The cloning and reconstitution of a Bovine Adenovirus Type 2 E3 deletion mutant and the sequencing and analysis of the Early 4 region.

by

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ABSTRACT

Recombinant Adenoviruses (Ads) have been shown to have potential applications in three areas: gene therapy, high level protein expression and recombinant vaccines. At least three different locations within the Ad genome can be deleted and subsequently used for the insertion of foreign sequences. These include the Early 3 (E3), Early 1 (E1) and Early 4 (E4) regions. Viral vectors of this type have been well studied in Human Ads 2 and 5, however one has not yet been constructed for Bovine Adenovirus Type 2 (BAV2).

The E3 region is located between 76.6 and 86 m.u. on the r-strand and is transcribed in a rightward direction. The gene products of the Early 3 region (E3) have been shown to be non-essential for viral replication, *in vitro,* but are required for host immunosurveillance. This study represents the cloning and reconstitution of a BAV2 E3 deletion mutant. A deletion of 1800bp was made within the E3 region of BAV2 and the thymidine kinase gene was subsequently inserted in the deleted area. The plasmid pdlE3-4tk1 (23.4Kbp) was constructed and used to to facilitate homologous recombination with the wild type BAV2 to produce a mutant. Southern Blotting and Hybridization results suggest the presence of a BAV2 E3 deletion mutant with thymidine kinase sequences present.

The E4 region of Human Adenovirus types 2 and 5 is located at the extreme right end of the genome (91.3 map units - 99.1 map units) and is transcribed in a leftward direction giving rise to a complicated set of differentially spliced mRNAs. Essentially there are 7 open reading frames (ORFs) encoding for at least 7 polypeptides. The gene products encoded by the E4 region have been shown to be essential for the expression of late viral genes, host cell shutoff and normal viral growth. We have cloned and

sequenced the right end segment between 90.5 map units and 100 map units of the BAV2 genome. The results show several open reading frames which encode polypeptides exhibiting homology to three polypeptides encoded by the E4 region of human adenovirus type 2. These include the 14kDa protein encoded by ORF1, the 34kDa protein encoded by ORF6 and the 13kDa protein encoded by ORF3. The nucleotide sequence, restriction enzyme map, and ORF map of the E4 region could be very useful in future molecular manipulation of this region and could possibly explain the slow growth rate of BAV2 in MDBK cells.

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CHAPTER 1: INTRODUCTION

The Adenovirus (Ad) was first discovered in 1953 when Rowe and colleagues surgically removed the adenoids from 53 children. The slowly progressive cytopathic effect of the virus gave the virus the name "adenoid-degenerating (Ad) agent". Since then, Ads have been isolated from every studied species of placental mammals, marsupials, birds and amphibians .

The classification of Ads is based on several properties. These include hemagglutination patterns, oncogenicity, DNA base composition and homology, relationship of T antigens and finally restriction enzyme patterns. Ads belong to the family Adenoviridae, which is further subdivided into two genera, *Mastadenovirus* and *Aviadenovirus*. Members of the genus *Mastadenovirus* infect human, simian, bovine, equine, porcine, ovine and canine hosts. The members of this group also possess a group specific antigen (Ag). Members of *Aviadenovirus* infect avian species.

Ads are nonenveloped icosahedral shaped virions which are approximately 65-80nm in diameter and made up of 13% DNA and 87% protein. Each virion contains a single molecule of double stranded DNA with a 55KDa protein covalently attached to each of its 5' ends. The genome is divided into early and late transcription units. The early regions, E1 to E4, are transcribed before the onset of DNA replication,

whereas the late transcription units are transcribed after the onset of DNA replication.

The early genes are particularly interesting because they encode protein products which are necessary for DNA replication and late gene expression. However, the gene products of the E3 region are not required for viral replication in cell culture, but are believed to be responsible for host immunosurveillence and important for the establishment of long term infection. These two properties of the E3 region make it a good candidate for deletion and subsequent insertion of 4-5Kbp of foreign sequences for the construction of a recombinant viral vectors.

A variety of literature exists on the construction of human Ad2 and Ad5 recombinant vaccines containing deletions within the E3 region. Ad vectors have been constructed that express glycoproteins from herpes virus (Johnson *et al..*, 1988), hepatitis B virus (Morin *et al.,* 1987), human immunodeficiency virus type 1 (Dewar *et al.,* 1989), vesicular stomatitis virus (Schneider *et al.*, 1989), rabies virus (Prevec *et al.,* 1990) and the respiratory syncytical virus (Collins *et al.*, 1990; Hsu *et al.,* 1991).

The primary objective of this study was to construct a recombinant BAV2 vector containing a deletion in the E3 region. If a recombinant BAV2 vector was rescued, future experiments could be designed to insert a gene for an antigen in the E3 region, as has been done for Ad2 and Ad5. The application of a recombinant BAV2 vector expressing a cloned antigen gene in the deleted area could be as a recombinant vaccine for cattle or sheep. Such a vaccine would be valuable because it would be relatively cheap, easy to administer and it would provide long lasting immunity.

Another region of the Ad genome which has been used for deletion and subsequent insertion of foreign sequences is the E4 region. A deletion

can be made between the start for E4 transcription and the right inverted terminal repeat as long as the virus is propagated in the cell line W162 which provides the missing functions of the E4 region, *in trans*. The function of gene products encoded by the E4 region have been studied using E4 deletion mutants. E4 deletion mutants of Ad2 and Ad5 have a 10-20 fold decrease in the final virus yield when compared to the wild-type as well as being inefficient for host cell shut off and late viral gene expression.

During this study, the putative E4 region of BAV2 was sequenced and subsequently analyzed for two reasons: first, BAV2 was observed to grow slowly in MDBK cell line. Therefore, the possibility that E4 may influence BAV2's growth was investigated by sequencing and subsequently analyzing the ORFs of the E4. Secondly, the sequence and restriction enzyme map of the E4 region would be a very valuable tool in future studies involving deletions within the E4 region.

CHAPTER 2: LITERATURE REVIEW

2.1 Adenoviruses

Ads are nonenveloped medium sized DNA viruses with an inner DNA protein core enclosed by an icosahedrally shaped protein capsid. This outer capsid consists of 20 triangular surfaces and 12 vertices. A fiber projection extends from each of the 12 vertices and the length of the fiber varies amongst serotypes (Norrby, 1966: 1969 and Horwitz, 1990). The protein coat is composed of 252 subunits called capsomeres. Of these 252 capsomeres, 240 are hexons and 12 are pentons (Ginsberg *et al.*, 1966 and Horwitz, 1990). Each penton contains a fiber projecting from a penton base on the surface of the capsid. The penton base is surrounded by 5 hexons, hence the name "penton". All other capsomeres are hexons because each one is surrounded by six other capsomeres. The double stranded DNA is sheltered in the core structure and contains a 55kDa terminal protein which is covalently attached to each of the the 5' ends of the viral genome.

Most detailed structural protein strudies have been carried out for human Ad types 2 and 5. The location of these viral polypeptides has been determined by surface labelling of the virion as well as analysis of degraded virions (reviewed by Horwitz, 1990). Figure 1 represents the various Ad2 capsid proteins and their positions relative to the core which shelters the double stranded genome. Polypeptide II is a 120kDa hexon

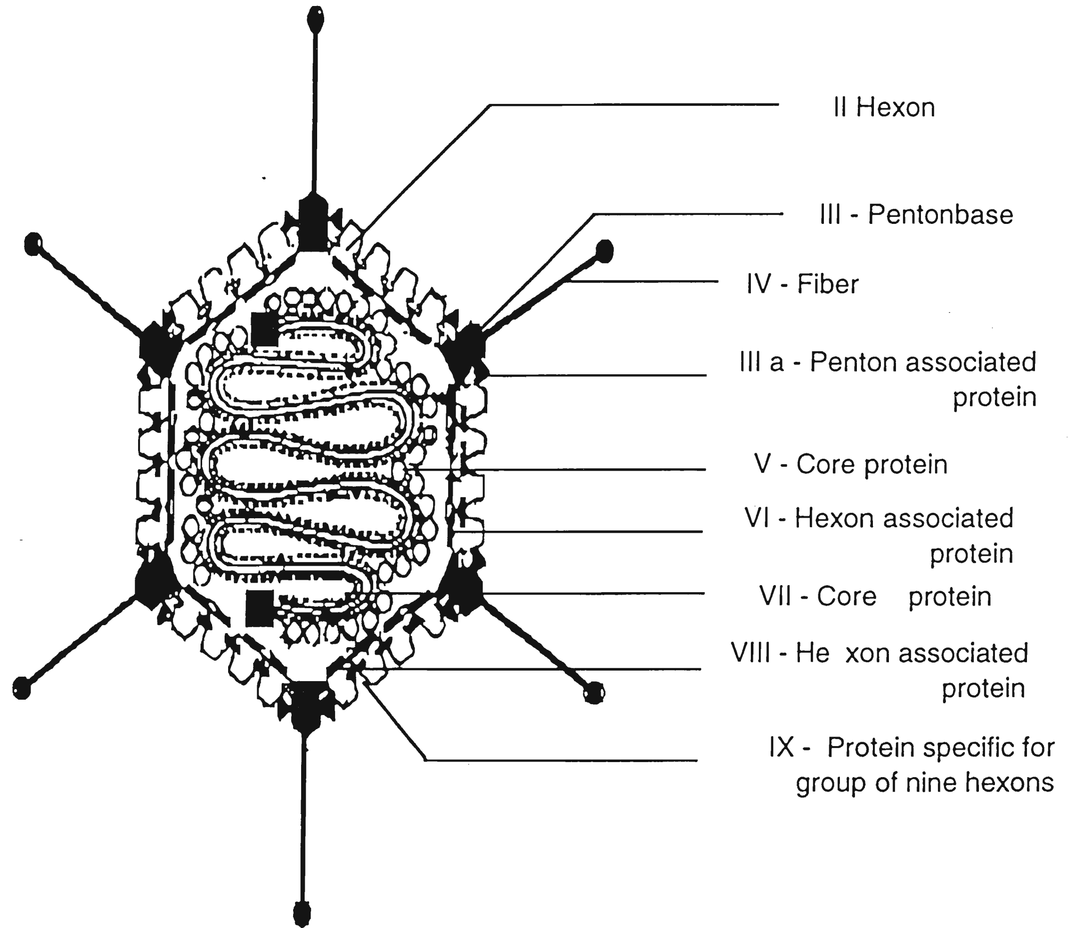


Figure 1: A diagrammatic representation of the adenoviral capsid proteins and their positions relative to the double stranded genome. (taken from Horwitz 1990)

polypeptide. The hexon capsomeres are made of a trimer of 3 identical polypeptides held together by. noncovalent interactions. The 100kDa protein is required for the assembly of the hexon trimer from monomers. Each hexon is associated with 6 other hexons through polypeptide IX (12kDa). Hexons are also associated with viral polypeptides VI (24kDa) and VIII (13kDa) to form the triangular surfaces of the viral capsid. Polypeptides V (48.5kDa), VI, VII (18.5kDa) and VIII are located within the core of the virion. Polypeptides V and VII are associated with the double stranded DNA molecule, whereas polypeptides VI and VIII are located within the internal surface of the hexon. The amino terminus end of the fiber is noncovalently attached to the penton base whereas, the carboxy knob is projecting away from the virion capsid (reviewed by Horwitz, 1990).

The various structural polypeptides characterize this group of viruses in various ways. Serological classification of Ads involve antigenic determinants which are present on the hexon, penton and fiber. Ad hemagglutinins are properties of the fiber and the penton base polypeptides and are related to the cytopathic effect (CPE) observed during Ad infection in cell culture (reviewed by Horwitz, 1990).

2.2 Classification

Ads belong to the family *Adenoviridae* which is subdivided into two genera *Mastadenovirus and Aviadenovirus.* The difference between the two genera is a group specific antigen present only in the genus *Mastadenovirus.* The genus *Aviadenovirus* includes Ads which infect avian hosts (fowl, turkey, goose, pheasant and duck). The genus *Mastadenovirus*

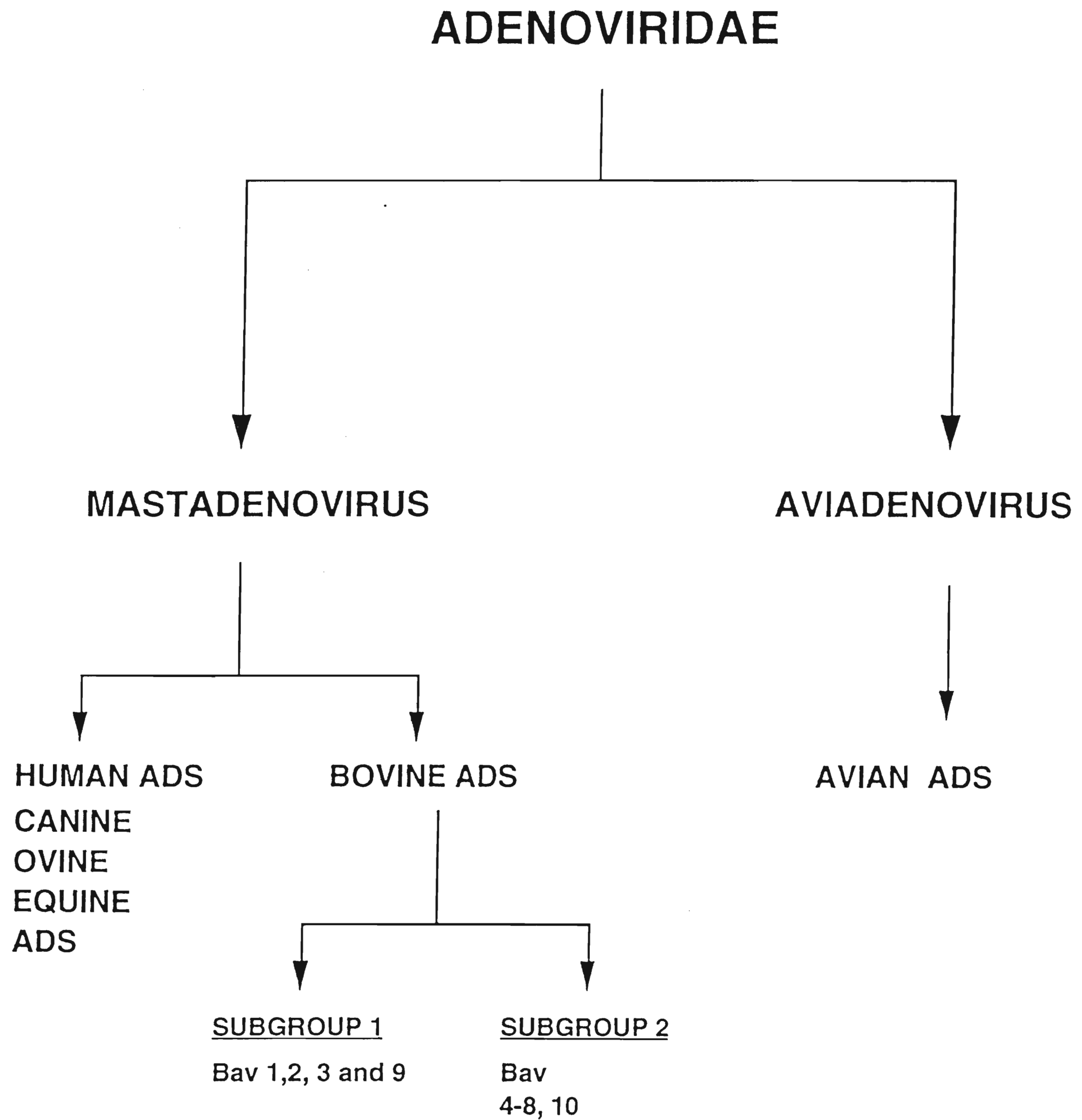


Figure 2: Diagrammatic representation of the classification of the family Adenoviridae.

consists of viruses isolated from mammals (man, monkey, cattle, pig, sheep, horse, dog, goat and mouse (Joklik, 1988) (See Figure 2).

Mastadenovirus is further subdivided into *Bovine Adenoviruses* (BAV) and other Ads which infect human, canine and ovine hosts. BAVS are further subdivided into two groups based on different serotypes. Group I includes serotypes 1, 2, 3, and 9. Group II includes serotypes 4-8 (Bartha, 1969 and Horwitz, 1990).

Ads have a large number of different hosts and over 80 different serotypes of mammalian adenoviruses and avian adenoviruses have been reported, forty- seven of them from human hosts (Herholzer, 1988). Forty-seven of the human serotypes are further divided into six subgenera (A to F) based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (SDS-PAGE) of polypeptides (See table 1) (reviewed by Wadell, 1994).

The six subgenera of human serotypes have been classified based on a number of characteristics such as hemagglutination patterns, GC content and restriction endonuclease digestion by *Sma* I (Table 1). Hemagglutination by Ads was first demonstrated by Rosen in 1958. The G+C content of the DNA of each subgenus differs: subgenus A 48%, subgenus B-1 and B-2 51%, subgenus C 58%, subgenus D 58%, subgenus E 58% and subgenus F 52%. It was generally found that low G+C content (48%) of group A correlated with tumor induction whereas group C (58%) are not oncogenic. *Sma* I cleaves the DNA at $^{5'}$CCC GGG, therefore fragments obtained after restriction endonuclease digestion are characteristic of the subgenera of human serotypes (reviewed by Wadell *et al.,* 1985).

Subgenus	Serotype	DNA Homology (%) Intrageneric	DNA Homology (%) Intergeneric	DNA G + C (%)	DNA Number of SmaI[a] fragments	Apparent molecular mass of the major internal polypeptides (kD) V	VI	VII	Hemagglutination pattern[b]	Length of fibers (nm)	Oncogenicity in newborn hamsters	Tropism symptoms
A	12,18,31	48-69	8-20	48	4-5	51.0-51.5 46.5-48.5[c]	25.5-26.0	18	IV	28-31	High (tumors in most animals in 4 months)	Cryptic enteric infection
B:1 B:2	3,7,16,21 14,[d]11,34, 35	89-94	9-20	51	8-10	53.5-54.5	24	18	I	9-11	Weak (tumors in few animals in 4-18 months)	Respiratory disease Persistent infections of the kidney
C	1,2,5,6	99-100	10-16	58	10-12	48.5	24	18.5	III	23-31	Nil	Respiratory disease persists in lymphoid tissue
D	8,9,10,13, 15,17,19,20, 22 to 30,32,33, 36,37,38,39, 42 to 47[e]	94-99	4-17	58	14-18	50.0-50.5[f]	23.2	18.2	II	12-13	Nil	Keratoconjunctivitis
E	4		4-23	58	16-19	48	24.5	18	III	17	Nil	Conjunctivitis Respiratory disease
F	40, 41	62-69	15-22	52	9-12	46.0-48.5	25.5	17.5	IV	28-33	Nil	Infantile diarrhea

Table 1: Properties and Characteristics of the human adenovirus serotypes of the subgenera A-F.

The DNA homology between serotypes of subgenera is only 4-23%. However, DNA homology between members of the same subgenus is greater than 48% (Green *et al.*, 1979). Sequence divergence between members of a single group has been found to be greatest in regions coding for the hexon capsomere and the fiber. The hexon and fiber are polypeptides to which the host's antibodies are directed, therefore variation would be expected here.

2.3 Ad infection

The normal life cycle of human Ads involves either a lytic infection or a latent infection. The lytic infection ultimately results in the destruction of the infected cell. An Ad lytic infection within group C is very efficient at the production of new virions and may result in the production of approximately 10,000 virions per infected cell (Green *et al.*, 1961). An Ad first attaches to a specific receptor on the host's cell membrane and then adsorbs and penetrates through the cytoplasmic membrane via clathrin coated pits which form endocytic vesicles. When the virus enters the cell, the pH drops to 5.5 resulting in the disruption of the endocytic vesicle and the release of the virus into the cytoplasm. The virus is then transported across the cytoplasm to the nucleus of the cell via microtubules where uncoating takes place. Parts of the genome are transcribed and the double stranded genome replicates. Viral mRNA are then tranported to the cytoplasm, where they are later translated into viral proteins on cytoplasmic polysomes. The viral proteins are then transported back to the nucleus where virions are assembled (reviewed by Horwitz, 1990).

A latent infection involves the virus persisting within the host cell without eliciting an immune response. Integration of Ad viral DNA into a host's genome at selective sites can result in "insertional mutagenesis" which seems to be associated with the initiation of viral malignant transformation (Doerfler and Bohm (eds) 1992). The oncogenic potential of Ads was first observed by Trentin *et al.* in 1962 and since then has been used as a characteristic for dividing Ads into subgenera.

2.4 Ad genome

2.4.1 DNA replication

The Ad genome consists of a double stranded molecule of approximately 36,000 bp in size (Joklik, 1988) enclosed in a protein capsid. The organization of the genome is similiar in all Ad groups. The viral genome is bracketed by inverted terminal repeats (ITR) which flank the coding regions and are used during Ad DNA replication. These ITRs consist of 54-166 nucleotides possessing the structure ABCD_____D'C'B'A'. All animal Ads studied have conserved this feature (reviewed by Horwitz, 1990) (see Figure 3).

DNA replication begins about 7 hours post infection. The replication of Ad DNA proceeds in a semi-conservative fashion beginning at origins of replication (ORI) located at nucleotides 1-18 in either ITR. DNA replication requires virally encoded pTP, DNA binding protein (DBP) and Ad DNA polymerase (140kDa) as well as a series of cellular factors (NFI, NFIII, and ORPA). The cellular factors bind to the ORI where they interact with pTP and Ad DNA polymerase to form a protein/DNA complex. Replication is initiated when the protein/DNA complex covalently attaches the pTP to dCTP to form a pTP-dCMP complex which presents a

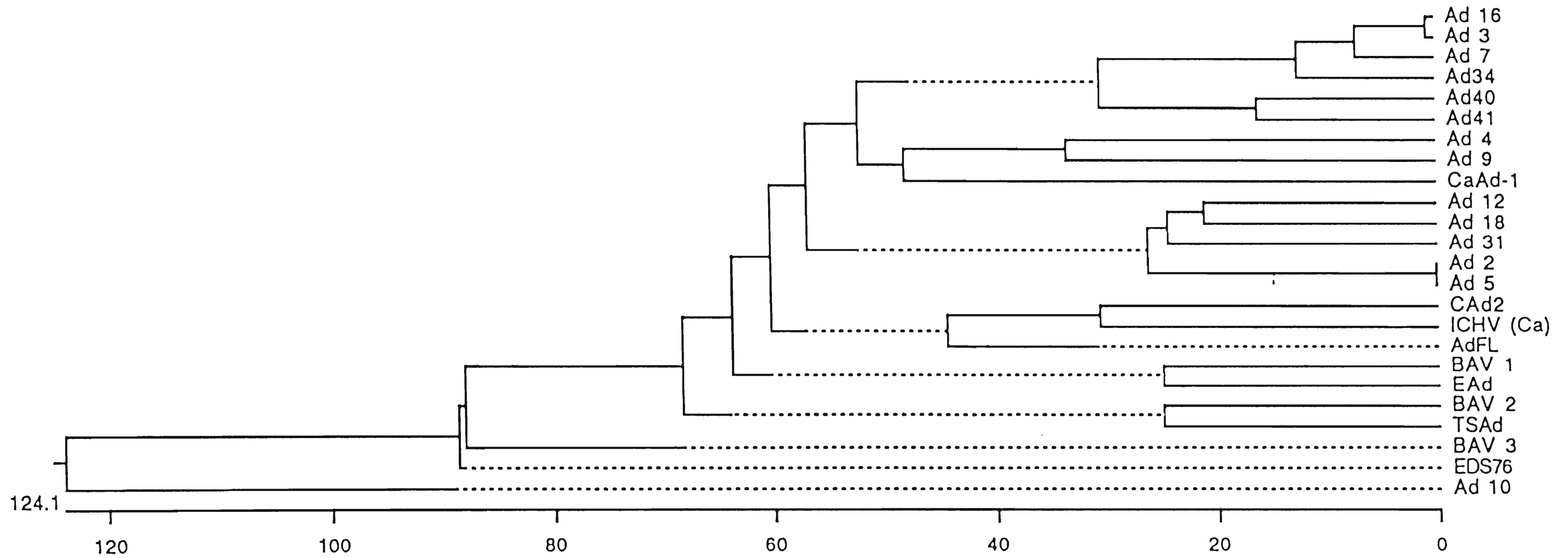


Figure3: Phylogenetic tree comparing the ITR's of Human Adenoviruses (Ad12, Ad18, Ad31, Ad3, Ad7, Ad16, Ad2, Ad5, Ad9, Ad10, Ad4, Ad34, Ad40, Ad41), Canine Adenoviruses (ICHV, CAd2, CaAd-1), Equine Adenovirus (EAd), Bovine Adenoviruses (BAV1, BAV2, BAV3), Rodent Adenovirus (AdFL), Shrew Adenovirus (TSAd) and Avian Adenoviruses (CELO, EDS76).

free 3'-OH group serving as a primer for elongation by Ad DNA polymerase. Elongation occurs until the displacement of one strand occurs as outlined in Figure 4. The displaced strand forms a panhandle structure through the annealing of complementary ITRs and subsequently serves as a substrate for the initiation of the synthesis of the double stranded genome.

