} pfu/ml following storage at –20°C for 150 days compared to a half-life of 34 days when using the traditional glycerol storage method.

The development of E1-deleted BAVs was patterned after the first generation Ad5 vectors. Thus, a bovine cell line, equivalent to the 293 packaging cell line had to be constructed. However, problems associated with the 293 cell line and first generation Ad vectors were addressed in our design of an ideal packaging cell line. This cell line must possess several features to allow for efficient generation of recombinant viral vectors. First, the cell line must be able to complement the E1 gene products that are missing from the viral vector, due to deletions

introduced to accommodate foreign inserts. The E1 gene products are the first proteins expressed after viral infection of a host cell and their major role is *trans*-activation of the other viral early and late promoters. Because Ads missing E1 region are replication-defective, the E1 products must be provided in *trans* in order for the E1-deleted BAVs to package as infectious virions. To meet that criterion we constructed the plasmid pCMV-E1, which contained BAV2 E1 region and used it to transfect primary embryonic cells in attempts to isolate transformed cell lines.

The second feature is that the cell line must not support wild-type reversion of mutant vectors through homologous recombination between the viral genomic DNA and the integrated viral DNA in the cellular genome. This has been shown to be a common problem with the human cell line 293. Even though the frequency of reversion is quite low, estimated at one in 10^5 to 10^6, one wild-type viral particle can give rise to 10^3 to 10^4 viral progenies upon infection of a host cell and can outgrow the mutants by exponential growth (reviewed in Smith, 1995). That type of viral stock cannot be used in gene therapy *in vivo* due to wild-type virus pathogenicity. In the present study, we specifically addressed the problem of wild-type revertants by constructing the plasmid pCMV-E1 in such a way that the CMV promoter replaces the E1 promoter. Replacing the promoter would eliminate the possibility of a crossover on the left end of the genome and consequently eliminate the unintentional generation of wild-type BAVs.

The third feature of an ideal packaging cell line is that they must be easy to transfect to recover virus from DNA. The difference in DNA transgene expression among cell lines lies in their ability to transport the DNA through the cell membrane and transfer it to the nucleus without degrading it (Orrantia and Chang, 1990). In a typical transfection experiment, the DNA is complexed with either calcium phosphate precipitate (Graham and Van der Eb, 1973) or with cationic lipids (Felgner *et al.*, 1987). In both methods of transfection endocytosis is the main type of DNA uptake (Zabner *et al.*, 1995). The DNA is then transported in endosomes that, depending on the cell line, may or may not fuse with lysozymes, which are known to be involved

in DNA degradation of foreign DNA. The basis behind the DNA escaping degradation by lysozymes is still not understood (Zabner *et al.*, 1995). However, this escape of the DNA has been mainly attributed to the difference between an efficient cell line and an inefficient cell line in transient DNA expression (Jordan *et al.*, 1996).

The constructed plasmid vector pCMV-E1 was then used to attempt to immortalize cells from primary cultures of bovine embryonic kidney and lung tissues. This attempt was unsuccessful due to several reasons. First, BAV2 E1 might have failed to transform primary bovine cells since both the virus and the cells belong to the same animal species. This seems to be the general rule for Ads as experimentally observed for human, mouse and monkey Ads. In other words, since bovine cells are permissive to BAV2, one should expect low levels of, or perhaps zero, transformation frequency of such cells by BAV2 E1. It should be noted here, however, that 293 cells were isolated as transformants of human embryonic kidney cells by human Ad5 E1 (Graham *et al.*, 1977), indicating that such transformation events, albeit rare, do happen. Second, to date BAV2 has not been shown to transform cells, of the BAVs, only BAV3 has been shown to transform cells (Lukash *et al.*, 1981),. The third possibility is that the bovine kidney and lung cells used for transfection might have already reached terminal differentiation, adding to the difficulty in isolating de-differentiated, transformed phenotype.

The inability of BAV2 E1 to immortalize primary cells *in vitro* urged us to attempt to construct a BAV2 packaging cell line from pre-established bovine cell lines such as MDBK. However, because these cells have been in culture for many generations, they will not depend on E1 expression for growth and since no selective pressure can be applied to retain E1 genes, any transfected E1 genes would be lost. The absence of E1 expression could also occur as a result of nucleotide methylation, which was previously observed when Ad DNA integrates in host-cell genome (reviewed in Doerfler, 1991). In order to assure the constitutive expression of the E1 region, the E1 genes were expressed under the control of the CMV immediate early promoter. In

addition, linking the E1 genes to a selectable marker would provide a means of selecting for cells that contain the plasmid contruct carrying the E1 genes. Therefore, the plasmid pCMV-E1 was modified further to carry the *neo* gene, which provide resistance to the drug G418, and accordingly named pCMV-E1-*neo*. Through selection with G418, the transfected cells were expected to express the drug resistance gene. Because the *neo* resistance gene and the E1 region of BAV2 were linked in the same plasmid construct, it would be safe to assume that G418 resistant cells also express the E1 genes.

We tested the idea that E1 expression might improve DNA uptake of a cell line. This hypothesis was based on the fact that 293 cells are very efficient in DNA uptake and constitutively epxress Ad5 E1 genes. The cell line MDBK, which is very inefficient at DNA uptake, was transfected with the plasmid pCMV-E1-*neo*. Through selection with the drug G418, 48 foci were isolated, out of which two cell lines, L-23 and L-24, were found to express the E1 region of BAV2. Subsequent testing of L-23 and L-24 cells showed that only 0.34% of the cells can take up trasnfected DNA, which was not different from parental MDBK cells, but far lower than what is customarily seen in 293 cells in which trasnfection rates reach about 67% of the cells. Therefore, these MDBK-based cell lines were not considered useful as a packaging cell line and a search for a new cell line was initiated.

For the purpose of establishing a BAV packaging cell line, several bovine cell lines were acquired and tested for transient expression of transfected DNA. The cell line CCL40 revealed efficiency of expressing transfected DNA comparable to 293 cells. As in the case of MDBK, these cells have also been grown in culture for several generations and will not depend on E1 expression. Consequently, the CCL40 cell line was transfected with pCMV-E1-*neo*, and underwent selection with the drug G418 resulting in two cell lines, namely ProCell 1 and ProCell 2. It was assumed that G418-resistant cells would have the resistance gene integrated in its genome as shown in many previous studies. In addition, it is assumed that G418-resistant cells

also contained the E1 BAV2 genes, since the trasnfection vector, pCMV-E1-*neo* was constructed in such a way as to link both genes under the control of separate promoters.

The cell lines ProCell 1 and ProCell 2 were not colony-purified; consequently, the site of integration was not determined. However, the result of a Southern blot analysis revealed that these two cell lines, generated from independent experiments, were a mixture of different cell lines (data not shown).

Expression of G418-resistance in the absence of E1 expression is possible in ProCell 1 and ProCell 2. This could be caused by partial insertion or DNA methylation in the regulatory sequences which has been previously observed in integrated foreign DNA (Doerfler, 1991). However, this study has shown high level expression of E1 as detected by a Northern blot analysis. The long term stability of E1 expression, however, remains to be determined.

Overlapping DNA sequences between the viral DNA that is used to establish the cell line and the DNA of the attenuated vector allow for double crossovers and the consequent generation of wild-type revertants. Therefore, it is necessary to have non-overlapping regions. That can be accomplished by designing the plasmid that is used to establish the cell line to have only those viral DNA sequences that were deleted from the plasmid used to rescue the attenuated virus. In our study, the plasmid pCMV-E1-*neo* carries BAV2 sequences from 1.1% to 17.5%. However, the plasmid vectors used in attempts to rescue E1-deleted viruses carried a deletion between 1.1% to 8.25%. Thus, these constructs do not allow for double crossovers and consequently eliminate the possibility of generating wild-type viruses.

Attempts to rescue a BAV2 viral vector carrying the *lacZ* gene in place of E1 did not result in the generation of a virus. These experiments were carried out using the constructed cells lines, ProCell 1 and ProCell 2. The failure to generate a virus from these transfections could be attributed to several reasons. First, this could have been caused by the failure to induce homologous recombination between two different DNA molecules to generate a single DNA

molecule which would get transcribed and translated to generate an infectious viral particle carrying the *lacZ* gene. Meanwhile, transfection experiments done with non-digested viral DNA did generate infectious wild-type viral particles. Second, the failure to recover a virus could have been an indication of lack of complementation of E1 products in the ProCell 1 and ProCell 2. However, the generation of defective E1 was unlikely since the expression cassette was constructed to carry wild-type E1 region with only the promoter deleted. Third, the deletions were based on examining the DNA sequence (Salmon and Haj-Ahmad, 1994; Esford and Haj-Ahmad, 1994) for open reading frames and comparisons to other known Ad polypeptides to determine the DNA sequences to be removed from E1 and E3 without removing any parts of the adjacent genes. However, there was the possibility that some genes, which may be necessary for the replication of the virus, were deleted, resulting in a non-viable virus.

Since virus rescue experiments using homologous recombination did not generate a virus, a different strategy was used to eliminate the need for homologous recombination. That strategy involved the modification of the vector cos-BAV2, which was previously constructed in our lab and carries the whole BAV2 genome. A cosmid vector, cosBAV2-*neo* was constructed, which carries the *neo* gene in the "original" E3 deletion between 75.8% to 81.4% viral map units. Attempts to rescue a mutant using this vector into CCL40 or CRL 6055 cell lines also failed. The cell lines CCL40 and CRL 6055 cell lines were assessed in the lab and they demonstrated DNA uptake efficiency of 37.5% and 28% respectively. Therefore, the DNA uptake efficiency of the cell lines could not be the cause of deficiency in virus generation. However, the E3 deletion made in this construct was the same size as the deletion in the plasmid pdlE3-5-Z plasmid. Therefore, the fact that both constructs did not generate a viable mutant virus could be attributed to the one property that is in common to both of these constructs, which is the size of the E3 deletion. Again, in this case, the DNA sequence was analyzed for open reading frames and homology to other Ad vectors. The "original" deletion showed an accurate removal of the E3

region without any parts of the adjacent genes. However, the same sequence analysis revealed that the E3 spontaneous deletion, accidentally identified in our virus stock, ranging from 24917 bp to 26417bp (Ojkic, 1998) has actually removed 29 bp from the right end of the 854 bp coding region of the neighboring protein eight (pVIII). This protein is associated with the outer capsid and such deletions would have been considered to be lethal to the virus, since altering the proteins associated with the outer capsid can alter the stability of the viral capsid as well as the attachment and the internalization stages of the virus life cycle.

Since, the E3 spontaneous deletion is a viable deletion, it would have been a better option to use in attempts to rescue viral recombinants instead of the "original" deletion. Attempts to use such a deletion would be more likely to generate a mutant virus. The strategy to construct such a vector is outlined in the Appendix, showing the plasmids constructed and the final plasmid needed to complete this strategy.

Future studies to evaluate and colony purify the cell lines ProCell 1 and ProCell 2 are necessary for the development of BAV2 as viral vectors. The site of integration and the integrity of the E1 sequences are to be determined in order to make predictions in terms of E1 complementation and the function of these cells as packaging cell lines. Also, the completion of the construction of cos-BAV2-E3-β, which contains the *lac*Z gene in place of the spontaneous deletion, would be needed for attempts to rescue an E3-deleted BAV2 virus. This rescue attempt seems to be more promising since it carries the spontaneous deletion present in the virus stock used in our lab.

XI. Summary

Based on the results obtained in this study, the following conclusions can be stated:

1) The plasmids pCMV-E1, which contain E1 region of BAV2 from 1.1% to 17.5%, and the plasmid pCMV-E1-*neo*, which contains the E1 region linked to the *neo* gene, were constructed.
2) Two cell lines, L-23 and L-24, based on MDBK cells, were constructed and shown to express E1 region of BAV2. However, these cell lines suffer low efficiency in DNA uptake.
3) Two cell lines, ProCell 1 and ProCell 2, based on CCL 40 cells, were also constructed and shown to express E1 region of BAV2. These cells were not colony purified, and they show efficiencies of expressing transfected DNA of about 34%.
4) The plasmids pdlE1E-Z and pdlE1E-Z-R, which carry the *lac*Z gene in place of the E1 region were constructed.
5) The plasmid pdlE3-5-Z, which carry the *lac*Z gene in place of the E3 region was constructed.
6) The BAV2 genomice clone, cosBAV2-*neo*, which carries the *neo* gene in place of the E3 region was constructed.

XII. References

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XIII. Appendix

A. Development of an E3 deletion BAV2 Genomic clone

1. Construction of cosBAV2-E3-*AscI*

The purpose of this study was to clone the spontaneous deletion, which is present in the viral stock currently used in our lab, with a uinque *AscI* restriction site at the exact position of the deletion in a cosmid vector that contains the whole BAV2 genome (Figure 40). Such vector can be used in attempts to rescue a mutant BAV2 virus that carries a foreign gene in place of deleted E3 region. The cloning strategy is very close to completion. All the plasmids were cloned up the cBAV-R-β.