2.4.2 Ad gene expression

The study of the Ad gene structure and gene control has lead us to understand of many of the principles we adhere to today. The definition that the start site of polymerase II transcription specified the mRNA 5' end, the discovery of RNA splicing, the finding that RNA cleavage rather than transcription termination generated the mRNA 3' end and the development of systems for *in vitro* transcription that allowed for the isolation of transcription factors were all recognized first using Ad systems (reviewed by Nevins, 1987).

Most of the Ad genes are transcribed in a series of steps regulated by viral promoters and cell encoded transcription factors. These transcription factors recognize specific nucleotide sequences located within the viral promoters which are situated upsteam and certain downstream regions of the viral genes (reviewed in Deofler, 1994).

The replication of the Ad is divided into 2 phases: early, corresponding to the events occurring before viral DNA replication and late, corresponding to events after the initiation of DNA replication. However, many early regions continue to be transcribed after the initiation of DNA replication. The late regions are then sequentially transcribed. During the early phases, 4 regions of the genome are expressed: Early region 1 (E1), which contains E1a and E1b; E2, which

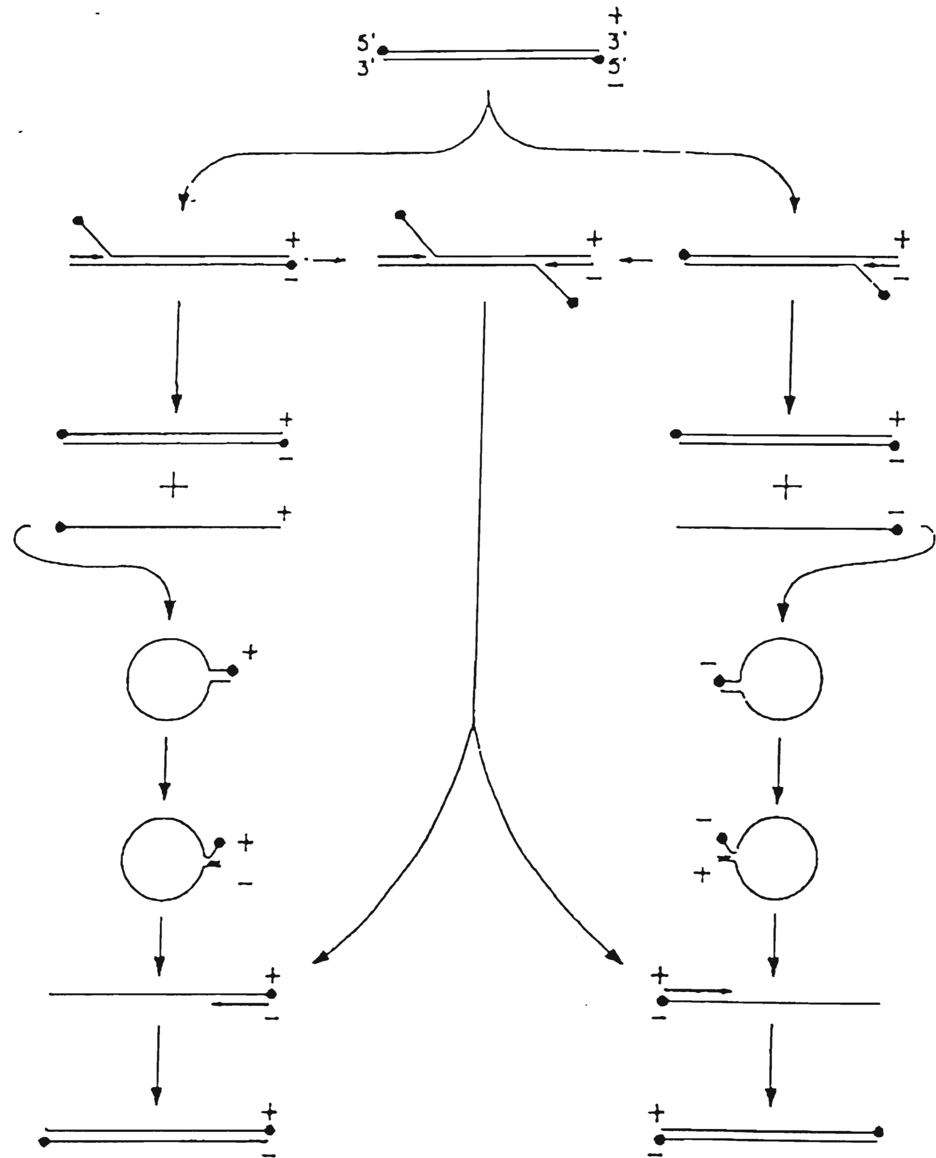


Figure 4: A proposed model for Adenoviral DNA replication (Joklik, 1988).

contains E2a and E2b; E3; and E4. The transcription of these regions is driven by promoters situated within the viral genome. After the initiation of DNA replication, the major late promoter (MLP) initiates viral transcription of all the late regions (L1 to L5) of the genome (reviewed by Sharp, 1984). The late regions are responsible for the production of structural virion proteins. Components of the MLP are located at about 17, 20, and 27 map units on the viral genome.

Specific viral functions can be encoded on one of the two viral DNA strands. The two strands have been termed rightward transcribed (or upper) or leftward transcribed (or lower) strand. The structure and the genome map of early viral mRNAs have been determined a number of ways including electron microscopy, S1 nuclease mapping, *in vitro* translation of hybrid RNA and complementary DNA cloning (Nevins, 1987). As mentioned above, during the early phase, four regions of the the Ad genome are transcribed. Three of these early regions (E1a, E1b, and E3) are transcribed in a rightward direction and three regions (E4, E2a and E2b) are transcribed in a leftward direction on the l strand (See Figure 5). The late regions (L1 to L5) are all transcribed in a rightward direction on the r strand.

2.5 E1 region

During the early times of infection, the E1 region is the first region to be transcribed. The primary transcript of this region is processed to give a complicated set of mRNAs responsible for the production of specific gene products having some important biological function (Graham *et al.*, 1974; Gallimor *et al.,* 1974 and van der Eb *et al.,* 1974), one of which is the transformation of cells.

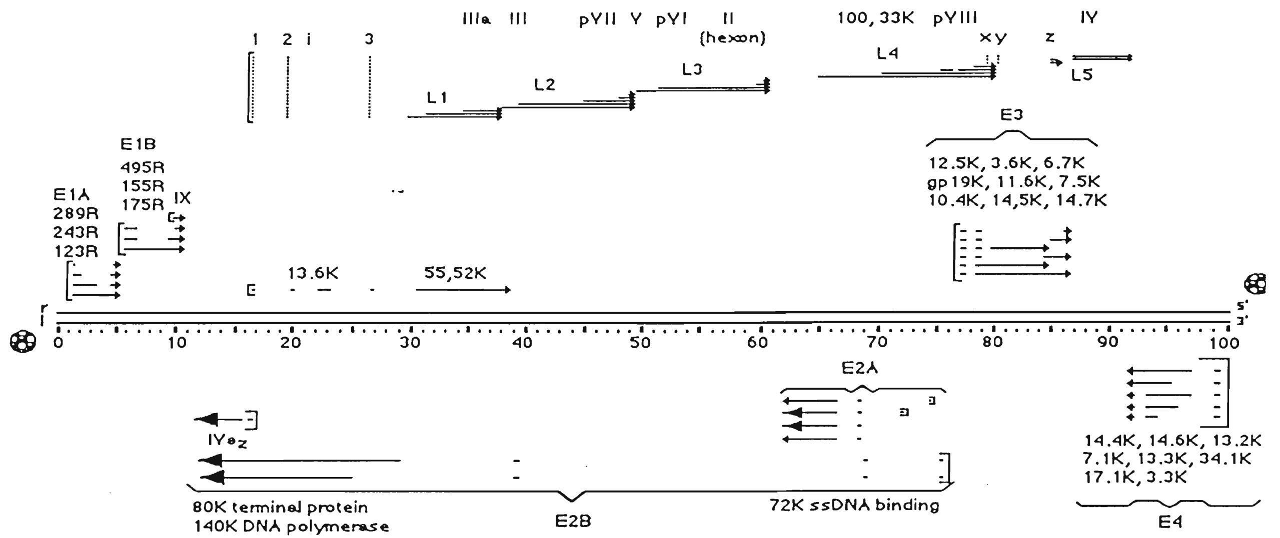


Figure 5: Transcription map of an Adenovirus. E1A, E1B, E3 and L1-L5 are transcribed in a rightward direction on the upper strand whereas, the E4, E2A and E2B regions are transcribed in a leftward direction on the lower strand. The various transcripts encoded by each region are represented by arrows. Each early region contains its own promoter and there is a MLP located at 16 m.u. which is responsible for the transcription of the late regions. The promoters are represented by [.]. (taken from Joklik 1988).

The expression of the E1 region is required for the expression of all other viral genes. The three major mRNAs transcribed from the E1a gene are: 12S, 13S, 9S. The 9S mRNA is transcribed only late in infection, whereas 12S and 13S are transcribed in both early and late times of infection. The gene products of the 9S mRNA and their functions are not well understood. In group C Ads, 12S and 13S encode two proteins. These are termed 289R and 243R based on the number of amino acids they are composed of (Perricaudet *et al.,* 1979; Ricciardi *et al.,* 1981 and Gaynor *et al.,* 1982). The two proteins are distinguishable by the unique 46 amino acids at positions 140-185 in the 13S mRNA. It is this unique region which is the *trans*-activator and is required for the expression of all other viral genes. Point mutations located within this unique region of the 13S mRNA have resulted in the production of virus which is impaired for growth and virus gene activation (reviewed in Moran and Mathews, 1987).

There are three domains of the E1a which are conserved among Ad serotypes. These include conserved regions [CR] 1, CR2 and CR3. It is the CR3 which encompasses the 46 amino acids which are unique to the 13S mRNA. CR1 and CR2 have been found to be responsible for cell transformation, the induction of DNA synthesis, repression of transcription functions of the E1a unit (Cogan *et al.,* 1992) and induction of TNF-independent cytotoxicity (reviewed by Gooding and Wold, 1990). The negatively acting regulatory component of the E1a region was demonstrated with protein synthesis inhibitors. Cycloheximide has been

shown to increase early viral RNA production in the absence of protein synthesis (Nevins, 1987).

The E1a region also exhibits nonconserved regions which have no effect on the *trans*-activation of other Ad genes when these regions are deleted. However, nonconserved regions such as the E1a C terminus have been shown to somehow affect the rapid nuclearization of E1a gene products (reviewed in Moran and Mathews, 1987).

Transcription of the E1b region results in the production of two mRNAs from two overlapping reading frames: 22S and 13S. The 22S mRNA encodes both 19kDa and 55kDa proteins. The 13S mRNA encodes a 19kDa polypeptide (Bos *et al.,* 1981). The 19kDa protein is multifunctional (reviewed by Gold *et al.,* 1990a) and plays a role in preserving the nuclear DNA integrity and cytoplasmic architecture which is destroyed by E1a gene products during productive infection (White, 1992). The infection of baby rat kidney cells with Ad12 mutants which lacked the gene for the19kDa protein illustrated this protein's function. Cells infected with this mutant resulted in degradation of the infected cell and viral DNA (Zhang *et al.,* 1992). This observed phenotype was believed to be a result of the inability of the 19kDa protein to preserve the structural components of the cells. More recently, it has been shown that the E1b encoded 19kDa protein protects the Ad infected cell against destruction from the tumour necrosis factor (Gooding *et al.,* 1991a; White *et al.,* 1992). This role led to the suggestion that this E1b gene product may be important in allowing the E1a to completely transform nonpermissive cells by preventing the host cell's destruction by TNF (White *et al.*, 1992). The 19kDa protein has also been shown to have *trans*-activation functions

for E1a, E1b, E2, E3, E4, and the cellular heat shock promoter (hsp70) (McGlade *et al.,* 1987; Yoshida *et al.,* 1987).

The 55kDa protein encoded by the E1b region is believed to be required along with the E1a gene products to produce full transformation of cells, *in vitro.* The 55kDa protein also forms a complex with the 34kDa protein produced by the E4 region. The formation of this complex is thought to be functional for the transport of cellular mRNA to the cytoplasm, thus preventing the accumulation of mRNA. Mutants that carry lesions in genes for either of the polypeptides display similiar lytic growth phenotypes: defects in viral DNA replication and late-gene expression (Cutt *et al.,* 1987).

2.6 E3 region

The E3 region is located between 76.6-86.2 m.u. on the r-strand of the viral genome. About nine overlapping mRNAs (a to i) are expressed by alternative processing of a single primary transcript that initiates from the E3 promoter (Chow *et al.*, 1979; Cladaras *et al.*, 1985; Wold and Gooding, 1991). Figure 6 illustrates the processed mRNAs and the two polyA sites (E3a and E3b) of the E3 region. Out of the nine proteins encoded by this region in group C adenoviruses, six have been identified. These are 6.7kDa (Wilson-Rawls *et al.*, 1990), glycoprotein 19kDa (Persson *et al.*, 1980), 11.6kDa (Wold *et al.*, 1984), 10.4kDa (Tollefson *et al.*, 1990a), 14.5kDa (Tollefson *et al.*, 1990b), and 14.7kDa (Tollefson and Wold, 1988; Wang *et al.*, 1988). Four of these proteins have been characterized. They include a 19kDa glycoprotein, 14.7kDa protein, 14.5kDa, and a 10.4 kDa protein (reviewed by Wold and Gooding, 1991).

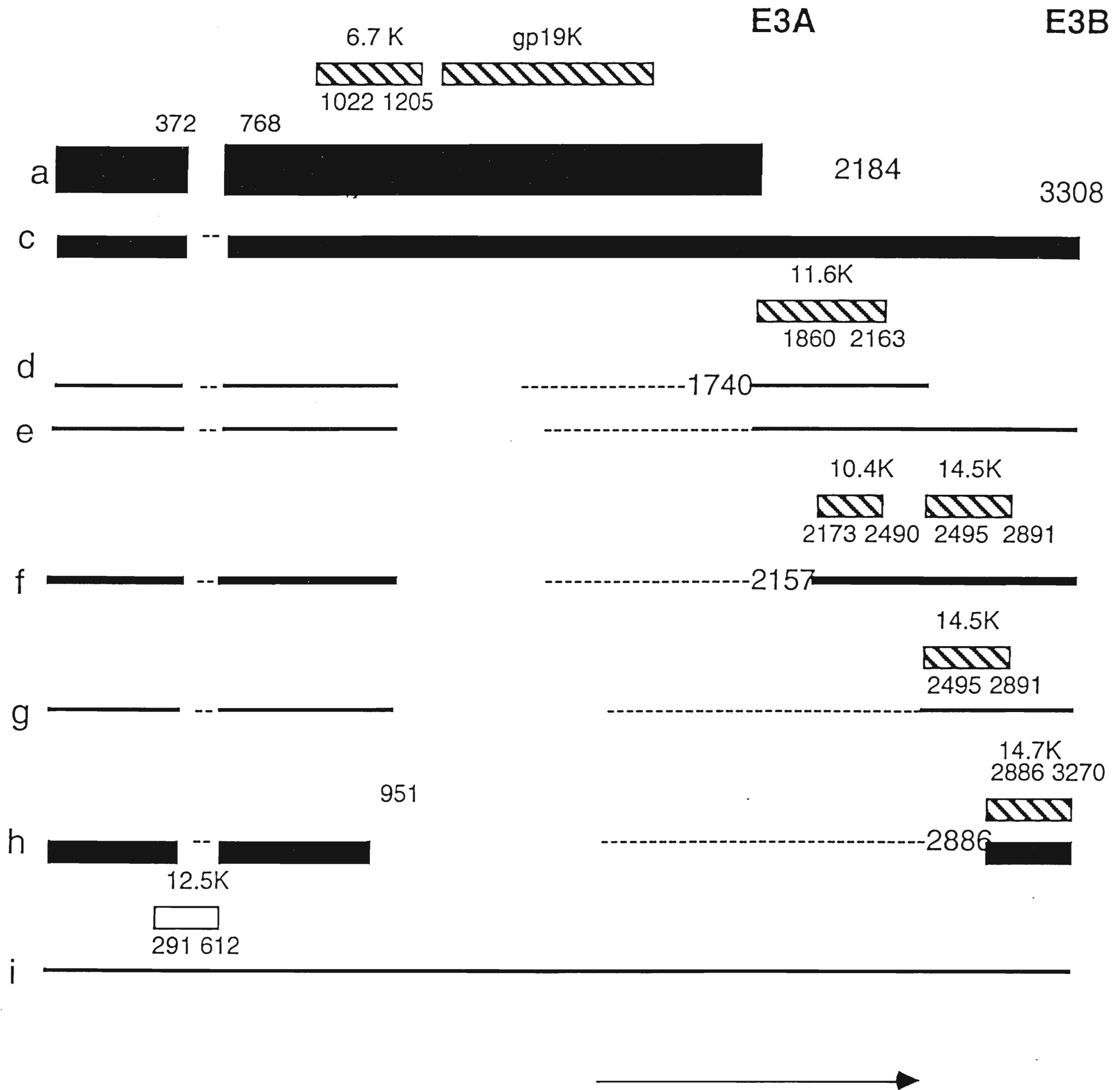


Figure 6: A diagrammatic representation of the transcripts of the E3 region. The exons of this region are represented by the solid arrows whereas the dashed lines represent introns. The protein products encoded by this region are represented by the cross-hatched boxes. (taken from Tolleffson *et all.* 1992).

The 19kDa glycoprotein was shown to complex with Class I Antigen of the major histocompatibilty complex (MHC) in the endoplasmic reticulum (ER). This blocks the transport of Class I antigens to the cell surface protecting the cell from destruction by the cytotoxic T cells. This was first demonstrated in Ad2 transformed rat cells (Kvist *et al.*, 1978). The net effect of gp19kDa being retained in the ER and binding to Class I antigens is to block the transport of Class I antigens to the cell surface. Therefore, this gene product plays a major role in viral oncogenesis. Transformed cells are permitted to grow because the immune system of the host will not recognize and destroy them. The 19kDa and 14.7kDa gene products are also thought to be involved in protecting latently infected cells from destruction by the immune system (reviewed by Wold and Gooding, 1991).

The 14.7kDa protein prevents the lysis of the infected cells by tumor necrosis factor (TNF). TNF is a multifunctional cytokine that is secreted by activated macrophages and lymphocytes and is responsible for regulating a wide variety of aspects of the immune system and the inflammatory response (reviewed in Beutler and Cerami, 1989). Gooding *et al.* (1988) showed that mouse fibroblasts are lysed by TNF when infected by adenovirus mutants that lack the E3 region. Uninfected cells were not lysed by TNF, nor were cells infected by the wild-type virus. The 14.7kDa protein is not the only gene product encoded by the E3 region which protects infected mouse cells from destruction from the TNF. The 10.4kDa and 14.5kDa proteins appear to exist in a complex and can also prevent TNF lysis (Gooding *et al.*, 1991b).

The 10.4kDa and 14.5kDa proteins are thought to function together to down-regulate the epidermal growth factor receptor (EGF-R) in adenovirus

infected cells. Normal binding of the epidermal growth factor to its receptor results in the internalization of this complex in clathrin-coated pits via endosomes which are subsequently degraded in lysosomes. This activity results in cellular metabolism, DNA synthesis and mitosis (reviewed in Wold and Gooding, 1990). An Ad infection mimics the EGF in that the EGF-R is still taken up via endosomes, internalized and degraded. Little is known about the mechanism of action of this complex (10.4kDa/14.5kDa), only that both are cytoplasmic membrane proteins, and which may localize to the cytoplasmic membrane where they interact with the EGF-R.

The deletion of the E3 region does not affect viral replication in cell culture. However, the gene products of this region are required to prevent destruction of the virally infected cells from the host's immune system and therefore play an important role in the establishment of long term infection *in vivo* (reviewed by Ginsberg *et al.,* 1989). Therefore, Ads have been attractive for their usage as recombinant viral vectors because foreign genes can be cloned in deleted E3 regions without the virus losing its ability to replicate in cells. However, their inability to counter host immune response results in a shorter duration of infection.

2.7 E2 region

The early region 2 is divided into E2a and E2b, each giving rise to their own gene products which are essential for the replication of viral DNA. The gene product encoded by the E2a region specifically controls transcription. The 72kDa DNA binding protein (DBP), appears to mediate the rapid decay of early mRNA. It has been observed that in the absence of this gene product, there is an overproduction of each viral mRNA.

E2b encodes polymerase and the terminal protein precursor (pTP) which are both required for the replication of adenoviral DNA. The pTP is 87kDa polypeptide which when complexed with the first deoxycytidyl triphosphate (dCTP), serves as part of the primer required for the growing DNA chain. The pTP is sequentially cleaved giving rise to the terminal protein (TP) which remains covalently linked to the 5' end of the DNA (review by Horwitz, 1990).

2.8 E4 region

The E4 region of the Ad genome is located at the extreme right end between 91.3 map units and 99.1 map units. The region is located on the l strand and is transcribed in a leftward direction giving rise to a complicated set of mRNAs (See Figure 7). There are six polypeptides encoded by this region's 7 open reading frames. Three polypeptides encoded by the E4 region have been identified and assigned to a specific open reading frames: an 11kDa protein (Sarnow *et al.*, 1982), a 34kDa protein (Sarnow *et al.*,1984) and a 19kDa protein. The 34kDa protein is known to form a complex with the large E1b protein which acts to inhibit host-cell protein synthesis at late times post infection (Sarnow *et al.,* 1984). Other polypeptides encoded by the E4 region have been identified but have not been assigned to any specific ORF.