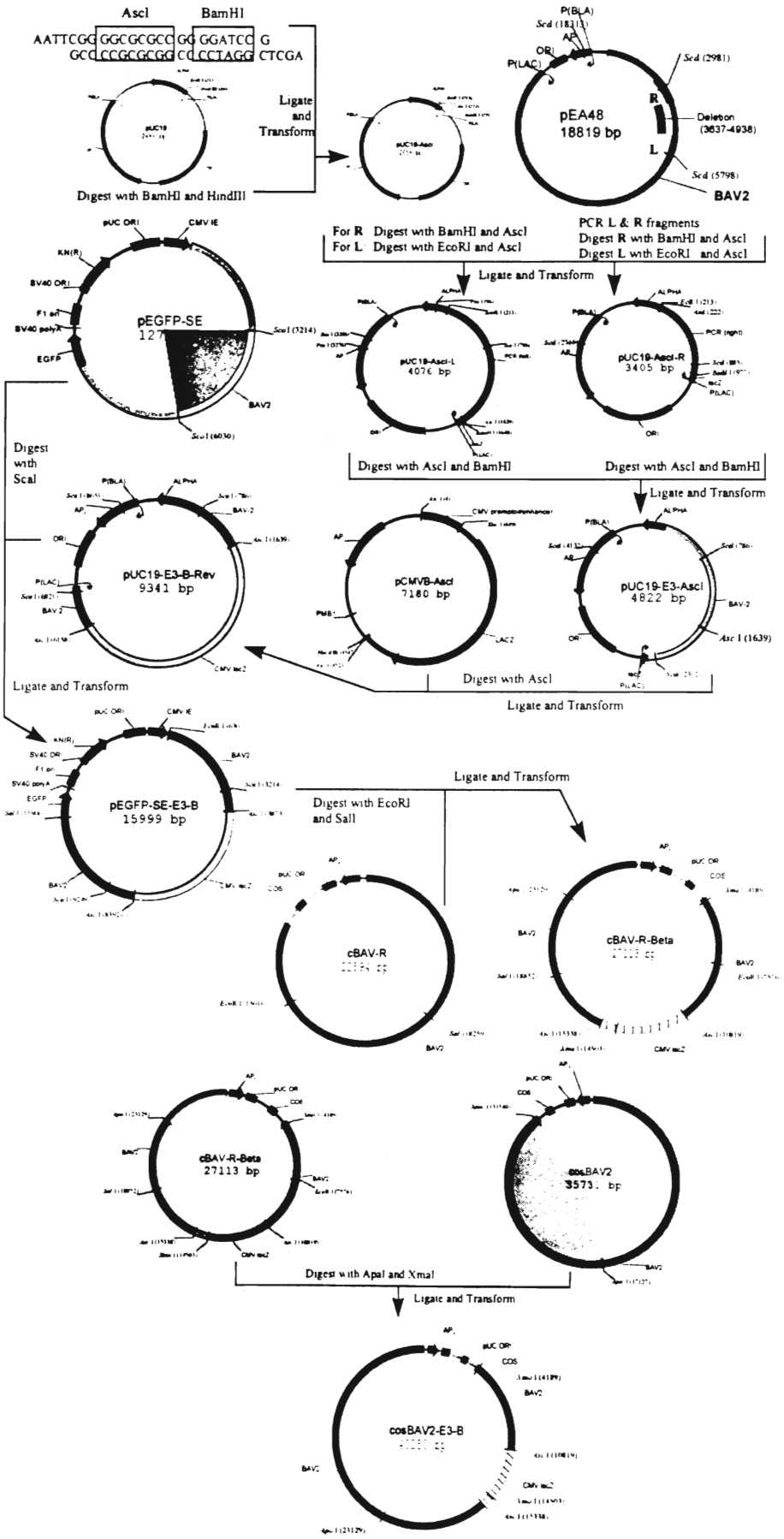
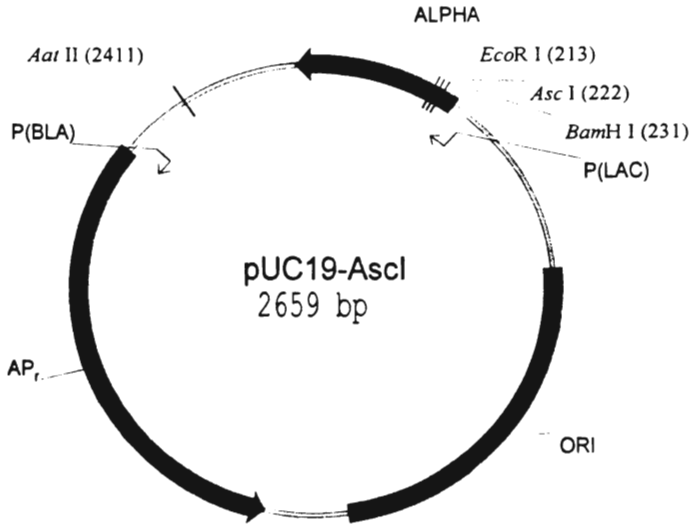


Figure 40: Construction strategy for cosBAV2-E3-B

I.



II.

Enzymes	AatII	AatII + AscI	EcoRI
Fragment Sizes (bp)	2,659	2,189	2,659
		470	

III.

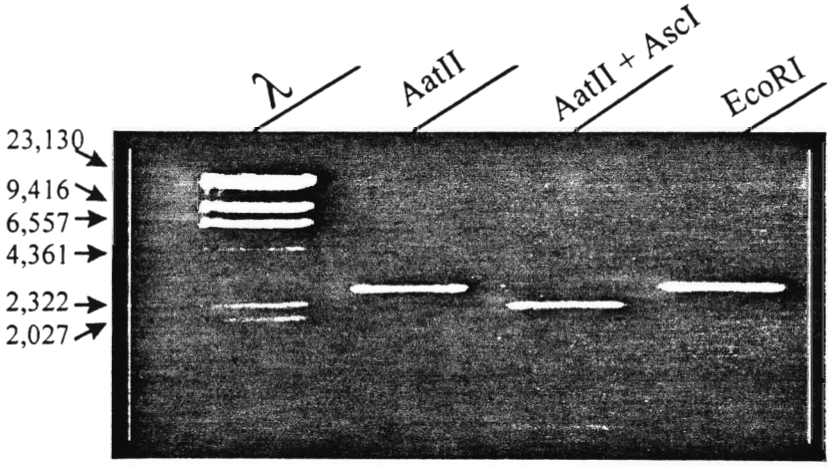
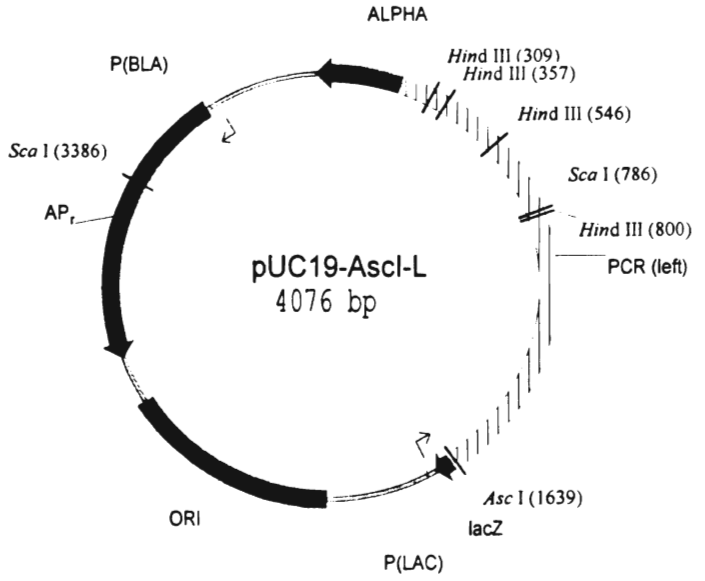


Figure 41: Restriction analysis of pUC19-*AscI*.

(I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, shows the plasmid pdlE3-5-Z digested with AatII, AatII + *AscI* and *EcoRI* respectively.

I.



II.

Enzymes	ApaII	AscI	HindIII
Fragment Sizes (bp)	1,246	4,076	3,585
	1,166		254
	951		189
	497		48
	216		

III.

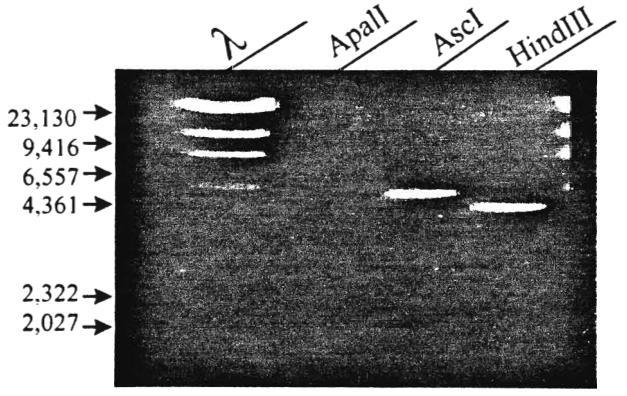
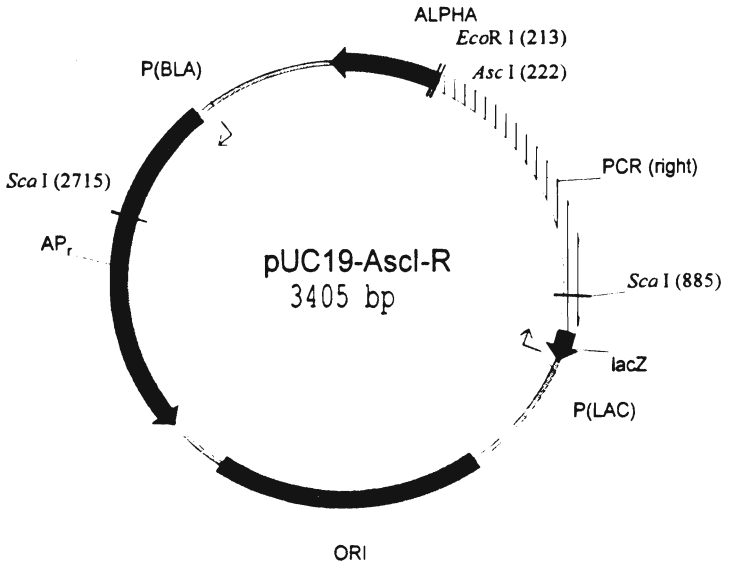


Figure 42: Restriction analysis of pUC19-*AscI*-L .

(I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, shows the plasmid pUC19-*AscI*-L digested with ApaLI, *AscI* and *HindIII* respectively.

I.



II.

Enzymes	ApaII	EcoRI	AscI
Fragment Sizes (bp)	1,662 1,246	3,405	3,405

III.

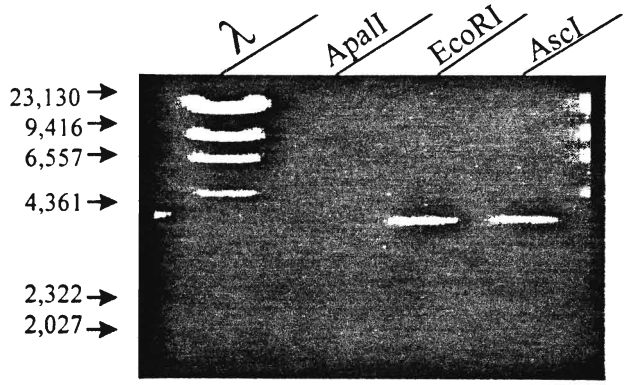
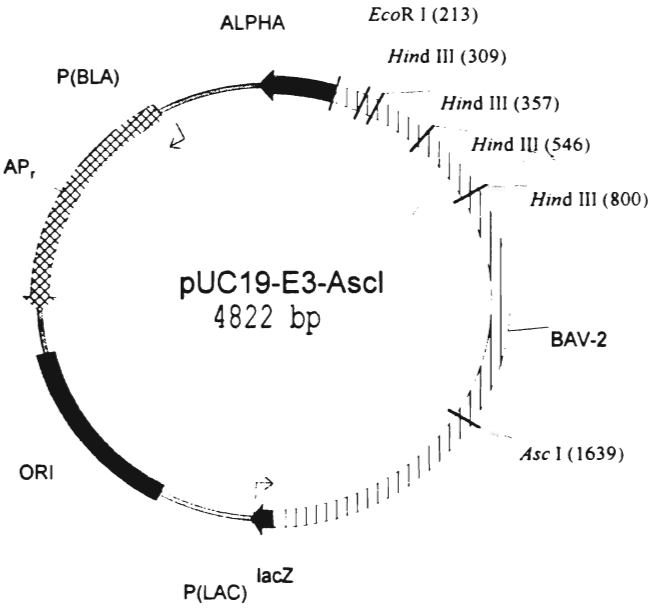


Figure 43: Restriction analysis of pUC19-*AscI*-R. .