The investigations into the function of the gene products of the E4 region has been facilitated by observing Ad E4 deletion mutants. (Sandler and Ketner, 1989, Yoder and Berget, 1986). Human Ad types 2 and 5 with a large deletion within the E4 region of their genome have been found to suffer a reduction in the accumulation of late mRNAs both in the nucleus and the cytoplasm of infected cells and have also failed to shutoff host

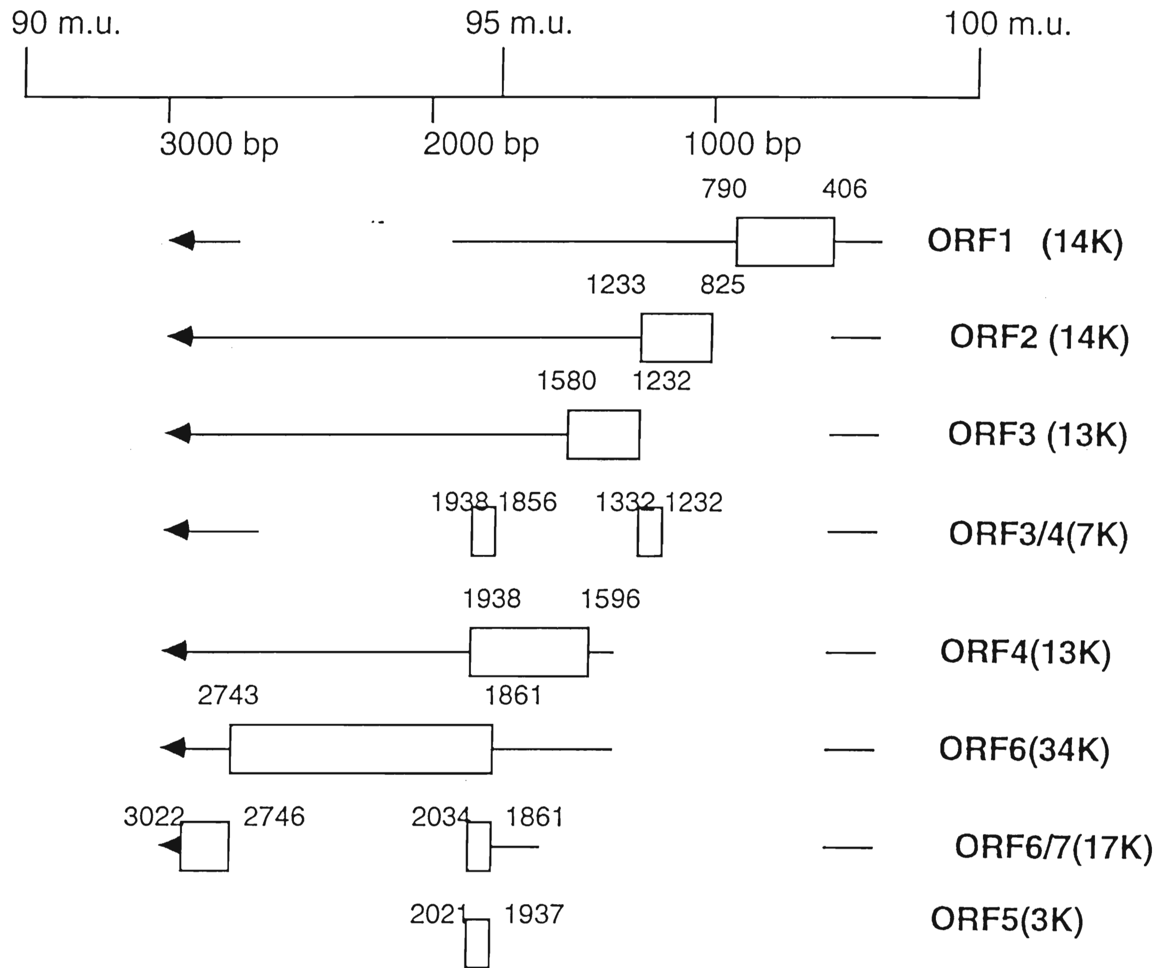


Figure 7: Physical map illustrating E4 mRNAs, coding regions and their location within the right end of Adenovirus type 5. The E4 mRNAs are transcribed in a leftward direction as represented by the arrows. The protein encoding sequences are represented by open boxes and the cap site and poly(A) site are represented at positions 324bp and 3136bp, respectively.

cell protein synthesis. These E4 deletion mutants are defective in late viral protein synthesis and DNA accumulation (Sandler and Ketner, 1989; Yoder and Berget, 1986). Viruses containing a deletion specifically in ORF6 which encodes the 34kDa protein were found to display a lag in the onset of viral DNA replication, decreased levels of late proteins, inefficient shutoff of host cell protein synthesis and a 10-20 fold decrease in the final virus yield. Viruses with deletions in ORFs other than ORF6 behaved like the wild type virus (Huang and Hearing, 1989).

The efficiency of viral replication is dependent on the production of large amounts of viral DNA and the synthesis of proteins which are necessary for assembly of the viral capsid during late times of infection. The synthesis of late mRNA is dependent on the production of several gene products during early times of infection. These products include those encoded by E1a, E1b and E4 regions. As previously mentioned, the E1a region encodes a protein which is 289 amino acids long, and is responsible for the *trans*-activation of the remaining early viral genes (Jones and Shenk, 1979, Kovesdi *et al.*, 1986 and Berk, 1986). It has been proposed that the E1a activation of the Ad E2 promoter involves the activation of a cellular factor, E2F (Nevins, 1986,1987). This activation of E2F results in the production of a new form of E2F which binds to two sites located on the E2 promoter, thus facilitating transcription. The same activation of E2F has been observed by gene products encoded by the E4 region of the Ad genome (Marton *et al.,* 1990).

2.9 Late regions

Five families of late transcripts are synthesized by the major late promoter (MLP) beginning at 16.45m.u. on the r strand and continuing through to approximately 99m.u. At least 12 mRNAs are processed from a primary late transcript. Each mRNA contains polyA signals at the 3' end and a tripartite leader is spliced to the 5' end. Although the onset of viral DNA replication represents the beginning of the late phase, the actual mechanism responsible for the switch from early to late is not well understood. In attempts to understand the switch from early to late, experiments involving Ad2 and Ad5 have been done. These experiments determined whether or not unreplicated DNA would be able to express late genes when added to a cell in which late viral transcription was already active. Ad5 was the primary infecting virus which was allowed to proceed to late transcription and Ad2 was subsequently added to cells. Late transcription did not occur from Ad2, suggesting that *cis*-acting factors play a role in the switch from early to late transcription.

Most of the proteins encoded by the late region are virion structural proteins. However, some nonstructural early proteins such as the DBP continue to be synthesized during late times of infection. Other nonstructural proteins produced during late times of infection include the 100kDa, 50kDa and 39kDa proteins which are involved in the assembly of progeny virions. The 100kDa phosphoprotein is a scaffold protein which is necessary for virion assembly. However, it is absent from the finished product. It binds to a hexon monomer and is then released upon the formation of a trimer (reviewed in Horwitz 1990). The 50kDa and 39kDa proteins act as scaffolds during the formation of the capsid.

Some late mRNAs are also produced early in Ad infection. L1 mRNAs are produced during the immediate early times of Ad infection. L2 and L3 mRNAs have also been detected during during early gene expression, whereas L4 and L5 have not (reviewed by Horwitz, 1990). Late polypeptides are synthesized at a maximum rate at 20 hours post infection.

2.10 Bovine adenoviruses

Bovine Adenoviruses (BAV) were first discovered by Klein *et al.* in 1959. Since then, BAVs have been isolated from a wide variety of hosts, including cattle and sheep (Belak and Palfi, 1974), free living buffalo (Baber and Condy, 1981), and fallow deer (Boros *et al.,* 1985). BAV infections result in symptoms characteristic of other adenoviral infections: respiratory tract disease, enteritis, conjunctivitis, keratitis and pyrexia (Darbyshire *et al.,* 1966; Mohanty, 1971; Mattsson, 1973).

There are ten serotypes of BAVS, differentiated based on serum neutralization and complement fixation tests. The ten serotypes are divided into two subgroups: subgroup I which includes serotypes 1, 2, 3, and 9 and subgroup II which includes serotypes 4, 5, 6, 7, 8, and 10. The members of subgroup I share the antigen common to all mastadenoviruses and have been shown to grow in bovine testicular and kidney cells and in continuous cell lines such as Madin-Darby bovine kidney (MDBK) cells (reviewed by Ishibashi and Yasue, 1984). Members of subgroup II do not carry a common antigen and they have been shown to only grow in bovine testicular, corneal or embryonic lung cells. (Bartha, 1969). A DNA homology study carried out by Benko *et al.* in 1990 showed that there is

extensive DNA homology between members of subgroup II, but very little homology was detected between members of subgroup I.

The genetic relatedness between members of subgroup I and subgroup II BAVs and between BAVS and human Ads has been investigated using DNA hybridization tests, hemagglutination test, ELISA and gel diffusion assay. The genetic relatedness of members of subgroup I to human Ads is variable and especially low for BAV1 and BAV2 (Belak *et al.,* 1983; Benko*et al.,* 1990). However, a close genetic relatedness was demonstrated between members of subgroup II BAVs by DNA hybridization, whereas no homology was detected between subgroups I and II or between subgroup II and human Ads (Benko *et al.,* 1990).

There are a limited number of DNA homology studies comparing BAVs to human Ads. Two regions of the Ad2 genome encoding for the hexon (polypeptide II and IV protein) have been found to be homologous to BAV7 (Hu *et al.,* 1984). Sequences located between 10 to 80m.u. of the BAV3 genome were found to share homology to the Ad2 genome. Consequently, the hexon protein was found to have greater than 80% identity to over 70% of the coding sequences.

The oncogenic potential of BAV3 and BAV8 have been demonstrated in newborn hamsters (Darbyshire, 1966; Mohanty, 1971). Newborn hamsters inoculated with BAV3 have been observed to produce undifferentiated sarcomas at the site of inoculation (Darbyshire, 1966). However, tumors were not observed in hamsters inoculated with BAV types 1, 2, 4, 5, 6, 7, and 9. As mentioned before, the genome of highly oncogenic types have a low G+C content, whereas nononcogenic types have a high G+C content. The genome of BAV3 was found to have 48% G+C compared to 62% and 61% for BAV1 and BAV2, respectively (Panigrahy *et*

al., 1977). Extensive DNA homology exists between BAV3 and Ad2, therefore it isn't surprising that the transforming region of BAV3 has been localized to the left end (Igarashi *et al.,* 1978; Elgadi *et al.,* 1993) of the genome as in Ad2.

Very few detailed studies have been published concerning the biological properties of BAVs (Mohanty, 1971; Adair and McFerran, 1976; Benko *et al.,* 1988). However restriction enzyme maps which are essential for detailed analysis of BAV genomes have been constructed for BAV1 (Bendo and Harrach, 1990), BAV2 (Belak *et al.,* 1983, 1986; Salmon *et al.,* 1993), BAV3 (Kurokawa *et al.,* 1978; Elgadi and Haj-Ahmad, 1992), and BAV7 (Hu *et al.* 1984). More detailed studies of BAVs are needed.

2.10.1 Bovine adenovirus type 2

BAV2 was first isolated by Klein *et al.* in 1960 from the feces of apparently healthy cattle. BAV2 infection results in respiratory and intestinal disorders in both bovine and ovine species. BAV2 has been divided into two subspecies based on hemagglutination properties and restriction enzyme patterns. Subspecies A refers to isolates similiar to that used during this study (strain #19) and were originally isolated from cattle, whereas subspecies B were originally isolated from sheep and were found to be closely related to ORT-III (Belak *et al.*,1983). Rusvai *et al.,* (1988) showed a close antigenic relationship between subspecies A and B when 8 monoclonal antibodies raised against BAV2 hexon cross reacted with both subtypes. The main difference between subgroups A and B seems to be within the E3 region sequences which encode for the fiber protein. Differences in the fiber protein would account for the

differences in host immunosurveillence and may explain why one subspecies is capable of infecting both bovine and ovine species.

2.11 Recombinant adenoviral vectors

Viral vectors can be utilized in two different ways; first, as high level expression systems for the production of an antigen or second, as live recombinant vaccines. Ads have been used as both expression vectors for the production of protein in mammalian cells and as vaccines for the immunization of humans and animals against many different diseases. More recently Ad vectors are being used as transducing viruses for gene therapy.

Ads are receiving attention as expression vectors and recombinant vaccines for several reasons: The viral genome is relatively stable and doesn't undergo rearrangement at a high rate. Therefore if a foreign gene is inserted, it will be maintained throughout the replication of the virus. The development of extensive restriction endonuclease maps has made the manipulation of the genome of Ads relatively easy using recombinant DNA techniques. Ads are capable of efficiently infecting nondividing cells and expressing large amounts of gene product from sequences cloned in deleted E1 and E3 regions. This makes them attractive for the purpose of both expression vectors and recombinant vaccines.

The early regions of the genome are important for the construction of recombinant viral vectors. There are three regions of the viral genome which can accept insertions of foreign DNA to generate a helper independent virus. These are E1, E3 and a short region between E4 and the

end of the genome (Graham and Prevec, 1991). The amount of DNA that can be packaged in virions is approximately 105% of the wild type genome which is approximately 2Kbp of DNA (Ghosh-Choudhury *et al.*, 1986). If any more that this is inserted into the viral genome the result is instability and rapid rearrangement of the genome to lose the expgenous DNA (Bett *et al.*, 1993). In order to insert larger segments of exogenous DNA into the Ad genome, the genome can be engineered by deleting viral sequences located within the E1 and E3 regions.

The E1 gene products are essential for the expression of the adenoviral genome and normal viral growth. The engineering of 293 cells (Graham *et al,* 1977). which provide the essential gene products encoded by the E1 region *in trans* has allowed deletions of up to 3.4Kbp of viral sequences within the E1 region to be made (Graham and Prevec, 1992).

Adenoviral vectors can be made with deletions in the E1 region. However, in order for the vector to be able to replicate in 293 cells 2 subregions must be retained in the E1 region. These two subregions are the left ITR (1-103bp) and the packaging signals (194-300). The protein IX gene and must also be retained for the viability of the vector. High expression vectors with inserted sequences in the E1 region are usually linked to a strong promoter such as the MLP. A typical construct designed for high level expression of a sequence in a deleted E1 region may encompass the MLP, sequences from the tripartite leader at the 5' end of the gene to be expressed and mRNA polyadenylation signals at the 3' end. Constructs of this kind have been shown to enhance the amount of expression as well as the stability of the mRNA (reviewed in Graham and Prevec, 1992).

The E3 region is non-essential for viral growth and replication of Ads. Therefore, deletions can be made in this area to further increase the amount of exogenous DNA which can be incorporated into the viral genome. However, the deletions made in this area cannot extend into sequences laying on either side of the E3 which code for structural proteins. A deletion within the E3 region can be up to 2Kbp without compromizing viral growth. With the development of extensive restriction endonuclease maps (Broker *et al.*, 1984; Tooze, 1981), deletions in the E1 and E3 regions can be made using recombinant DNA techniques. With deletions in the E1 and E3 regions of the viral genome up to 7-8Kbp of exogenous DNA can be added. Haj-Ahmad and Graham (1986) developed an Ad5 vector containing a deletion in the E1 and E3 regions with the capacity to accept 7.5Kbp of foreign DNA.

2.11.1. The use of Ads as transducing viruses

Ads have become increasingly popular for their use as transducing viruses mainly because they do not require target cell replication for transfer and expression of exogenous genes. Therefore, they are good candidates for *in vivo* gene delivery in slowly replicating cells such as hepatocytes and endothelial cells for which gene delivery and expression was virtually impossible. Other characteristics which make Ads attractive transducing agents are: they can be engineered to accept up to 7.5Kbp of exogenous DNA, there is no association of human malignancy with Ad infection, and live Ads have been safely used before as a human vaccine (reviewed in Horwitz, 1990).

Studies are now showing the potential use and safety of Ads as a tool to be used for somatic gene therapy of pulmonary diseases such as

cystic fibrosis. The aim of somatic gene therapy for cystic fibrosis is the introduction of the therapeutic gene (ie. cystic fibrosis transmembrane conductance regulator or CFTR) into respiratory epithelium. Ads seem to be good candidates for this delivery for several reasons: they have a normal tropism for respiratory epithelia, they are capable of infecting non-proliferating cells and they have been shown to successfully transfer the cDNA of the CFTR gene *in vivo* in many animal species (Rosenfeld, 1991; 1992, Li *et al,* 1993). *In vitro* studies have shown the delivery and expression of reporter genes such as lacZ and the gene encoding for the enzyme luciferase to a variety of different cell types by the use of an Ad with the gene in place of the normal E1 coding sequences (Engelhardt *et al.,* 1993, Li *et al.,* 1993, Rich *et al.,* 1993, Bout *et al.,* 1994).

The first report of administration of a recombinant Ad to humans involved an E1 deficient Ad encoding CFTR. The recombinant Ad2 was administered to the nasal airway epithelium of 3 individuals with cystic fibrosis. The transfer of the CFTR was successful and alleviated symptoms experienced by the three patients (Zabner *et al.,* 1993).

The possibility of using recombinant Ad vectors to transfer genes *in vivo* has been investigated extensively using animal models. Reporter genes such as those encoding for ß-galactosidase and a-1-antitrypsin have been successfully transferred and expressed in a variety of cell types using an adenoviral vector as the vehicle of transport. *In vivo* gene delivery and expression have been successfully accomplished in hepatocytes (Jaffe *et al.,* 1992; Li *et al.,* 1993), cells of the central nervous system (Bajocchi *et al.,* 1993; La Salle, 1993), malignant cells (Brody *et al.*, 1994), endothelial cells (Rosenfeld *et al.*, 1991; Lemarchand *et al.* 1993).

2.11.2. The use of Ads for live recombinant vaccines

The use of Ads as live recombinant vaccines is also receiving a lot of attention. Recombinant Ads containing a cloned gene coding for a foreign antigen offer many advantages. Such vaccines are valuable because they would be relatively cheap, easy to administer and they would provide long lasting immunity.

Ad5 recombinants carrying inserts of HSV, VSV and rabies glycoprotein coding sequences in the E3 region have been shown to raise neutralizing Ab in rhesus monkeys, cows, pigs, dogs, foxes, skunks, racoons and mice (reviewed in Graham and Prevec, 1992). Ad vectors have been constructed that express the gene for various glycoproteins including those of the herpes virus (Johnson *et al.*, 1988), hepatitus B virus (Morin *et al.,* 1987), human immunodeficiency virus type 1 (Dewar *et al.*, 1989), vesicular stomatitis virus (Schneider *et al.,* 1989), rabies virus (Prevec *et al.,* 1990), and the respiratory syncytical virus (Collins *et al.*, 1990; Hsu *et al.,* 1991). Glycoprotein gene inserts are primarily driven by the E3 promoter or the MLP. Expression in all of the above cases was enough to elicit the production of neutralizing Ab to immunize the organism against subsequent challenge with the antigen.

2.12 Objectives of this investigation

The objectives of this study were:

(1) To make a precise deletion in the E3 region of BAV2.

(2) To insert the thymidine kinase (tk) gene of HSV-1 into the deleted E3 region.

(3) To reconstruct the BAV2 genome containing the tk in the deleted E3 region.

(4) To rescue a mutant virus containing tk in the deleted E3 region.

(5) To sequence the putative E4 region and analyze the open reading frames of this region.

CHAPTER 3: MATERIALS AND METHODS

3.1 Bacterial Strains

Escherichia coli strain DH5α (BRL) was used for all recombinant plasmid engineering.

3.1.1 Propagation and Maintenance of Bacterial Cultures

Bacteria were grown in sterile Luria broth (LB; 10% Bacto-tryptone, [Difco]; 5% Bacto-yeast extract, [Difco]; 10% NaCl, [Sigma], pH 7.0). The bacteria were inoculated into an appropriate volume of LB containing appropriate antibiotics for selection. They were then placed into a 37°C shaking water bath (Gyrotory Water Bath Shaker; Model G76) overnight. In order to colony purify the bacteria, the overnight culture was streaked onto LB agar plates containing the appropriate antibiotic. For short term storage, the plates were placed in 4°C and for long term storage, 1ml aliquots were stored in 15% glycerol (Fisher) at -20°C.

3.1.2 Transformation

Transformation was carried out as described by Sambrook *et al.* (1989). Overnight culture of bacteria were diluted 1:100 into LB medium and grown at 37°C to an OD_{600} of 0.3 to 0.4. The culture was then aliquoted into sterile polypropylene tubes and centrifuged at 3000 rpm (1100xg; IEC Centra-7R) for 15 minutes at 4°C. The cell pellets were then

resuspended in 25ml of ice-cold transformation buffer (75mM CaCl2, fisher; 5mM Tris.HCl, [Sigma]; pH 7.6) and placed in 4°C cold room on ice overnight. The tubes were centrifuged again as described above and resuspended in 1ml of ice cold transformation buffer. One hundred µl aliquots were transferred into 1.5ml microcentrifuge tubes.

Transforming DNA (1-10µg) was added to the competent cells and incubated on ice for 30 minutes, with occasional mixing (every 10 minutes) of the tube contents. The cells were then heat-shocked for 45 seconds at 42°C and diluted in 900µl of SOC media (2% bacto-tryptone, 0.5% yeast extract, 10mM NaCl, 2.5 mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ and 20mM glucose). One hundred µl aliquots were then plated on LB agar plates containing the appropriate selective antibiotic. The plates were incubated at 37°C overnight to obtain transformant colonies.

3.1.3 DNA ligation

DNA ligations were carried out either overnight at 16°C or at room temperature for 2-3 hours. A typical ligation consisted of 10-50ng of digested plasmid DNA, 100ng of linearized pUC19, 0.5µl (400U/µl) of T4 DNA ligase, 2µl of 10X ligase buffer (10mM $MgCl_2$; 20mM dithiothreitol; 50mM Tris; 1mM ATP; pH 7.5, supplied by NEB) and the reaction mixture was brought to a final volume of 20µl with sterile water.

3.2 Plasmid DNA preparations

3.2.1 Extraction of plasmid DNA

Mini-prep DNA isolations were carried out as described by Birnboim and Doly (1979). Transformants were inoculated into 3ml of LB containing the appropriate antibiotic. In this study, ampicillin (25-

50μg/ml) was used. The cultures were then incubated at 37°C in a shaker-water bath overnight. The culture was then collected into a 1.5ml microcentrifuge tube and centrifuged for 20 sec at 12,000 rpm (12,000xg). The resulting pellet was resuspended in 100μl of lysozyme solution (10mM EDTA, 50mM glucose, 25mM Tris.HCl, pH 8.0) that contained 2mg/ml lysozyme (Sigma). The tube was then incubated on ice for 20-30 minutes followed by the addition of 200μl of alkaline SDS (0.2N NaOH, and 1.0% SDS) at room temperature for 15 minutes or until the solution became clear. One hundred and fifty μl of 3M NaAc (pH 4.8) was added and the sample was then incubated on ice for a further 30-45 minutes. The sample was centrifuged for 5 minutes at 12,000 rpm and the supernatant was collected into a fresh sterile 1.5ml microcentrifuge tube. Two volumes of ice-cold 95% ethanol was added to the supernatant and the resulting solution was mixed and incubated at -20°C for 20-30 minutes. The sample was centrifuged at 12,000 rpm for 5 minutes and the resultant DNA pellet was resuspended in 300μl of autoclaved distilled water and ethanol precipitated again. The resulting pellet of DNA was dried at 37°C and resuspended in TE buffer (10mM Tris, 1mM EDTA; pH7.5) or sterile distilled water.

3.2.2 Large scale plasmid DNA extraction and purification

For a large scale plasmid DNA extraction, bacterial transformants were grown overnight in 500ml of LB at 37°C in a shaker-water bath. The extraction of plasmid DNA was carried out according to the procedure outlined above for the mini-prep DNA isolation, except that the bacterial culture was centrifuged at 4000 rpm (1900xg) for 10 minutes and the pellet was resuspended in 10ml of lysozyme solution, followed by the

addition of 20ml of alkaline-SDS and then 15ml of NaAc pH 4.8. The resultant DNA pellet was resuspended in 1ml of TE buffer or sterile autoclaved water.

The plasmid DNA was purified by using a cesium chloride (CsCl) gradient using the procedure taken from Sambrook *et al.* (1989). For every ml of DNA solution, 1g of CsCl was added. This was then mixed gently until the solid dissolved. An aliquot of 0.8ml of ethidium bromide (EtBr; 10mg/ml, Sigma) was added to every 10ml of DNA/CsCl solution. The final density of the solution (by weight measurement) was always between 1.55-1.59 g/ml. The solution was centrifuged in 3ml Beckman Quick-Seal centrifuge tubes and centrifuged in the Beckman TL-100 ultramicrocentrifuge at 65,000 rpm (179,000xg; rotor SN1533) for 20 hours at 22°C. After centrifugation the red band could be visualized using UV light. The band was then removed using a sterile syringe and the EtBr was removed by a series of sequential extractions with isoamyl alcohol. The band was placed in a fresh sterile tube and an equal volume of isoamyl alcohol was added and mixed. The top layer was removed and again an equal volume of isoamyl alcohol was added and mixed. This procedure was then repeated until the pink colour had disappeared from both the lower aqueous phase and the upper organic phase. Any residual CsCl was removed from the DNA with the addition of 6 volumes of sterile autoclaved water and the DNA was precipitated out of solution with the addition of 3 volumes of ice-cold 95% ethanol. The sample was left in -20°C for no longer than 10 minutes and centrifuged. The DNA pellet was resuspended in TE buffer or water.

3.2.3 Restriction enzyme digestion and analysis

The enzymes used during this study (purchased from Promega, Boehringer Mannheim or New England Biolabs) were used according to the manufacturer's instructions. Digestions were usually set up for 4-16 hours at 37°C, unless otherwise specified by the manufacturer. A typical digestion consisted of 10µl of DNA (1-10µg of DNA), 2µl of 10X buffer and 0.5µl of enzyme (20U/µl). The reaction mixture was then brought to a final volume of 20µl and placed at the appropriate reaction temperature (usually 37°C).

Once samples were digested, the fragments generated could be visualized using agarose gel electrophoresis. Five µl of loading buffer (20% glycerol, 2% SDS, 0.05% bromophenol blue) was added to the samples and they were loaded into the wells of a 0.9% agarose gel. The fragments were then electrophoretically separated at 1-10 V/cm in TAE buffer (40mM Tris, 20mM acetic acid, 1mM EDTA; pH 8.0). When the loading buffer had migrated a sufficient distance for the separation of the DNA fragments, the gel was then stained with EtBr (0.5µg/ml) for 10-30 minutes. The gel was then illuminated using a UV transilluminator and photographed using a Polaroid Land camera with Polaroid type 57 film. The sizes of the DNA fragments were graphically estimated using the lambda-*Hin*dIII digest (N.E.B) as a molecular weight marker.

3.3 DNA sequencing

The DNA sequencing of the *Eco*RI D fragment was carried out using the dideoxy nucleotide (ddNTP) chain termination protocol described by Sanger *et al.* (1977). The "Sequenase Version 2 Kit" (United States Biochemicals) was used and reactions were set up according to the

supplier's recommendations. Plasmid DNA was first denatured by adding one-tenth the volume of 0.2M NaOH, 0.2mM EDTA and incubating 1 hour at 37°C. Then the sample was neutralized with the addition of one-tenth of the volume of 3M NaAc pH 4.8 on ice. The denatured DNA was then precipitated by adding 2 volumes of ice-cold 95% ethanol and centrifuging in microcentrifuge for 2 minutes. The concentration of the resulting single stranded DNA was determined using a spectrophotometer, more specifically by its absorbance at 260nm. (1 OD unit is equal to approximately 35µg of ssDNA/ml). Approximately, 3-5µg of denatured DNA was annealed to 5-9ng of a 16-mer primer in 10µl of 1X Sequenase buffer (200mM Tris.HCl, (pH 7.5), 100mM $MgCl_2$ and 250mM NaCl). Primers were either universal (pUC19 specific sequences adjacent to the multiple cloning site) or they were custom synthesized by Vetrogen Corp. (London, Ontario). Annealing was carried out at 65°C for 2 minutes, followed by gradual cooling to 30°C over a time period of approximately 30 minutes.

Primed DNA synthesis was initiated with the addition of 6µM of DTT (dithiothreitol), 0.2µM of each of the dNTPs (dCTP, dGTP, dTTP and [a-^{35}S]-dATP) and 3.25 units of Sequenase Version 2.0 T7 DNA polymerase. Following primer extension at room temperature for 5 minutes, synthesis was resumed in the presence of additional dNTPs and one ddNTP. An aliquot of 3.5µl of the reaction mixture was added to each of the four sequenase termination mixtures (80µM of each of the four dNTPs, 8µM of one of the dideoxy-NTPs, 50mM NaCl). The termination reactions took place at 40°C for 5 minutes and 4µl of stop/loading dye (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was added to each tube and stored at -20°C.

The sequencing gel is prepared in 60 ml containing the following chemicals: 6% Long Ranger (J.T. Baker), 8M urea and 1X TBE (89mM Tris, 89mM Boric acid, 2mM EDTA; pH 8.0). Approximately, 500µl of 10% ammonium persulfate .and 50µl of TEMED (N,N,N',N'-tetramethylethlene diamine; Bio-Rad) were added to the gel solution to facilitate polymerization of the gel. After pre-running the gel for approximately 1 hour, a shark tooth comb was inserted between the plates to form wells, where the samples were loaded. The sequencing reactions were then heated to 75-80°C prior to loading the gel and 2.5µl was loaded into each well. Electrophoretic separation was carried out at a constant current of 1.25A/cm in 0.6X TBE buffer for 1.5-2 hours. Gels were lifted off the glass plates by adsorption to 3mm Whatman filter paper, covered with plastic wrap, and vacuum dried at 80°C for 20 minutes in a Bio-Rad model 583 gel drier. Dried gels were then exposed to Kodak X-OMAT X-ray film for 72 hours for the ^{35}S labelled samples.

3.4 Cell line

Madin-Darby bovine kidney (MDBK) cells were grown and maintained in 150mm plates in a-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS; Gibco Laboratories), 0.29µg/ml L-glutamine, 0.225% sodium bicarbonate and 1.5% antibiotic-antimycotic (10,000 units/ml penicillin, 25µg/ml amphotericin B and 10,000 µg streptomycin; Gibco Laboratories). The cell line was maintained at 37°C incubator at constant atmospheric pressure in a CO_2 incubator.

3.5 Viral strain propagation

Semiconfluent 60mm dishes of MDBK cells were infected with approximately 1ml of BAV2 (~10^7 pfu/ml) and maintained in aMEM supplemented with 5% .FBS. Infected cells were left at 37°C in the CO2-incubator until approximately 80-90% cytopathic effect (cpe) was observed. The viral DNA was isolated via Hirts extraction (Hirts, 1967).

3.5.1 Hirts extraction

Once maximum CPE of infected cells was observed, the viral DNA was isolated as described by Hirts (1967) and modified by Haj-Ahmad and Graham (1986). Approximately, .75-1ml of lysing buffer (0.01M Tris; 0.01M EDTA;.4% Sodium dodecyl sulphate (SDS); pH 8.0). with 1X Pronase (20X stock; 10mg/ml in 0.01 M Tris, pH 8.0) was added directly to the plates and incubated for 2-3 hours at 37°C, followed by extraction with phenol:chloroform:isoamyl alcohol (25:24:1 v/v). The aqueous phase was collected into a fresh tube and the DNA was precipitated with the addition of 2 volumes of ice-cold 95% ethanol and incubated at -20°C for 15 minutes followed by centrifugation for 5 minutes in the microcentrifuge. The ethanol was aspirated and the DNA pellet was dried at 37°C for 5-10 minutes. The pellet was then resuspended in an appropriate volume of TE buffer on ice and stored at -20°C.

3.5.2 Plaque-assay

A tenfold serial dilution using pooled virus in PBS^{++} was used for the assay. One hundred µl aliquots of 10^{-3}, 10^{-4} and 10^{-5} dilutions were used to infect 60mm plates containing a monolayer of MDBK cells, plated at $1X10^6$ cells/plate. The infection was initiated by removing the

medium from the plates and subsequently adding 0.1ml of the virus onto the plates. After 1-2 hours incubation at 37°C, the cells were overlayed with 10ml of 1:1 mixture of Noble Agar (2%) and 2X MEM. Prior to the overlay, the noble agar and 2X MEM were brought to 42°C and mixed. Once the agar solidified, the plates were placed at 37°C in the CO_2 incubator until plaques appeared. Each plaque contained the progeny of a single virus and made it possible to obtain a population of genetically identical viruses.

Once plaques appeared (10-12 days), each individual plaque was picked using a sterile pipet and placed in a sterile 4ml glass tube in 1ml of PBS^{++}. The plug was then mixed by pipetting up and down using the pipet. Then 100µl of this plaque-purified virus was used to infect cells as described before. The viral stocks were stored at 4°C and long term storage was in 10% glycerol at -20°C.

3.6 Infection of mammalian cells with BAV2 and subsequent transfection with plasmid DNA

Transient transfections were carried out by using calcium phosphate-DNA precipitate formed in HEPES (Graham &van der Eb 1973). Plasmid DNA purified by a CsCl/ethidium bromide density gradient (Sambrook et al. 1989) was dissolved in 0.1X TE buffer (1mM Tris, 0.1mM EDTA; pH 8.0) at a concentration of 1 µg/µl. The following solution was made using sterile conditions: (442.5µl TE Buffer, 5µl of plasmid DNA (1µg/µl), 2.5µl of carrier DNA (25µg), and 50µl of 2.5M $CaCl_2$. This 500µl solution was then added slowly to an equal volume of HEPES (N-2-hydroxyethypiperazine-N'-2-ethanesulfonic acid) while bubbling with a sterile pipet. This mixture was then incubated at room temperature for 30 minutes and subsequently 500µl was added to each 60mm dish

containing 5ml of α-MEM supplemented with 5% fetal bovine serum (Gibco) and a cell density of $1x10^5$/plate. After 4 hours of incubation at 37°C, the medium was removed and 5ml of fresh medium was added. The plates were subsequently incubated at 37°C.

3.7 Southern blot and hybridization

Southern blotting and hybridization with a radiolabelled probe were carried out according to Southern (1975) as modified by Ausubel *et al.*, (1992).

3.7.1 Transfer of DNA to nitrocellulose membrane

Viral DNA was digested overnight in approximately 50µl followed by the addition of 5µl of loading buffer. The sample was then separated in a 0.8% agarose gel, with the appropriate molecular weight markers and controls. The gel was stained with 0.5µg/ml EtBr and photographed. The gel was then placed in 500ml of 0.2N HCl for approximately 10 minutes, making sure that the gel was completely covered and periodically rocked. The gel was then washed several times with distilled water and placed in denaturing solution (1M NaCl/0.5M NaOH) for 2 X 15 minutes. This was followed by 2 X 15 minute incubation in a neutralization solution (1M NH_4OAc). During incubation, a nitrocellulose membrane (Dupont) was cut to fit the gel and then placed on top of distilled water until it became saturated, followed by an incubation period of 5-10 minutes in 20X SSC. Two pieces of 3mm Whatman paper were cut slightly smaller than the gel and a wick was prepared by cutting one piece of 3mm Whatman approximately 2 cm wider than the width of the gel. The wick was placed in an agarose gel container and saturated with 6X SSC. The gel was

positioned on top of the wick with the nitrocellose membrane placed right on top of the gel. The two pieces of 3MM Whatman filter paper were then placed on top of the membrane, so that the paper didn't overhang the membrane. A 2-3cm thick stack of paper towels was then placed on top of the Whatman filter paper along with a gel tray and 0.2-0.4 kg of weight. The ends of the tray were covered with plastic wrap and the DNA was allowed to transfer to the membrane overnight.

After the transfer was complete, the nitrocellulose membrane was dried at room temperature. The DNA was fixed onto the membrane by exposing the side of the membrane carrying the DNA to a source of UV irradiation (254nm) using a UV Stratalinker 1800 (Stratagene).

3.7.2 Prehybrization, Hybridization and Washing

Prehybrization was carried out at 68°C. Essentially, the nitrocellulose membrane was placed in a solution containing 6X SSC, 5X Denhardt's reagent (50X Denhardt's contains 1% of each of the following: Ficoll [Pharmacia], polyvinylpyrrolidone, bovine serum albumin [Sigma]), 0.5% SDS and 100µg of denatured herring sperm DNA for approximately 1 hour. The volume of the prehybridization solution used was approximately 0.2 ml for every square centimeter of nitrocellulose membrane.

After prehybridization, the solution was completely removed and 0.2 ml of the hybridization mixture was added for every square centimeter of nitrocellulose membrane. The hybridization solution used consisted of 6X SSC, 0.5% SDS, 100µg of denatured herring sperm DNA. The radiolabelled probe was then added to the hybridization mixture and the hybridization was allowed to proceed for 2 hours at 68°C.

After hybridization the nitrocellulose membrane was washed for 15 minutes at 68°C by replacing the hybridization solution with 6X SSC;0.5% SDS. The membrane was then washed a second time by replacing the first wash with 1X SSC;0.5%SDS at 68°C.

When the washing of the membrane was complete, the membrane was removed and dried by placing it on Whatman paper. The membrane was then covered with a piece of plastic Saran wrap and exposed to X-ray film as previously described. The DNA could usually be detected after 16-24 hours of exposure at -70°C with a Cronex intensifying screen.

CHAPTER 4: RESULTS

This project was multifaceted and proceeded in a series of steps to reach the final objective. The starting plasmid was pEA48 (supplied by Kirsty Salmon) which contains the BAV2 *Eco*RI A fragment and the E3 region (refer to Figure 30 for *Eco*RI map of BAV2). First, pEA48 was used as a template for subcloning which was necessary to make the deletion within the E3 region feasible. Secondly, a precise deletion was made within the E3 sequences of the BAV2 genome and a reporter gene (tk) was inserted. Thirdly, the BAV2 genome was reconstructed in recombinant plasmids which were used to facilitate homologous recombination with the wild-type (wt) BAV2 genome in MDBK cells. Hybridization techniques were then used to detect the presence of tk sequences. Finally , in order to understand the poor growth rate of BAV2 in MDBK cells, the E4 region of BAV2 was cloned and sequenced. The resulting sequence and ORFs were then compared to known Ad E4 sequences and proteins.

4.1 Construction of the recombinant BAV2 plasmids

During this study, 9 new recombinant BAV2 plasmids were constructed and analyzed using restriction enzyme digestions. The resulting fragments were electrophoretically separated on a 0.9% agarose gel along with a sample of a molecular size marker (lambda phage DNA digested with *Hin*dIII). The size of the fragments were determined using a

standard curve constructed from the distance migrated versus the known size of the fragments of the molecular size marker. The sizes of the fragments generated from these digestions were then compared to the expected sizes as determined from the physical restriction enzyme map predicted for each plasmid. Of the 9 new recombinant BAV2 plasmids constructed, 2 are subclones of pEA48 and 5 are of various sizes containing a precise deletion within the E3 region. Of these 5 plasmids, 2 contain a reporter gene (tk) in place of the E3 region. The remaining two recombinant plasmids contain the right and left end of the BAV2 genome cloned into the vector pUC19.

4.1.1 Subcloning of BAV2 genome

The plasmid pEA48 was chosen as the starting plasmid due to the availability of an extensive restriction enzyme map for this plasmid (Salmon, 1993). Two plasmids were subcloned: pSE1 and pPE1. The strategy for the construction of pSE1 is diagrammatically represented in Figure 8. The restriction endonuclease *Sal*I cuts pEA48 in two places. Shotgun ligation followed by transformation and subsequent screening produced the plasmid pSE1 (Figure 9). This plasmid was then used in a similiar strategy (digestion followed by shotgun ligation) to construct the plasmid pPE1 (Figure 10)

4.1.1.1 Restriction enzyme analysis of pSE1 and pPE1

The plasmid pSE1 containing from 65.2 to 90.5 map units of the BAV2 genome was digested with the following enzymes: *Eco*RI, *Pst*I, *Sal*I, *Sal*I+*Pst*I, and *Eco*RI+*Pst*I (Figure 9). *Eco*RI and *Sal*I each cut the plasmid only once therefore generating one band with a size of 10.9Kbp (lanes 3 &

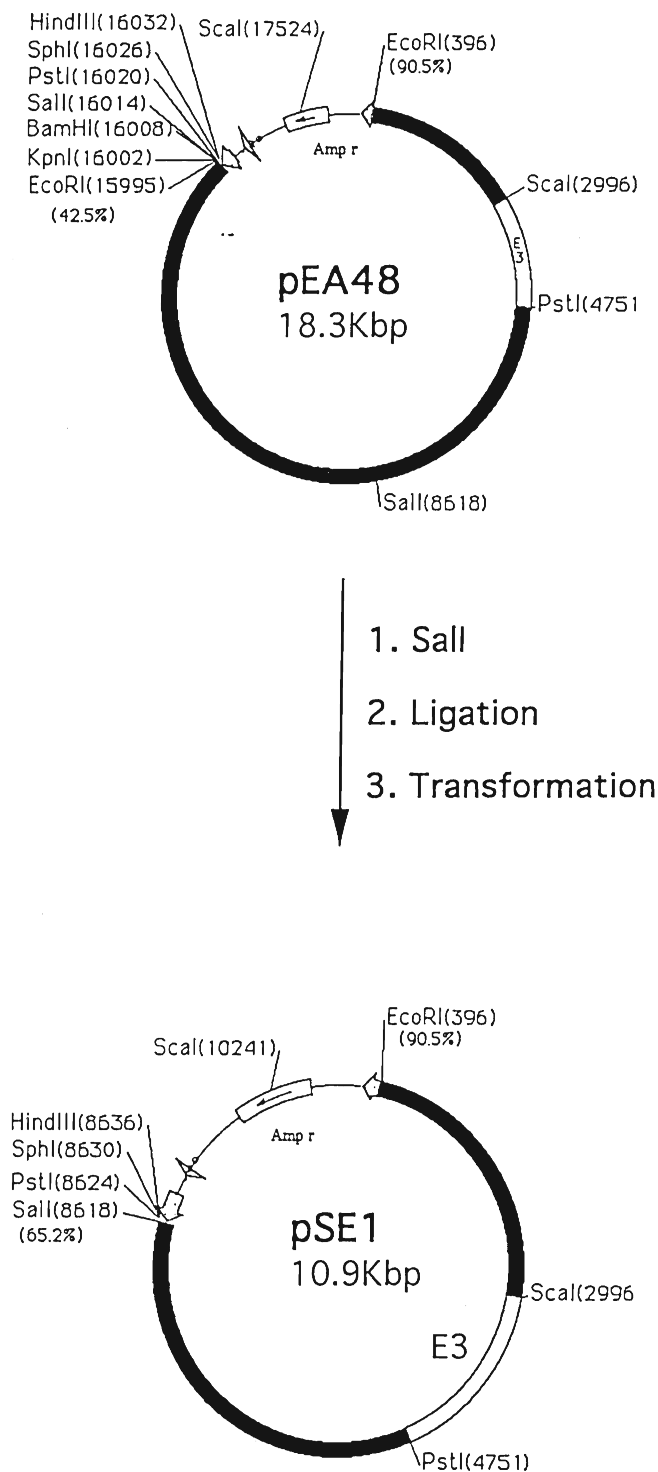


Figure 8: Strategy for the deletion of the fragment between *SalI*(65.2%) and *SalI* in the multiple cloning site in pUC19.

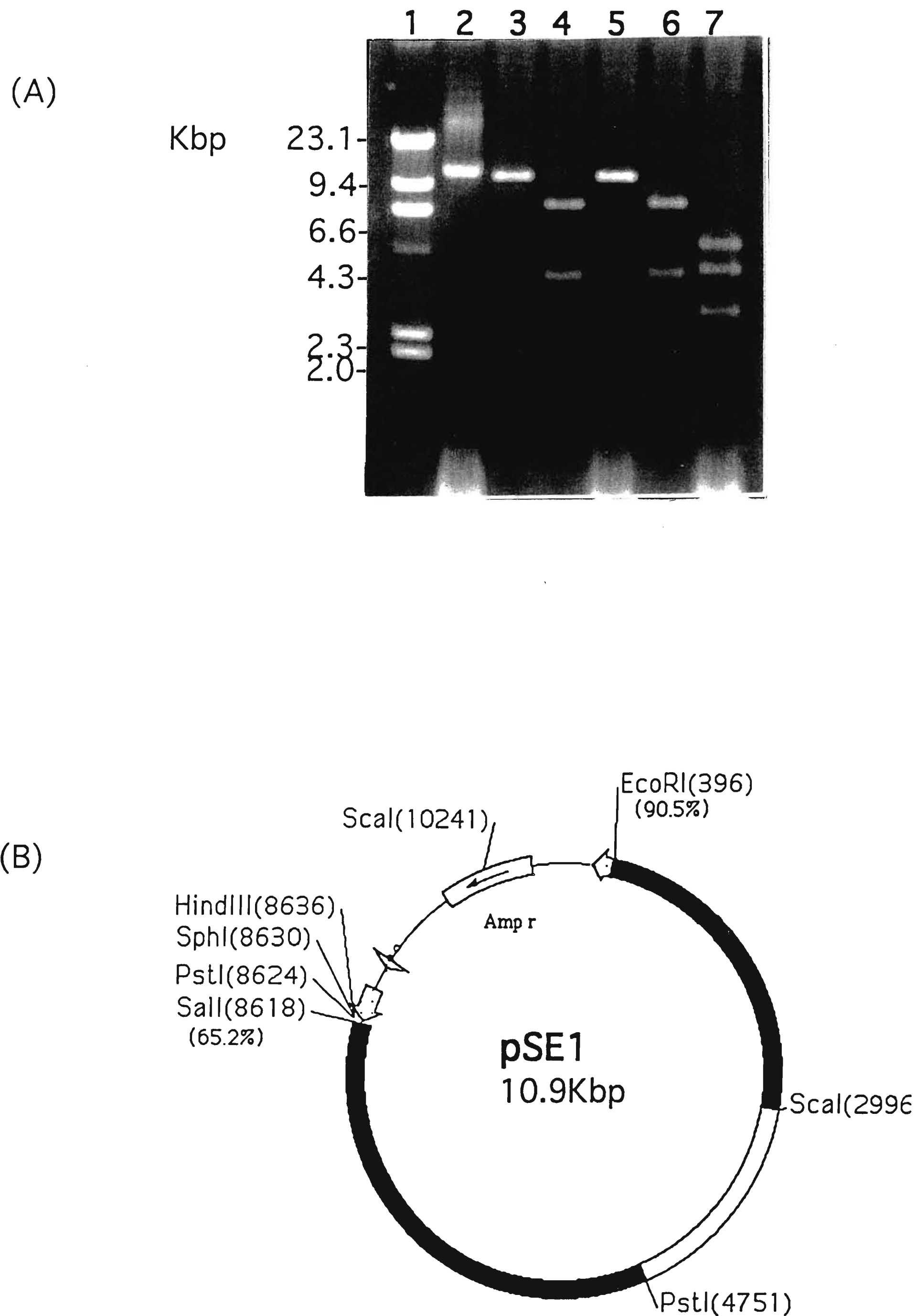


Figure 9 : (A) Photograph of a 0.9% agarose gel stained with ethidium bromide. Lane 1 is a lambda marker (*Hind*III digest). Lane 2 represents the uncut plasmid pSE1 and lanes 3-7 are pSE1 digested with *Eco*RI, *Pst*I, *Sal*I, *Sal*I and *Pst*I and *Eco*RI and *Pst*I, respectively.
(B) Restriction enzyme map of the plasmid pSE1.