(I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, shows the plasmid pUC19-*AscI*-R digested with ApaLI, *EcoRI* and *AscI* respectively.

I.



II.

Enzymes	AscI	HindIII	EcoRI
Fragment Sizes (bp)	4,822	4,331	4,882
		254	
		189	
		48	

III.

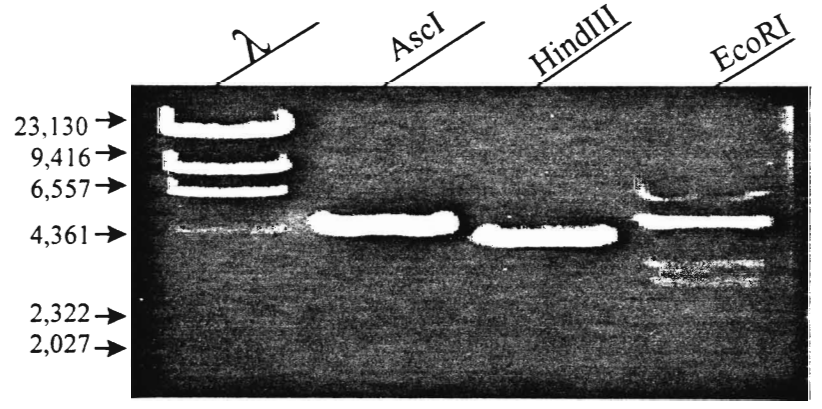
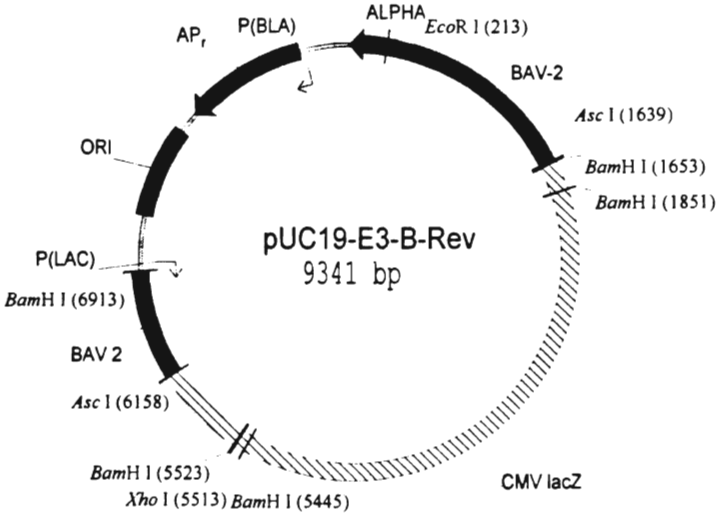


Figure 44: Restriction analysis of pUC19-E3-*AscI*.

(I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, shows the plasmid pUC19-E3-*AscI* digested with *AscI*, *HindIII* and *EcoRI* respectively.

I.



II.

Enzymes	AscI	BamHI	EcoRI	XhoI
Fragment Sizes (bp)	4,822	4.081	9,341	9,341
	4,519	3,594		
		1,390		
		198		
		78		

III.

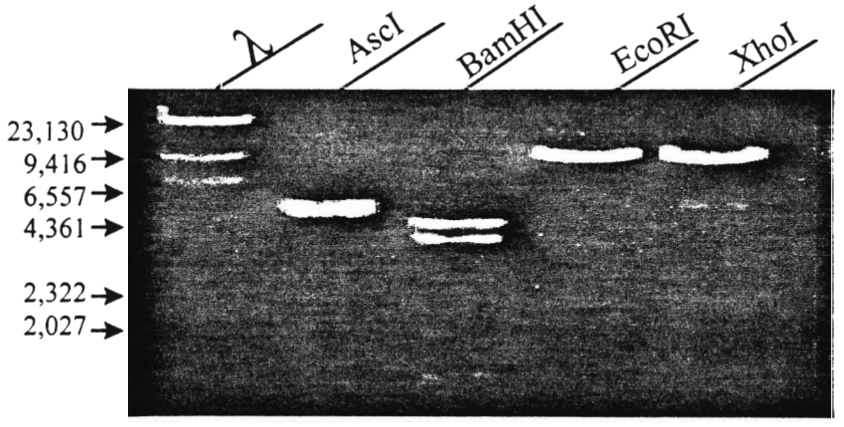


Figure 45: Restriction analysis of pUC19-E3-B-Rev.

(I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, shows the plasmid digested with *AscI*, *BamHI*, *EcoRI* and *XhoI* respectively

B. Development of an E1 deletion BAV2 Genomic clone

1. Cloning Strategy of cosBAV2-E1-Z

The purpose of this study was to construct a cosmid vector that carries the *lac*Z gene in place of deleted E1 region. This vector's construction is based on the construction of the vector cosBAV2, which contains the whole BAV2 genome and was previously cloned in our lab. The final construct cosBAV2-E1-Z would be used in attempts to rescue a BAV2 viral vector carrying the *lac*Z gene in place of the E1 coding region. This mutant virus requires the packaging cell lines ProCell 1 or ProCell 2, which would provide the E1 products to support viral growth. Such construct would eliminate the dependence on homologous recombination to generate a modified genomic DNA. Thus, making the rescue attempt experiments relatively easy where only one type of DNA has to be transfected. All of the plasmids, up to the plasmid cosBAV-CD, were constructed. Therefore, this experiment is only two steps away from completion.