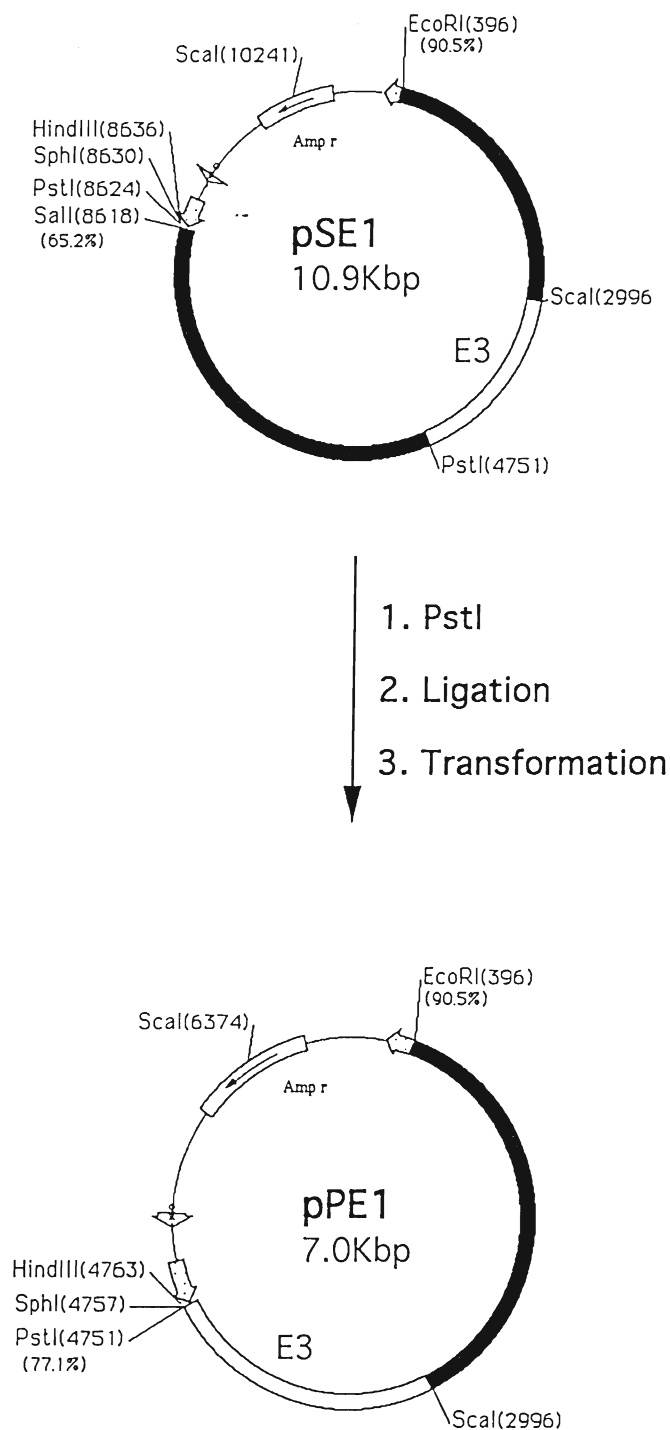


Figure 10: Strategy for the deletion of the fragment between *Pst*I (77.1%) and *Pst*I in the multiple cloning site of pUC19.

5, respectively). *Pst*I generated two bands (lane 4); 7.0Kbp and 3.9Kbp. Digestion with *Sal*I and *Pst*I together generated the expected two bands (lane 6); 7.0Kbp and 3.9Kbp, whereas a double digestion with *Eco*RI and *Pst*I generated 3 bands..(lane 7); 4.3Kbp, 3.9Kbp, and pUC19, 2.7Kbp. The construction of pSE1 was confirmed using restriction enzyme digestions and the restriction map of pSE1 is shown in Figure 9B.

pPE1 was shown to contain the BAV2 genome spanning from 90.5 to 77.1 map units by digesting the plasmid with *Pst*I, *Eco*RI and *Eco*RI+*Pst*I (Figure 11A). The enzymes *Pst*I and *Eco*RI each cut the plasmid once (Figure 11B), therefore generating a band of 7Kbp in size (lanes 3 & 4). When pPE1 was digested with both *Eco*RI+*Pst*I, 2 bands were generated; a fragment 4.3Kbp and pUC19; 2.7Kbp (lane 5). The construction of pPE1 enabled us to proceed to the next step involving the deletion of the E3 region. This plasmid contains two unique restriction enzyme sites, *Pst*I and *Sca*I, which flank the E3 region and made the deletion of the E3 region feasible.

4.1.2 Deletion of the E3 region

The availibility of the nucleotide sequence of the BAV2 E3 region (Esford,1994) enabled a precise deletion (1800bp) to be made within this region. The strategy involved digesting pPE1 completely with *Pst*I + *Sca*I and ligating in the presence of a synthetic linker (Procyon Biopharma Inc. London, Ontario) with compatible sticky ends for *Pst*I and *Sca*I. The synthetic linker contained a multiple cloning site with several unique restriction enzyme sites designed to facilitate the insertion of foreign DNA sequences in recombinant plasmids. The deletion of the E3 region was confirmed by both restriction enzyme analysis and by sequencing

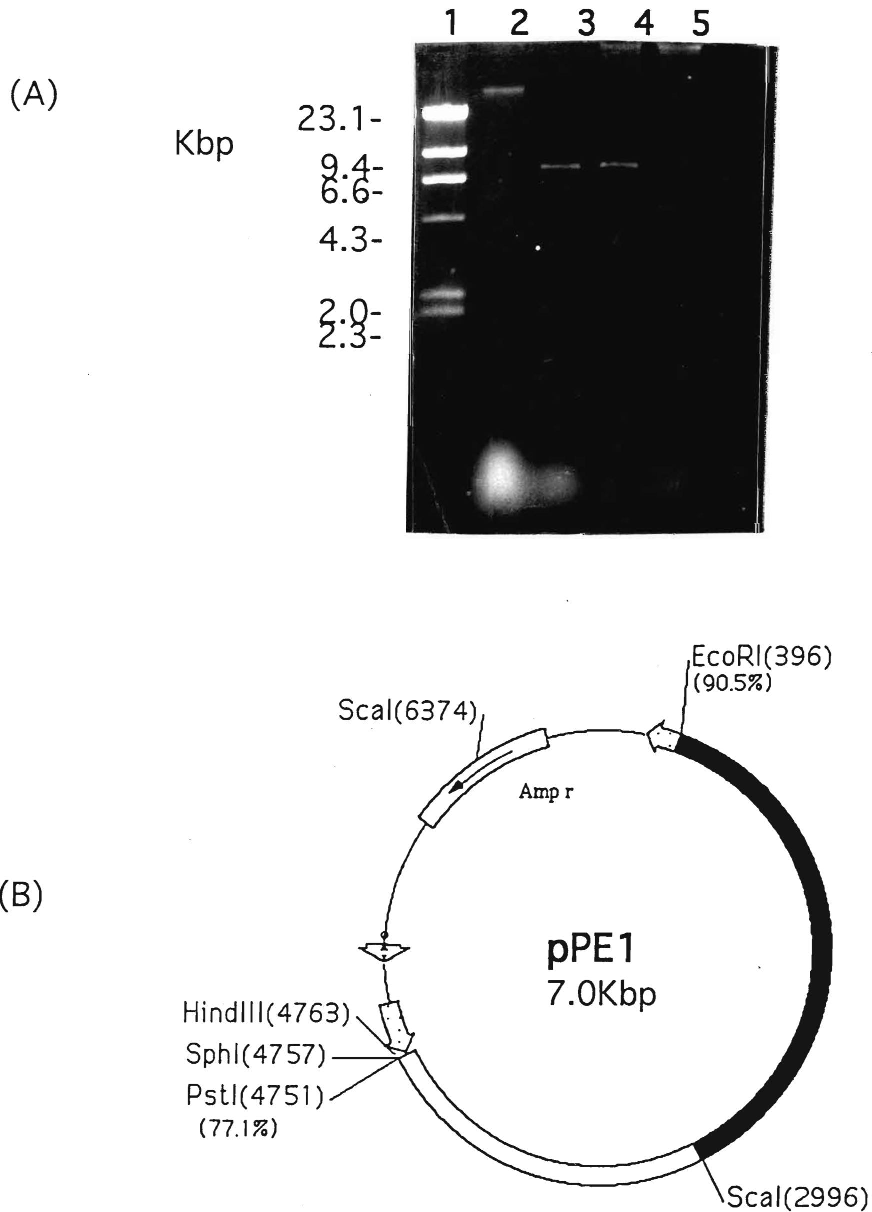


Figure 11 : (A) Photograph of a 0.9% agarose gel stained with ethidium bromide. Lane 1 is a lambda marker (*Hind*III digest). Lane 2 is uncut pPE1 and lanes 3-5 are pPE1 digested with *Pst*I, *Eco*RI and *Eco*RI+*Pst*I, respectively.
(B) Restriction enzyme map of the plasmid, pPE1.

(Figure 16A & B). The sequence analysis revealed that the deletion was precisely made between *Pst*I(77.1m.u.) and *Sca*I(82.5m.u.) and the multiple cloning site was successfully inserted.

4.1.2.1 Restriction enzyme analysis of pdIE3-1

The confirmation of the construction of the plasmid, pdIE3-1 involved the use of several restriction enzyme digestions: *Eco*RI, *Pst*I, *Bam*HI and *Xho*I (Figure 13A). Each of these restriction endonucleases digest pdIE3-1 only once, generating 1 band approximately 5.3 Kbp in size (lanes 3-6, respectively). The physical restriction enzyme map of pdIE3-1 is shown in Figure 13B.

4.1.3 Reconstruction of BAV2 genome in recombinant plamids

The next step following the deletion of the E3 region involved reconstructing the left end of the BAV2 genome in recombinant plasmids. This reconstruction was required to provide a large area for homologous recombination to occur between the plasmid DNA and the wtBAV2 genome in subsequent steps of this project. Two plasmids pdIE3-3 and pdIE3-4 were constructed using shotgun ligation strategies as oulined in Figure 14 and 17, respectively.

4.1.3.1 Restriction enzyme analysis of pdIE3-3 and pdIE3-4

pdIE3-3 was confirmed to contain from 65.2-90.5 map units (E3 deleted) of the BAV2 genome using the following restriction enzyme digestions: *Bam*HI, *Bam*HI + *Sal*I, *Eco*RI, *Pst*I, *Eco*RI + *Kpn*I and *Eco*RI+*Bam*HI (Figure 15A). *Bam*HI and *Eco*RI each cut pdIE3-3 once to generate 1 band of approximately 9.1Kbp (lanes 3 & 5, respectively). When pdIE3-3 was

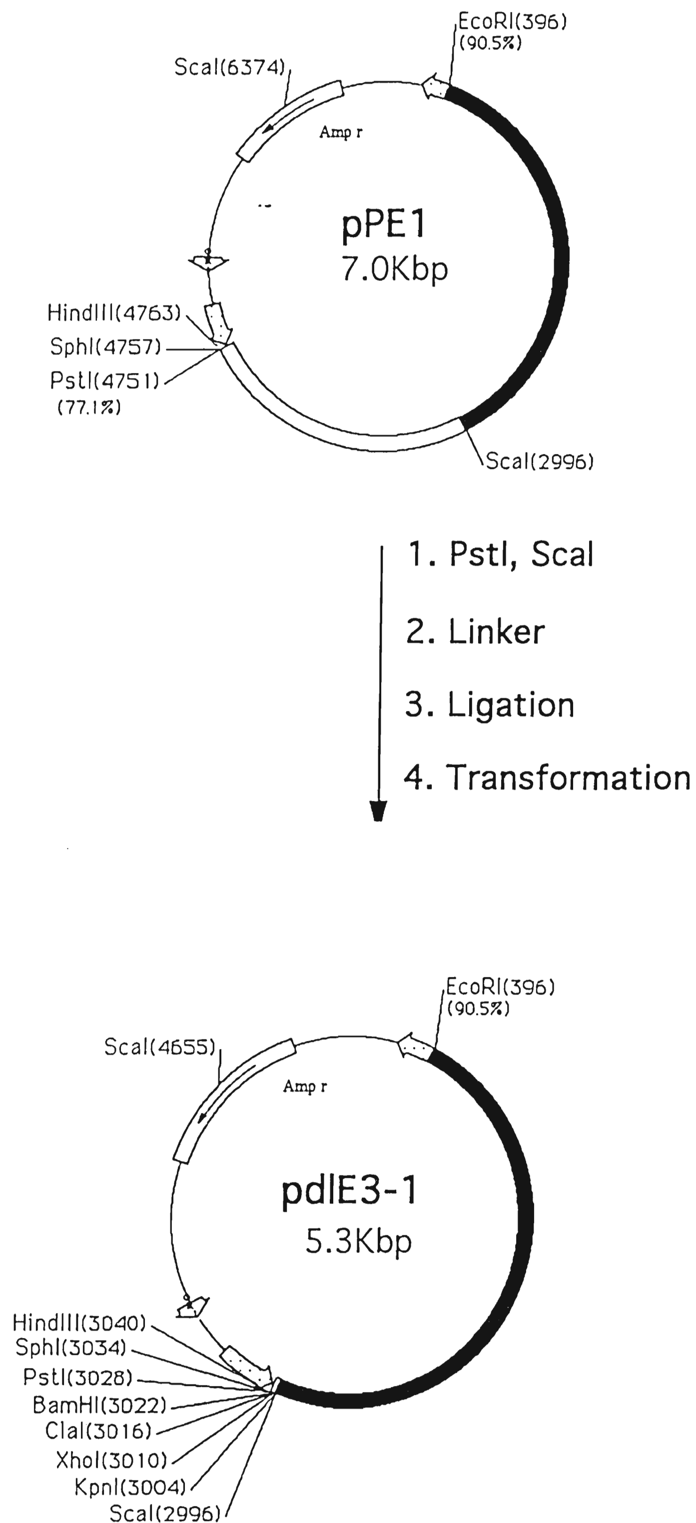


Figure 12: Strategy for the deletion of the E3 region between *Pst*I (77.1%) and *Sca*I (82.5%) and the insertion of a linker(*Sca*I, *Kpn*I, *Xho*I, *Cla*I, *Bam*HI, *Pst*I).

(A)

(B)

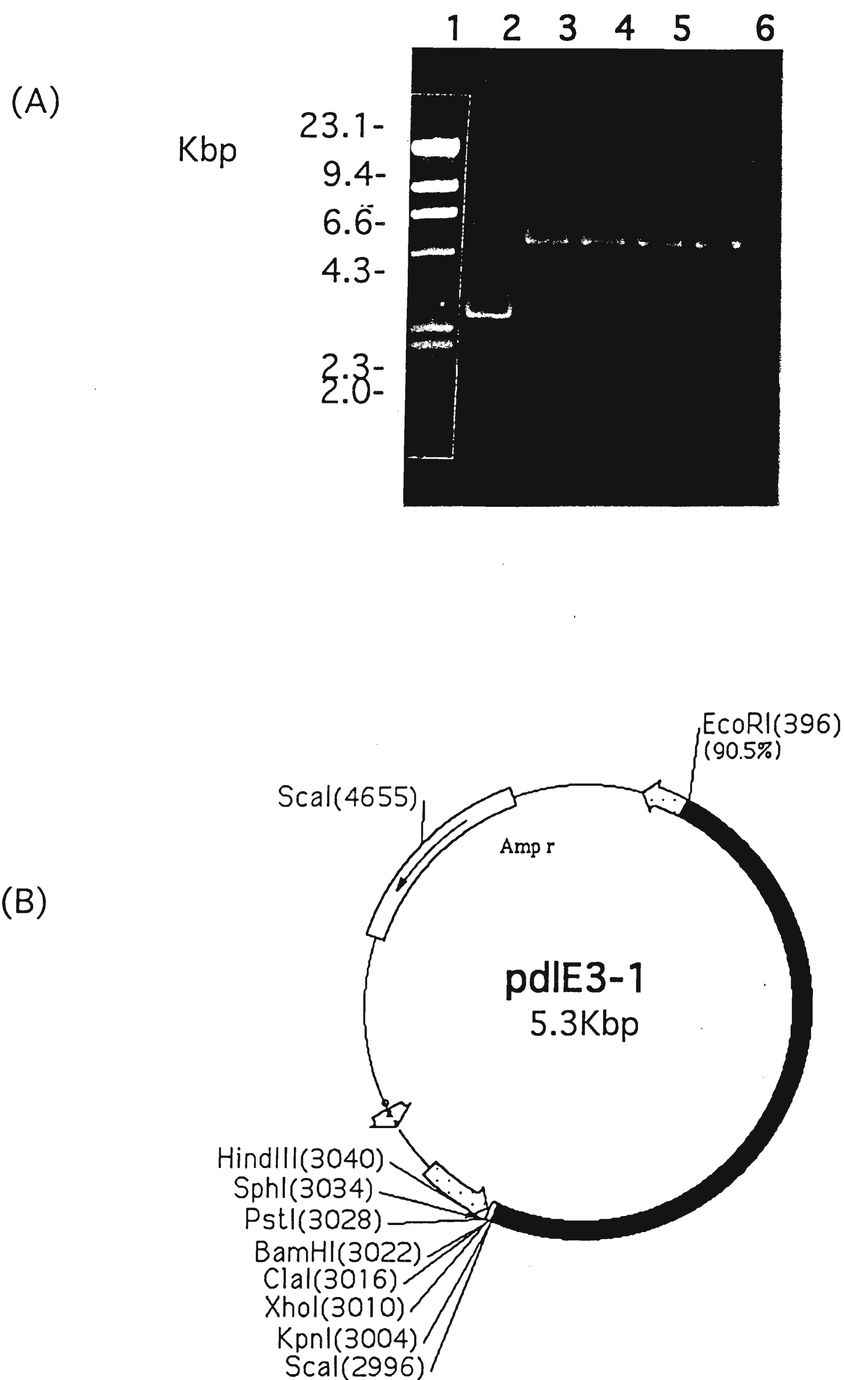


Figure 13 : (A) Photograph of a 0.9% agarose gel stained with ethidium bromide. Lane 1 is a lambda marker (*Hin*dIII digest). Lane 2 is uncut pdIE3-1 and lanes 3-6 are pdIE3-1 digested with *Eco*RI, *Pst*I, *Bam*HI and *Xho*I, respectively.
(B) Restriction enzyme map of the plasmid, pdIE3-1.

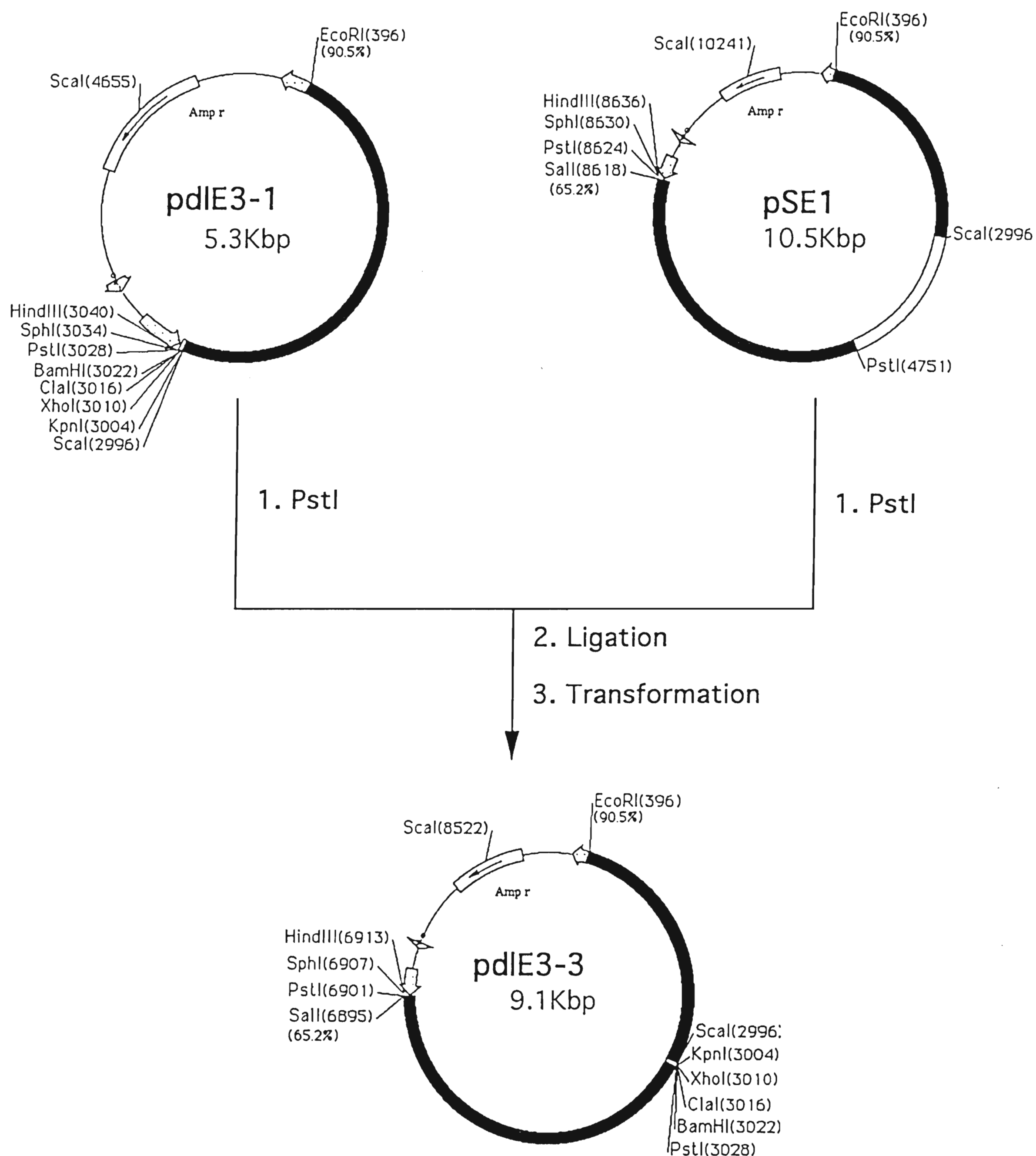


Figure 14: Strategy of the reconstruction of the right end of BAV2 by digesting pSE1 and pdlE3-1 with PstI and ligating.