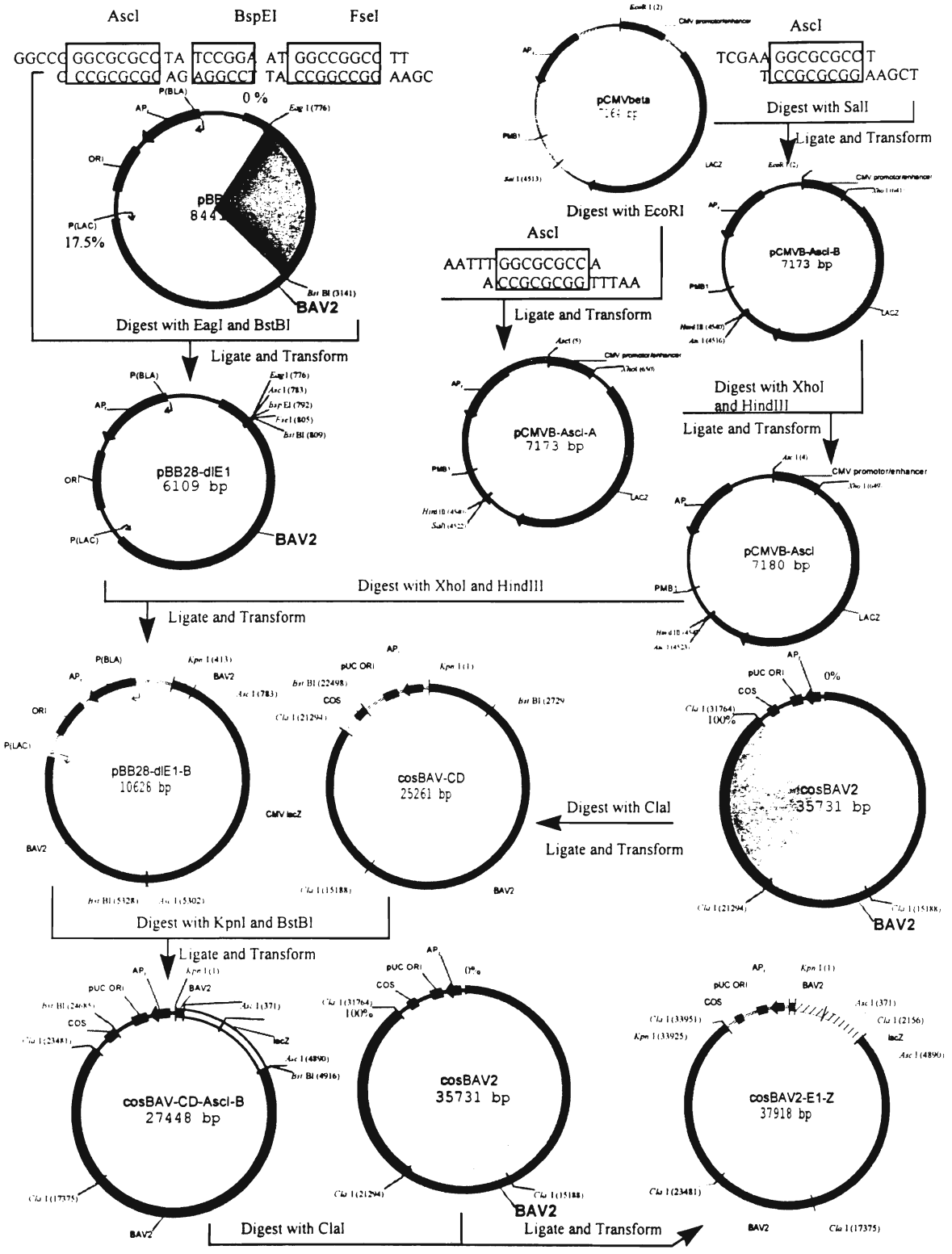
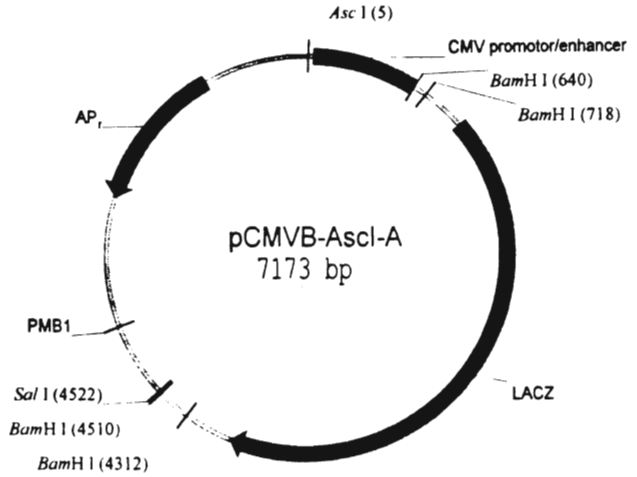


Figure 46: Construction strategy for cosBAV2-E1-Z

I.



II.

Enzymes	AscI	BamHI	SalI
Fragment Sizes (bp)	7,173	3,594	7,173
		3,303	
		198	
		78	

III.

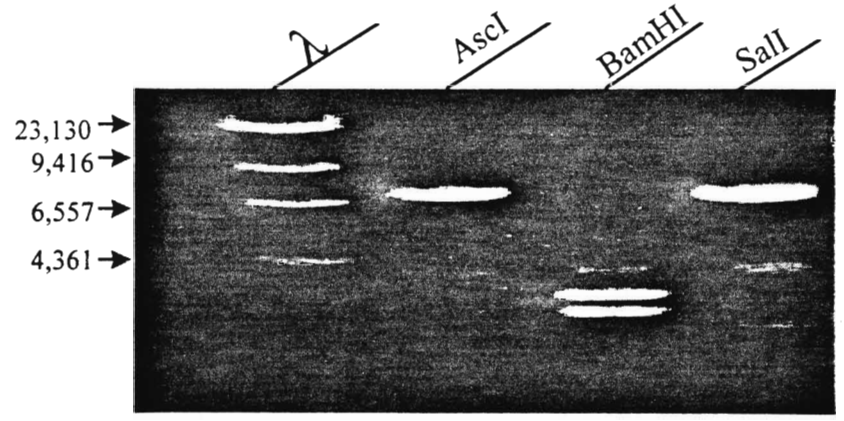
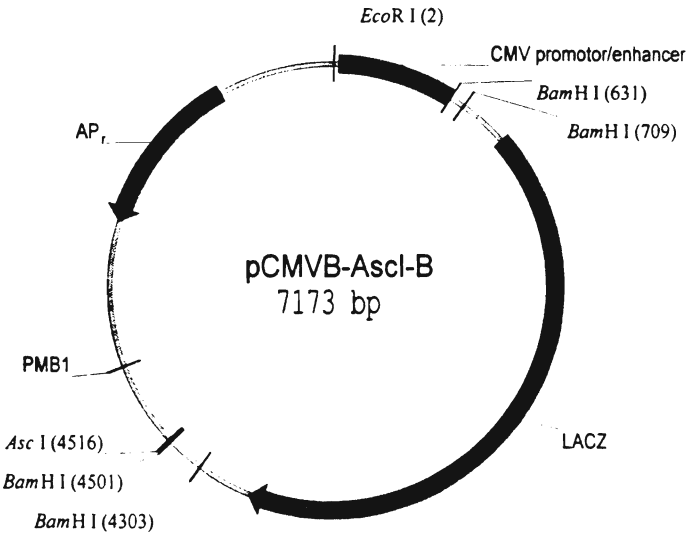


Figure 47: Restriction analysis of pCMVβ-*AscI*-A

(I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, shows the plasmid digested with *AscI*, *BamHI* and *SalI* respectively

I.