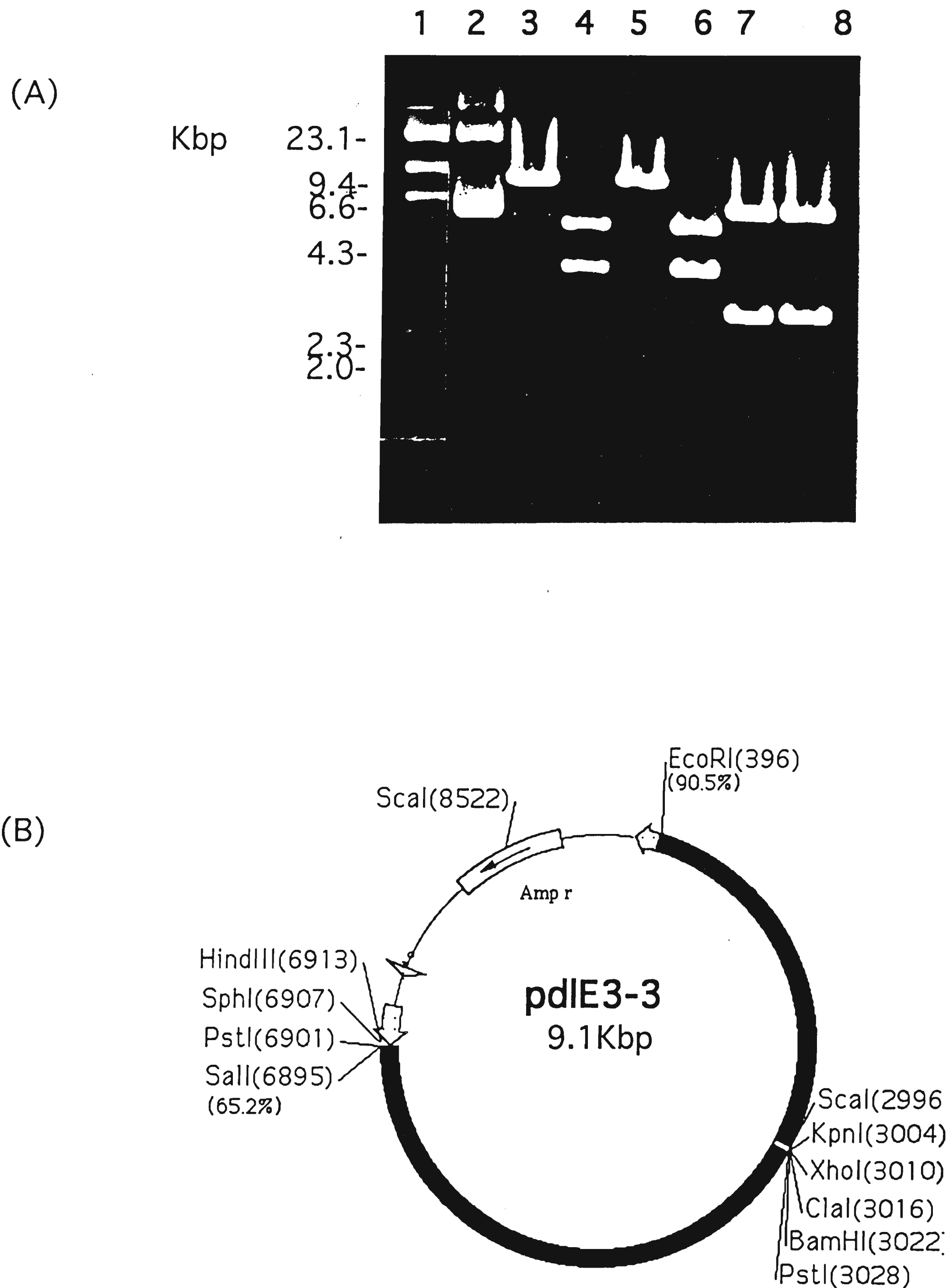


Figure 15 : (A) Photograph of a 0.9% agarose gel stained with ethidium bromide. Lane 1 is a lambda marker (*Hin*dIII digest). Lane 2 represents the uncut plasmid pdIE3-3 and lanes 3-8 are pdIE3-3 digested with *Bam*HI, *Bam*HI and *Sal*I, *Eco*RI, *Pst*I, *Eco*RI and *Kpn*I and *Eco*RI and *Bam*HI.
(B) Restriction enzyme map of the plasmid pdIE3-3.

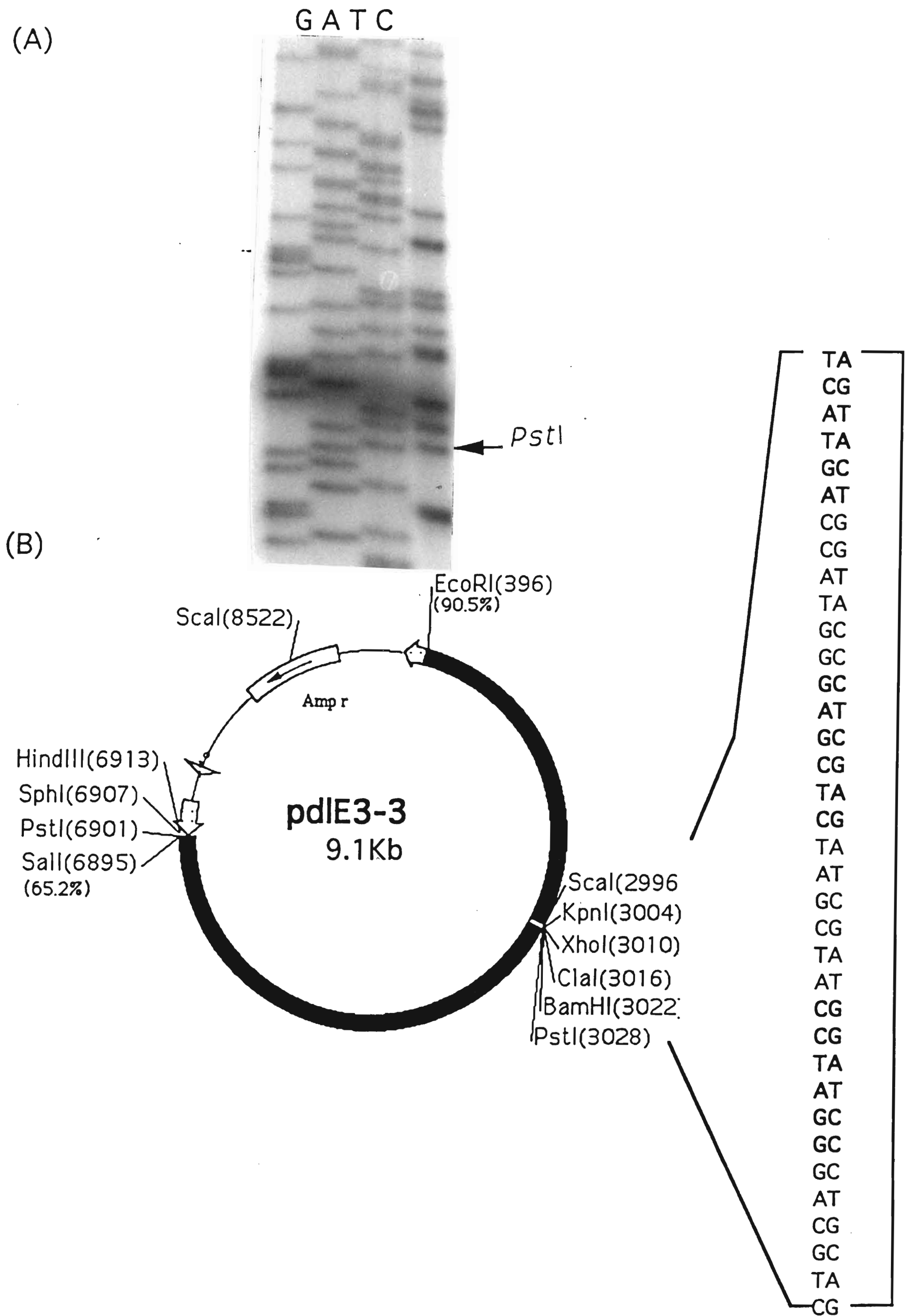


Figure 16: A photograph of a sequencing gel (6% Long Ranger) showing the nucleotide sequence of the multiple cloning site in the deleted E3 region. Panel B shows the restriction enzyme map and 36 bp corresponding to the restriction enzyme sites within the MCS.

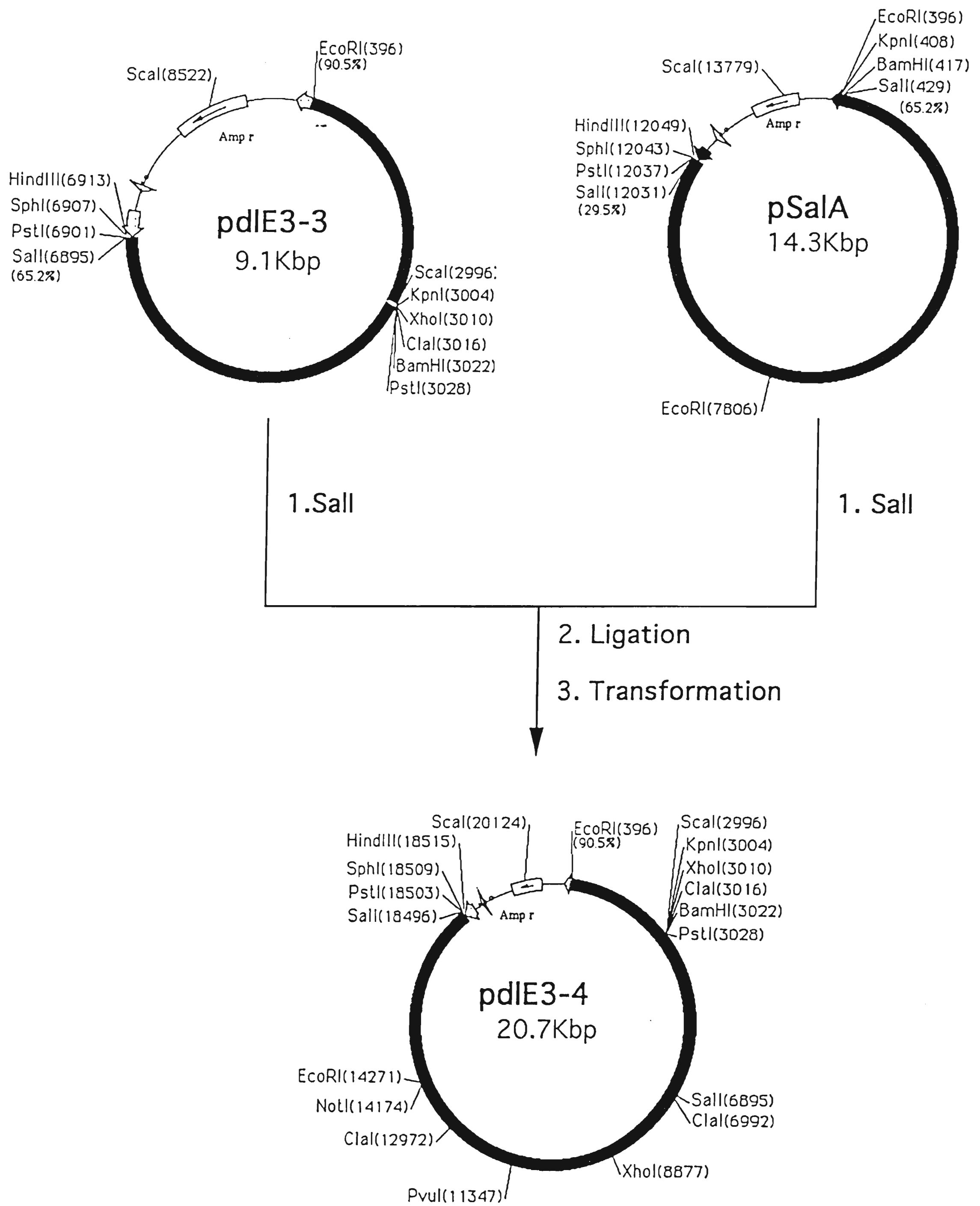


Figure 17: Strategy of the reconstruction of BAV2 genome with the insertion of the Sal A fragment (65.2 m.u.-29.5m.u.). The genome now extends from 29.5 m.u. to 90.5m.u. with the E3 deleted.

digested with *Bam*HI+*Sal*I and *Pst*I, 2 bands were generated in each case: 5.2Kbp and 3.9Kbp (lanes 4 and 6, respectively). The two remaining digestions, *Eco*RI+*Kpn*I and *Eco*RI+*Bam*HI, generated two bands: 6.5Kbp and 2.6Kbp (lanes 7 and 8, respectively). The physical restriction enzyme map of pdlE3-3 is shown in Figure 15B.

The plasmid pdlE3-4 was shown to contain the BAV2 *Sal*A fragment by digesting the plasmid with the following enzymes: *Sal*I, *Eco*RI, *Bam*HI and *Bam*HI + *Eco*RI (Figure 18A), and analyzing the fragments generated. When pdlE3-4 was digested with *Bam*HI, the one expected band was generated at 20.7Kbp (lane 6). Both *Sal*I and *Eco*RI, each recognize two sites within pdlE3-4, therefore generating two bands at 11.6Kbp, 9.1Kbp and 13.9Kbp, 6.8Kbp, respectively (lanes 3 & 4). The double digestion with *Eco*RI+*Bam*HI generated the expected 3 fragments: 11.2Kbp, 6.9Kbp and 2.6Kbp (lane 5) and confirmed the orientation of the *Sal*A fragment within the plasmid, pdlE3-4. The physical restriction enzyme map of pdlE3-4 is shown in Figure 18B.

4.1.4 The cloning of a reporter gene (tk) in the deleted E3 region

The strategy to clone the tk gene of HSV in place of the deleted E3 sequences was slightly different from those used in previous steps (Figure 19). The strategy involved the use of the plasmid pdlE3-4 digested with *Bam*HI. *Bam*HI generated a single band from a cut within the multiple cloning site. The digested pdlE3-4 was combined with *Pvu*II digested tk173 and a synthesized *Bam*HI linker (Procyon Biopharma, London, Ontario) and ligated with T4 DNA ligase. The ligated DNA was used to transform competent *E. coli* strain DH5α. The recombinant plasmids were isolated from transformed *E. coli* and screened for the presence of the tk

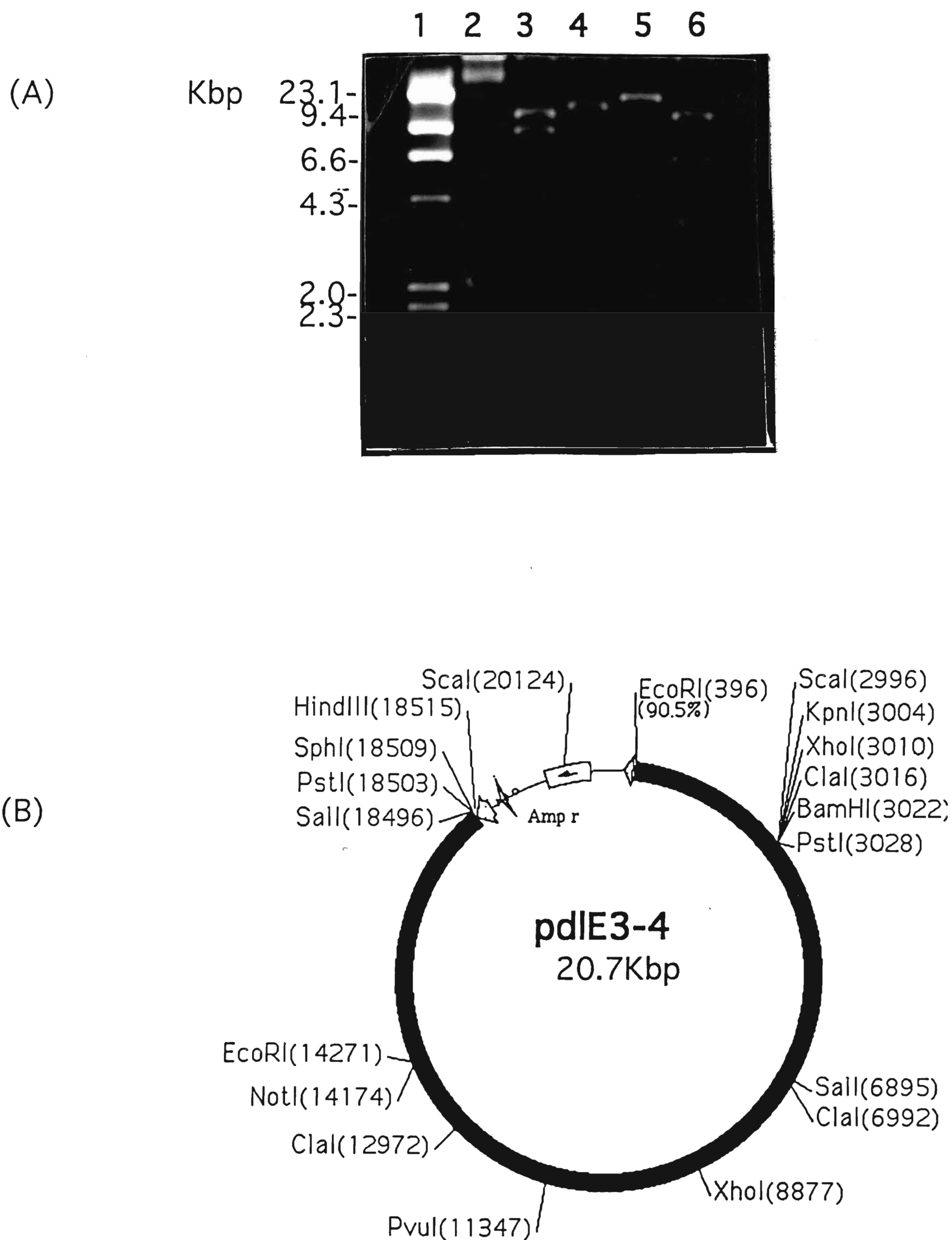


Figure 18 : (A) Photograph of a 0.9% agarose gel stained with ethidium bromide. Lane 1 represents a lambda (*Hin*dIII digest). Lane 2 represents the uncut plasmid pdIE3-4 and lanes 3-6 represent pdIE3-4 digested with restriction enzymes *SalI*, *Eco*RI, *Bam*HI and *Eco*RI and *Bam*HI,respectively.

(B) Restriction enzyme map of pdIE3-4.

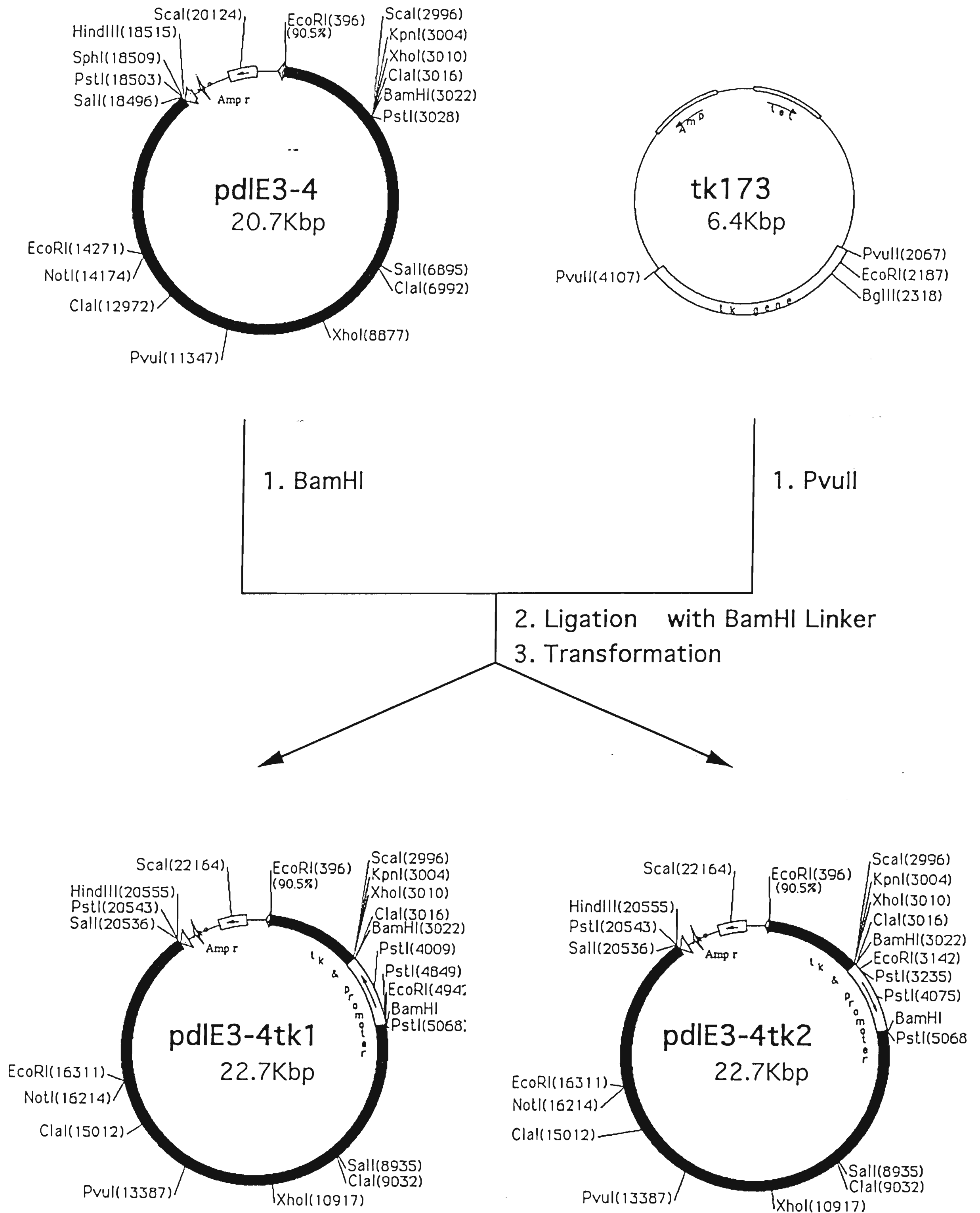


Figure 19: The insertion of the thymidine kinase (tk) gene in the unique BamHI site. The tk gene is inserted in both orientations with its own promoter.

gene using restriction enzyme analysis. Out of the 250 colonies screened, two were found to contain the tk sequences. The plasmid, pdlE3-4tk1 (Figure 20B) has the tk gene in the orientation parallel to the deleted E3 sequences, whereas in pdlE3-4tk2 (Figure 21B), the tk gene is in the anti-parallel orientation.

4.1.4.1 Restriction enzyme analysis of pdlE3-4tk1 & pdlE3-4tk

The plasmids, pdlE3-4tk1 and pdlE3-tk2 were confirmed by digestion with the following restriction enzymes: *Bam*HI, *Eco*RI and *Sal*I (Figure 20A &21A). When both plasmids were digested with *Bam*HI, two bands were generated (Figure 20A & 21A; lane 3); a large fragment approximately 20.7Kbp and the tk gene, 2Kbp. Digestion with *Sal*I generated a doublet in both cases; 11.6Kbp and 11.1Kbp (Figure 20A, lane 5 and Figure 21A, lane 6). Unlike *Bam*HI and *Sal*I, *Eco*RI cuts within the tk gene and was used to confirm the orientation. *Eco*RI generates 3 fragments for pdlE3-4tk1: 11.3Kbp, 6.9Kbp and 4.5Kbp (Figure 20A, lane 4), whereas 3 fragments differing in size were generated for pdlE3-4tk2 (Figure 21A, lane 4): 13.1Kbp, 6.8Kbp and 2.8Kbp. A double digestion of pdlE3-4tk2 with *Eco*RI & *Bam*HI generated 4 bands (Figure 21A, lane 5): 11.2Kbp, 7.0Kbp, 2.6Kbp and 1.9Kbp. In addition to these, a short fragment (120bp) is expected to be generated, however it can not be seen clearly on the agarose gel due to its small size.