II.

Enzymes	AscI	BamHI	EcoRI
Fragment Sizes (bp)	7,173	3,594	7,173
		3,303	
		198	
		78	

III.

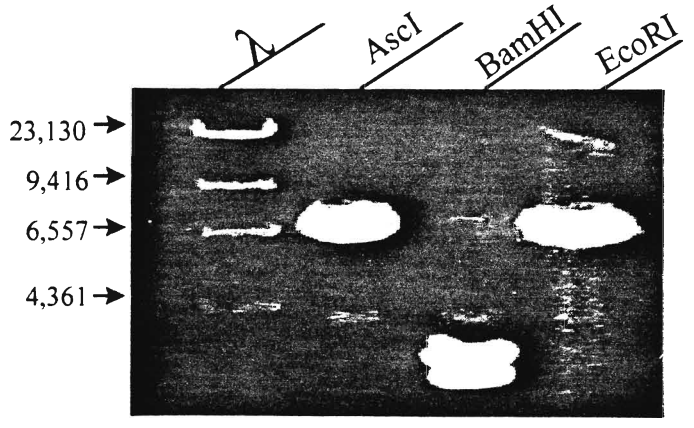
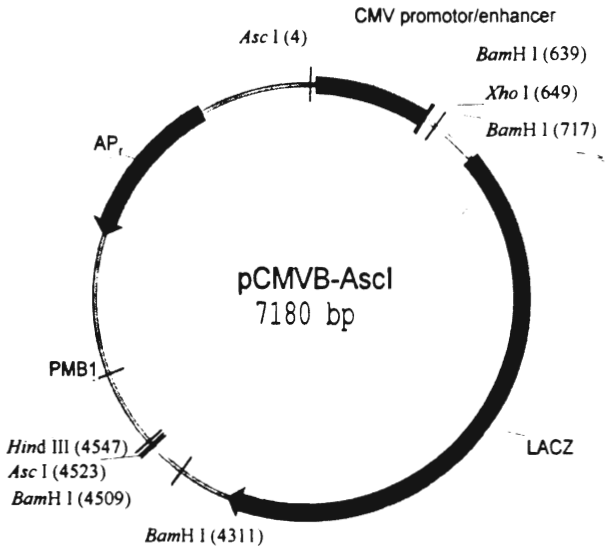


Figure 48: Restriction analysis of pCMVβ-*AscI*-B

(I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, shows the plasmid digested with *AscI*, *BamHI* and *EcoRI* respectively.

I.



II.

Enzymes	AscI	BamHI	HindII	XhoI
Sizes(bp)	4,519	3,594	7,180	7,180
	2,661	3,310		
		198		
		78		

III.

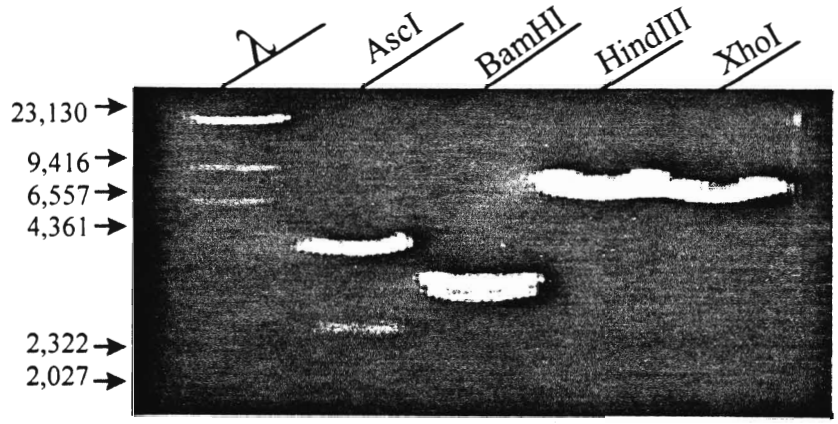
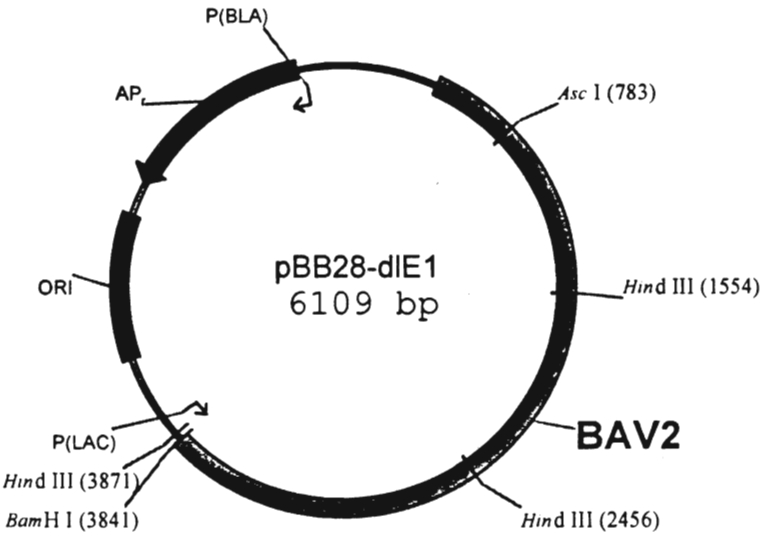


Figure 49: Restriction analysis of pCMVβ-*AscI*

(I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, and 5 show the plasmid digested with *AscI*, *BamHI*, *HindIII* and *XhoI* respectively.

I.



II.

Enzymes	AscI	EcoRI	HindIII
Fragment Sizes (bp)	6,109	6,109	3,792
			1,415
			902

III.