4.1.5 Cloning of the right and left end of BAV2 genome

To increase the library of recombinant BAV2 plasmids and provide alternative strategies for rescuing a BAV2 vector, the right and left end of the BAV2 genome were cloned to generate the plasmids pdlE3-5 and

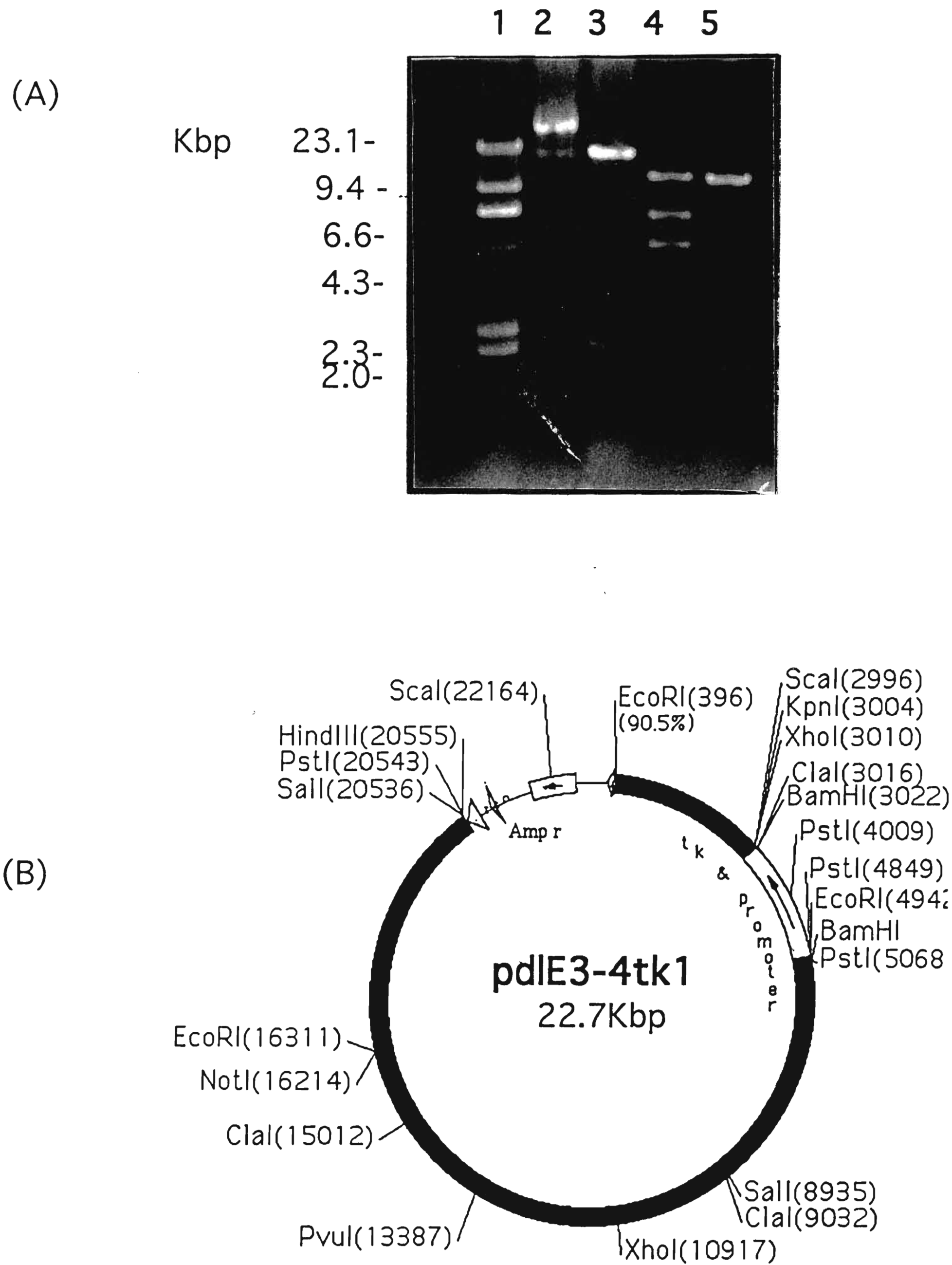


Figure 20 : (A) Photograph of a 0.9% agarose gel stained with ethidium bromide . Lane 1 is a lambda marker (*Hind*III digest). Lane 2 represents the uncut plasmid, pdlE3-4tk1 and lanes 3-5 are pdlE3-tk1 digested with *Bam*HI, *Eco*RI, and *Sal*I.

(B) Restriction enzyme map of the plasmid pdlE3-4tk1

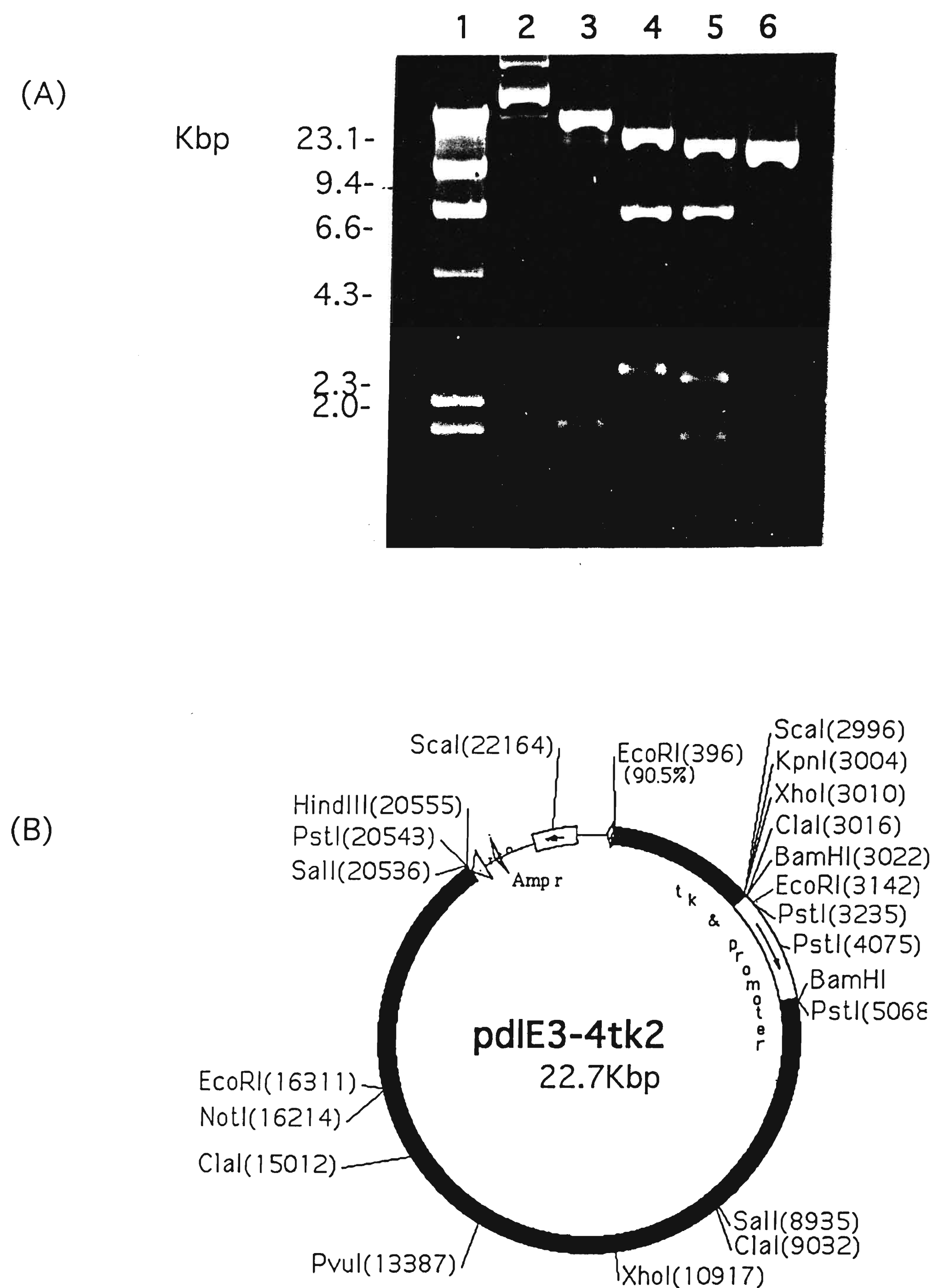


Figure 21 : (A) Photograph of a 0.9% agarose gel stained with EtBr Lane 1 is a lambda marker (*Hind*III digest). Lane 2 represents uncut plasmid, pdlE3-4tk2 and lanes 3-6 represent the plasmid digested with *Bam*HI, *Eco*RI, *Eco*RI and *Bam*HI, and *Sal*I.
(B) Restriction enzyme map of plasmid pdlE3-4tk2

pdlE1-E, respectively. However, these plasmids were not used for any rescue attempts during this study. The strategy to clone the left end involved using *Sal*I digested pBBSK (provided by Jeff Kormos) combined with *Sal*I digested pEB and ligated with T4 DNA ligase (Figure 22). The recombinant plasmid, pdlE1-E was isolated from transformed *E. coli*. This plasmid was confirmed to contain 0-42.5m.u. of the BAV2 genome with a deletion in the E1 region using the following restriction enzyme analysis: *Sal*I, *Eco*RI and *Sal*I+*Eco*RI (Figure 23A). *Sal*I and *Eco*RI each generated 2 fragments: 9.6Kbp and 4.2Kbp and 7.1Kbp and 6.7Kbp, respectively (lanes 3 & 4). The correct orientation was confirmed by the analysis of the *Eco*RI digestion. An *Eco*RI restriction enzyme site is situated within the fragment cloned in the unique *Sal*I site. When this plasmid was digested with both *Eco*RI and *Sal*I, 3 bands were generated: 6.7Kbp, 4.2Kbp and 2.9Kbp (lane 5). Figure 23B is the physical restriction map of pdlE1-E.

The strategy employed to clone the right end of BAV2 was similiar to that used for the left end (Figure 24). The plasmid, pdlE3-5 was shown to contain 65.2 -100m.u. of the BAV2 genome with a deletion within the E3 region through analysis of the fragments generated from the following restriction enzyme digestions: *Eco*RI, *Sal*I and *Pst*I (Figure 25A). *Sal*I cuts the plasmid once, generating one band at 12.6Kbp (lane 4). *Eco*RI generates two bands: 9.1Kbp and 3.5Kbp (lane 3), whereas *Pst*I generates 4 bands: 5.3Kbp, 3.9Kbp, 2.9Kbp and a small fragment at 520bp (lane 5). The resulting DNA fragments from the *Pst*I digestion of pdlE3-5 suggested the presence of an additional *Pst*I site at approximately 99 m.u. which was later confirmed when the sequence of the right end was analyzed.

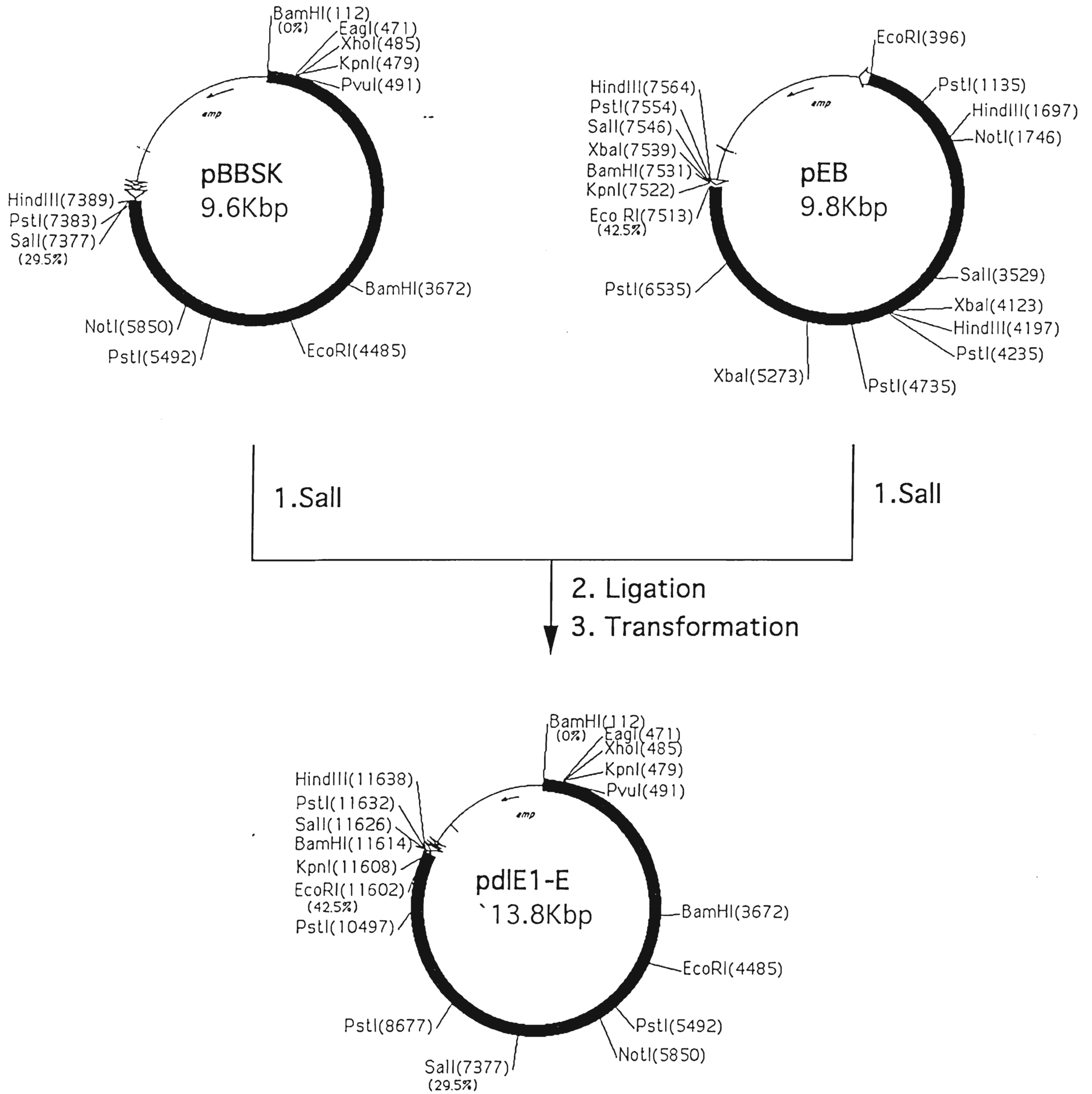


Figure 22: Strategy used in the construction of pdlE1-E from plasmids pBBSK and pEB.

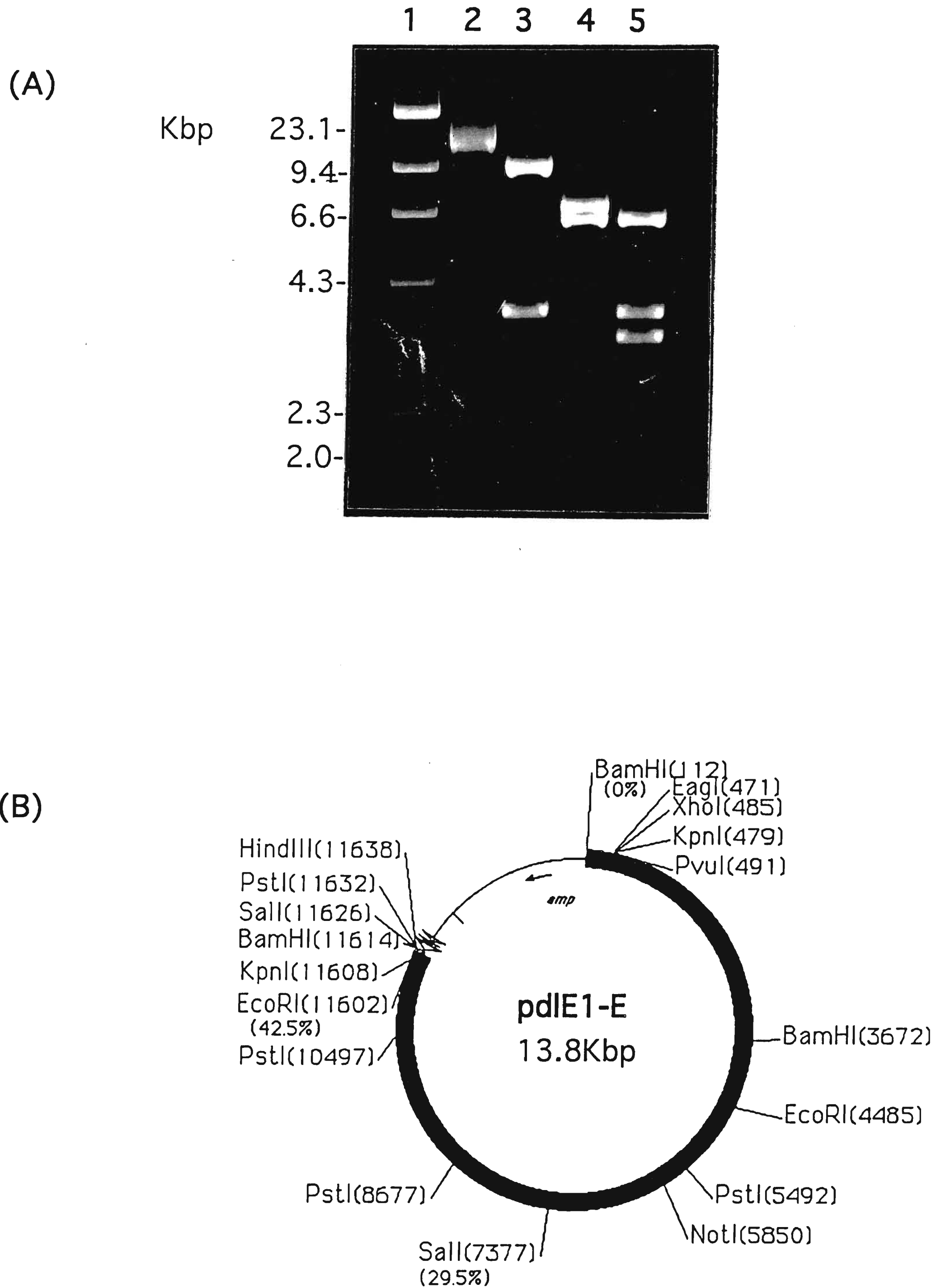


Figure 23: (A) Photograph of an agarose gel stained with ethidium bromide. Lane 1 is a lambda marker (*Hind*III) digest. Lane 2 is uncut pdIE1-E and lanes 3-5 represent pdIE1-E cut with *Sal*I, *Eco*RI and *Sal*I+*Eco*RI, respectively.
(B) Restriction enzyme map of pdIE1-E.

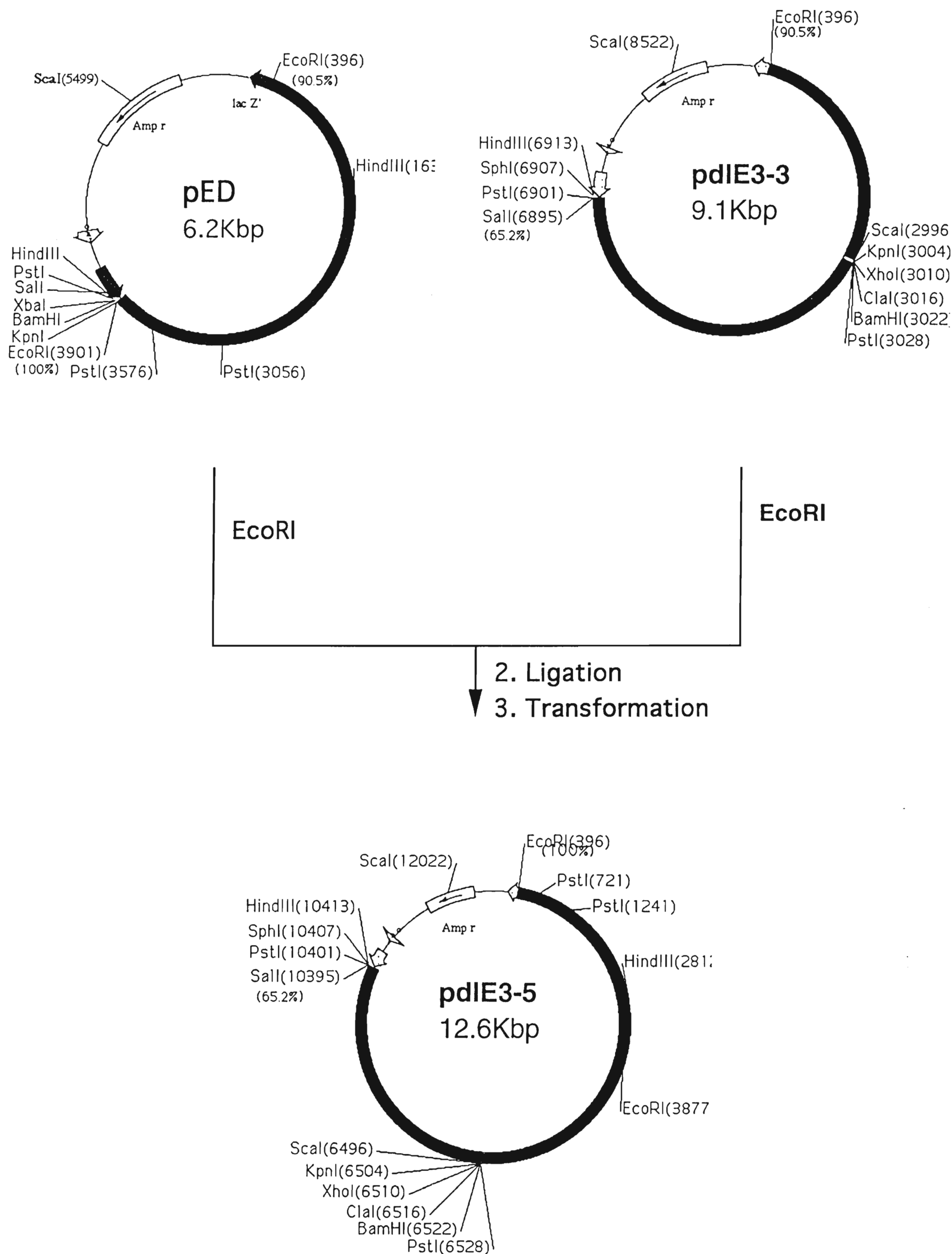


Figure24: Strategy of the cloning of the *Eco*RI D fragment in the unique *Eco*RI site of pdIE3-3. The new plasmid , pdIE3-5 now extends from *Sal*I (65.2m.u.) to *Eco*RI (100m.u.).