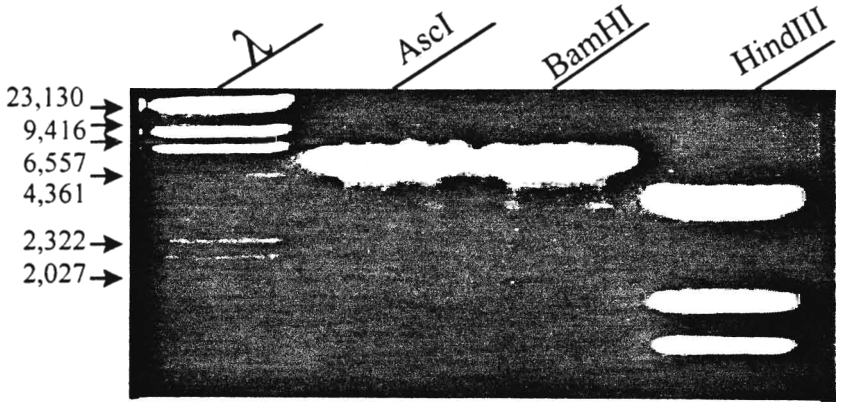
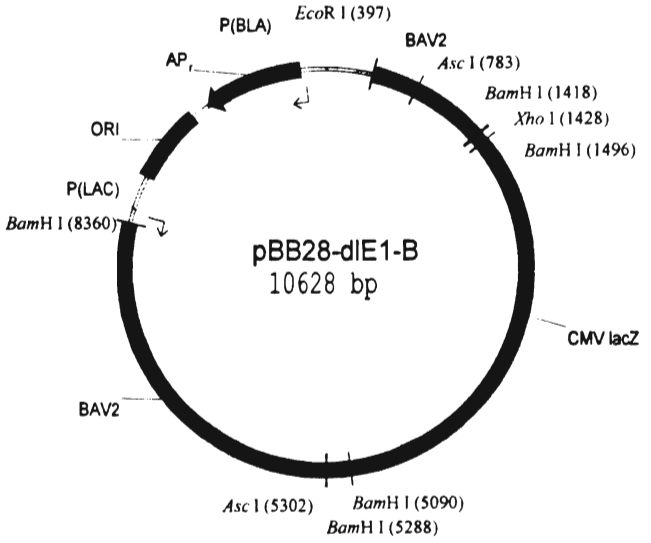


Figure 50: Restriction analysis of pBBdlE1

(I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, show the plasmid digested with *AscI*, *BamHI* and *HindIII* respectively.

I.



II.

Enzymes	AscI	BamHI	EcoRI	XhoI
Sizes (bp)	6,109	3,686	10,628	10,628
	4,519	3,594		
		3,072		
		198		
		78		

III.

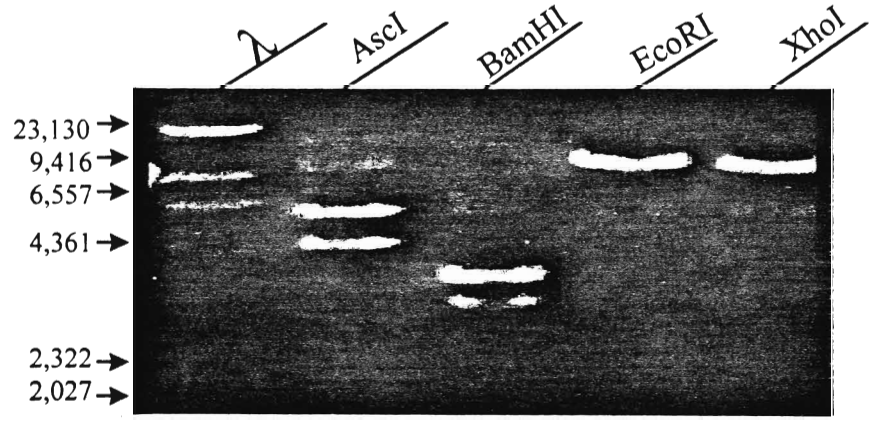
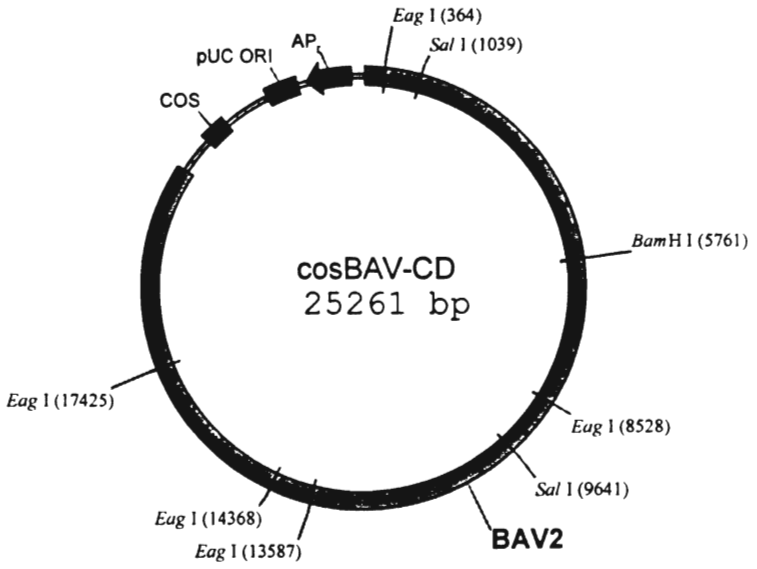


Figure 51: Restriction analysis of pBBdlE1-B

(I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, and 5 show the plasmid digested with *AscI*, *BamHI*, *EcoRI* and *XhoI* respectively.

I.



II.

Enzymes	BamHI	SalI	EagI
Fragment Sizes (bp)	25,261	16,659	8,200
		8,602	8,164
			5,059
			3,057
			781

III.

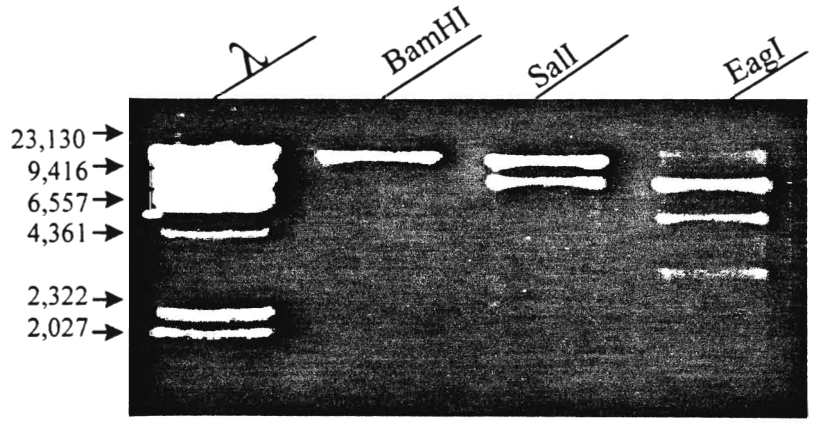


Figure 52: Restriction analysis of cosBAV-CD

(I) Restriction endonuclease map of the plasmid (II) Table of the expected DNA fragment sizes and (III) A picture of an agarose gel electrophoresis. Lane 1 shows the λ marker used to estimate the DNA fragment sizes, lanes 2 , 3, 4, shows the plasmid digested with *BamHI*, *SalI* and EagI respectively.