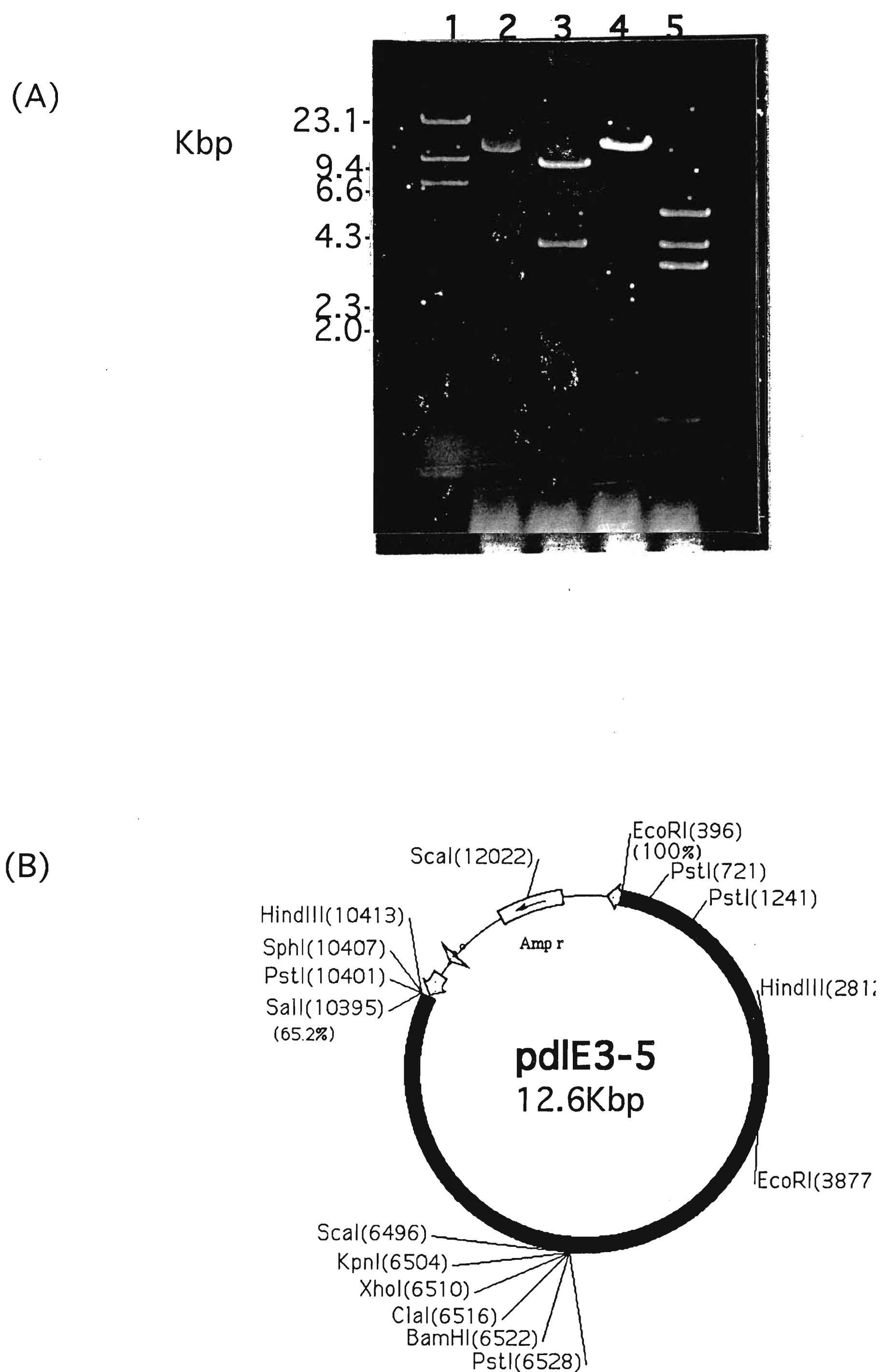


Figure 25 : (A) Agarose gel stained with ethidium bromide. Lane1 is a lambda marker (*Hind*III digest). Lane 2 is uncut pdIE3-5 and lanes 3-5 are pdIE3-5 cut with *Eco*RI, *Sal*I, and *Pst*I, respectively.
(B) Restriction enzyme map of pdIE3-5.

4.1.6 Attempt to rescue a BAV2 E3 deletion mutant

The strategy used involved infecting MDBK cells with wtBAV2 followed by transfection with CsCl purified plasmid, pdlE3-4tk1 (Figure 26). The recombinant plasmid, pdlE3-4tk1 is large enough to facilitate homologous recombination with the wtBAV2 genome in MDBK cells. A ^{32}P labelled tk173 probe was hybridized to Hirts extracted pooled viral DNA that was digested with *Bam*HI and *Bam*HI + *Eco*RI. The DNA was extracted after the second round of infection to eliminate any residual plasmid DNA from the transfection. The DNA fragments that hybridized to the ^{32}P-labelled tk173 probe appear as bands (Figure 27). The sizes of the DNA fragments were determined using a lambda *Hind*III digested marker (lane 1) and *Pvu*II digested tk173 (lane 2). When *Bam*HI digested viral DNA was probed with ^{32}P-labelled tk173 one band, approximately 2Kbp in size (lane 4), co-migrated with the tk gene of *Pvu*II digested tk173 (lane 2). The larger band also generated is the result of partial or undigested viral DNA. When the viral DNA was double digested with *Eco*RI and *Bam*HI, a band slightly lower than 2Kbp was generated. This is expected because there is an *Eco*RI site located within the tk promoter. The sizes of the higher bands observed were determined to be approximately 6Kbp and 4.3Kbp from an agarose gel (data not shown). Therefore, it is reasonable to assume that these bands are a result of partially digested viral DNA. For example, if the *Bam*HI digestion was incomplete and the enzyme did not cut some viral DNA at 82.5 m.u. (See Figure 27), then it is possible to obtain a ^{32}P labelled fragment at approximately 4.4 Kbp.

To further investigate the results represented in Figure 27, a ^{32}P -labelled tk173 probe was hybridized to Hirts extracted DNA from plaque purified virus that was digested with *Bam*HI. Of the 70 plaque isolated, 4

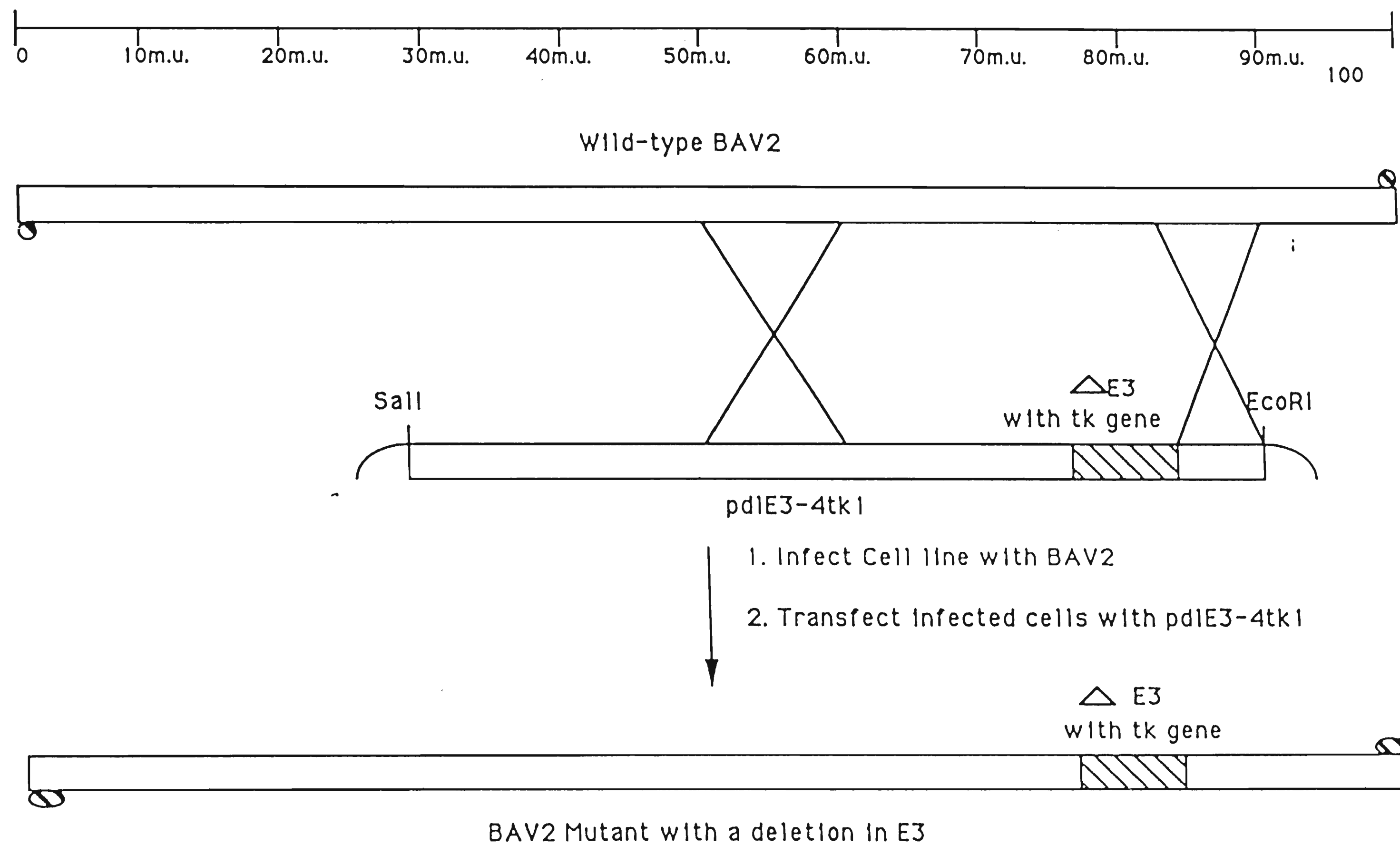


Figure 26 : Strategy used in the attempt to rescue a BAV2 mutant containing a deletion in the E3 region with the thymidine kinase gene in its place.

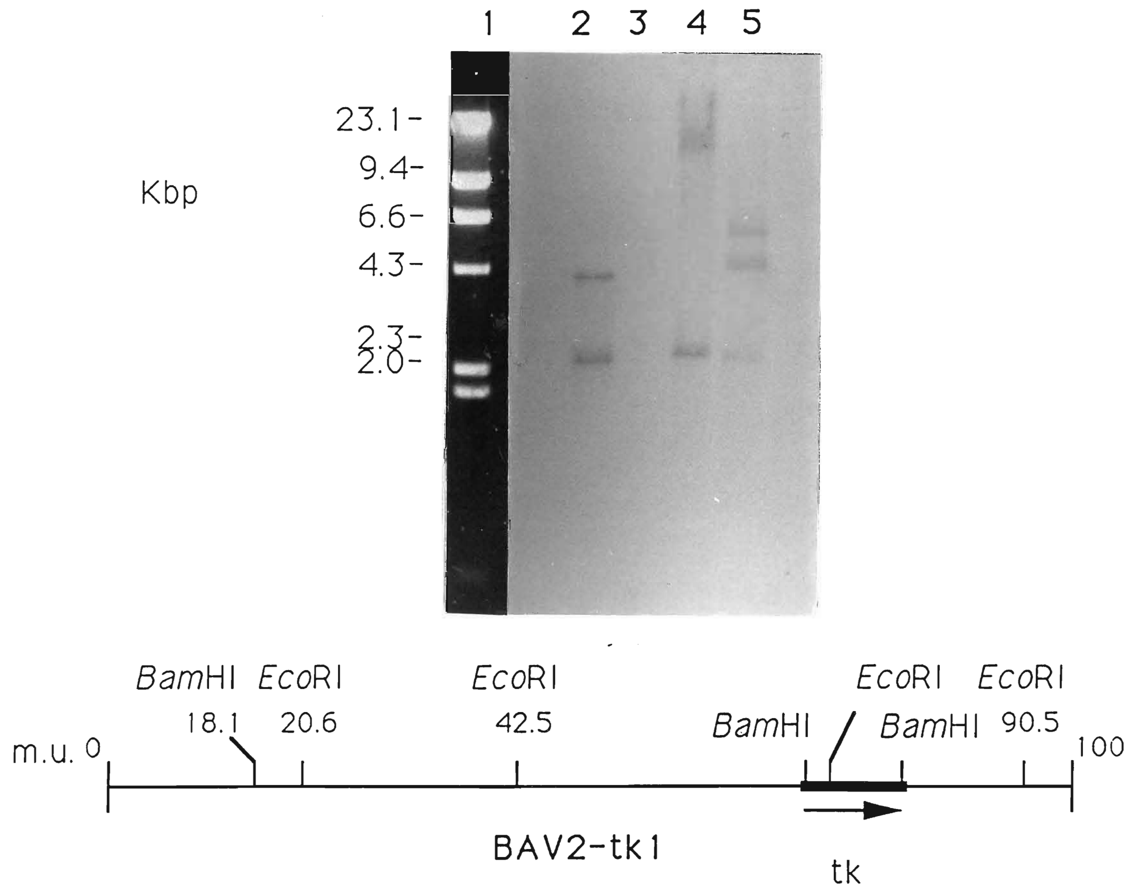


Figure 27: Photograph of an autoradiogram of Hirts extracted viral DNA, digested with *Bam*HI (lane 4) and *Bam*H+*Eco*RI (lane5) hybridized to radioactive labelled.tk173. Lane 1 is a lambda marker (*Hind*III digest), lane 2 is *Pvu*II digested tk173 and lane 3 is *Bam*HI digested cell line DNA

showed one band slightly higher than the tk gene from *Pvu*II digested tk173 (lane 3) (See Figure 28A; marked with*). ^{32}P-labelled BAV2 DNA was hybridized to the samples so that the viral DNA fragments would appear as bands (Figure 28B). When hybridized to the ^{32}P-labelled BAV2 DNA, two additional fragments were apparent in every lane containing plaque purified viral DNA digested with *Bam*HI. The approximate size of one band is 5.8Kbp, however the size of the larger band which is greater than 23.1Kbp is to large to estimate.

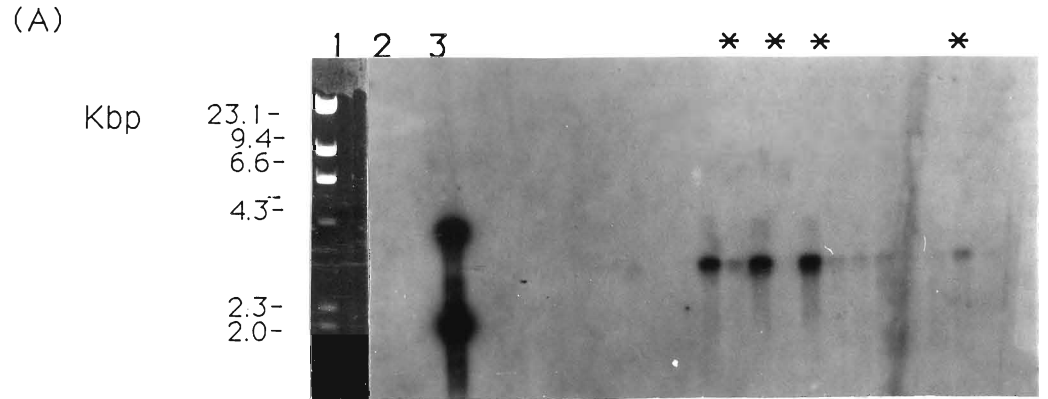
4.2 Sequencing of the E4 region

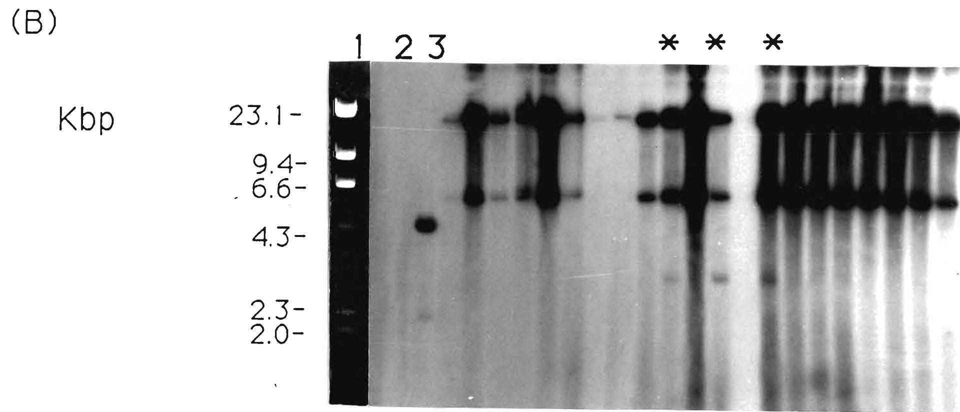
4.2.1 Sequencing strategy.

Sequencing of the E4 region started with the plasmid, pED, which contains the *Eco*RI D fragment (90.5%-100%) of BAV2 cloned into the vector pUC19. Universal primers (specific for pUC19 sequences) were used to initiate the sequencing and custom synthesized oligonucleotides (Procyon Inc. London, Ont) were used to continue the sequencing of the entire fragment (Figure 30). Vent polymerase was used to resolve compressed areas. Figure 29 is the resulting nucleotide sequence from 90.5 to 100 map units of BAV2 corresponding to the E4 region and the right ITR.

4.2.2 Organization and homology of the putative E4 region

Within the 3550 bp fragment, there are 40 ORFs capable of coding for polypeptides of 25 or more amino acids. A homology search of the nucleotide and predicted amino acid sequences using the European Molecular Biology Laboratory (EMBL) nucleic acid and protein data base and





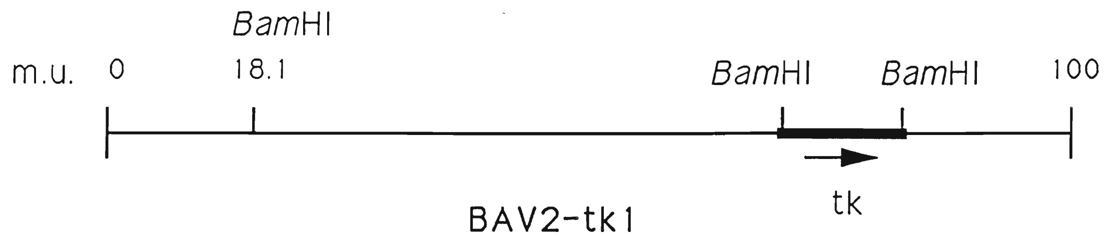


Figure 28: (A) Photograph of an autoradiogram of plaque purified viral DNA, digested with *Bam*HI and hybridized to radioactive labelled. tk173. Lane 1 is a lambda marker (*Hind* III digest), lane 2 is *Bam*HI digested cell line DNA, lane3 is *Pvu*II digested tk173 and the remaining lanes represent plaque purified viral DNA.
(B) Photograph of an autoradiogram, representing the samples in (A) hybridized to radioactive labelled BAV2 DNA.

Figure 29: Nucleotide sequence of the BAV2 genome from 90.5 m.u. to 100 m.u. Potenial "TATA" boxes are represented in bold, as are the polyadenylation signals and the first ATG of 6 ORF which share identity to proteins encoded by the E4 region of other Adenoviruses.

100% 5' ORI NFI binding site
CATCATCAATAATATACGGTGCATTTTGTGCGTGATGACGTATACAGCGTACGTAAGGGA 60

GGAGCTTGCAAGCGTGGGAAACTTGACCTTTGAACGGGCGCGGTGAAGTGGGTTGGACAA 120

CCTTTGAGCATGAGGTGCTGGAATTATTGACCTTTACACAGGCGGAGTTCAGTTTCGCTA 180

CGTCAGTGGACTTGCCGGCAAGCCCAGTACTTTTCCAGTCAGTGACAGTGACGCCTAAGG 240

"TATA"
GTGGAGTACATCCTTTAAATACAGCAGTGACGACTTTAGACTCAGCACTGCGCCTCACTC 300

"cap" ORF1 start
ACAGCCAGTTTTACCATTCTGCCACTACTTAACTTTAGATGCTCTTCAGCTTTTTGTTGA 360

GAGCGGCTTTGCCATCTGCTGTGCTTCCTAAGCGAGCTTCTGCAGGAGCTGCTGGATATG 420

ACTTGTGTGCTTCTACTTATGTAGAAGTTCCAGCTGGAGCAAGAGTGCTAGTTCCTACTG 480

GATTAAAAATTTCAATTCCAGAAGGACATTATGGACCGTACGTCCGCGATGTCTGGGCTT 540

GCTGTTACTCAGGCTTTAATGTGGGAGCTGGCGTGATCGACTGCGACTACAGGGGAGAGC 600

TGTTTGTATTAATTTTTAATCATGGAAACCAAAATTACCTTATTTCGCCAGGACAGCGAG 660

ORF1 end
TGGACGCACGTTATTATTGAAAAACTCTGACTCTGTGTGGAGTTGGACAGCATCGACGAC 720

CACACTGAAAGAGGTGCTAGCGGCTTTGGGTCTACTGGATTATATTGGCTCCTGTTGTCA 780

ATGGGGAAATTATAAAAATTTTAATTCCTTCATCTTTAACTAACTCTGTGCATAGCGAAC 840

GACCTCAAGATTTTGCTACATCTGTGGCTTCAATGGCTGAGTGTTGTGGCCTCTAAGCTG 900

ATTATATAGCGCTCGGCAATAATGTGTTATTTAAGTTCTTACTGCACTCATCAGCCAAAC 960

TGTTATGTTGTAGTCCTCCCTGTTTGTAGGAGGAGCTTCAACAGATTCTTTACTTACTAA 1020

TAATGGCGAATATGAAGATTTTTTAATTAATGAGCTACAAGAATATTTGCATGAAGCAGT 1080

GTTTGATTACACTCGCCTACTTGGAGAAGACATTTCCCTAGAAGACTGCAAATTTATACA 1140

AGTTAGCTCAGTATAAGTTTTACATGTGGTATGATGCATTTGTCAATAAATGGTTTCTTT 1200

ORF2 start
AAGAAAATCTGCTGAAATTGTTTCTATCATTAATTATGTTACCAATCAACTCGAGAAGTC 1260

TTTTATAGTTTACTATCAACATTTAATGAGGAAAGTTCACAACGTTGGATTTACTTTGTC 1320

TACGTTCATCAATTGCAGCTGCAGAGAGCACGGATGGGTCGCCGATCTGTGCATGAGCTT 1380

TTGCTTTGTGGAGAACATCTATCAAGACTTGAGAAGGTACTTATTGACTTATACTTTGAA 1440

ORF2 end
CATGGACAATTTAAACATCAAAAGTAGTGACTATAACTGCCGCAGTTCTTTATCTAATCA 1500

CTGCATTGGATATGTGAAGTCACCAAAATCATGCGACATGTTTTGCTATGTGTTTAGAAC 1560

TTCCGATTCTTTGGGAGTTTATTTTATGTCACCATGAAACTATCTTTTTAAAAAATTATT 1620

TATGTAATTGCGTAGAGATTGAAATGTGCTCTGTGTATGATAAATTTTTATTACCTGATT 1680

ACTCCTCACTTGCTGCTAGGTGGGGCTTACATTGCCACTGTACAGATAGACAAACTTTAC 1740

AATGTTTTGCTGCTAGGCGTGTCATAGAAGCTTTAGTTAAAGAGGTTTTAAAGGGCACAA 1800

TGTATAATCAGATGTTTTGGGATTATAGACAATTTGTAAACAGAGGCTTGCTGATACTTT 1860

ORF3
ATGTTTGTTGGCAGTTATTATGTTAGAAGAGTTCATTTTATTGTAATTAAAGTTATTTTA 1920

GACAAAGATGTGGAAAAAAATAAGGAAGTTGCAATTTGGGGAAGCAGCTTATATTAATGGC 1980

CTGTATAGCAATTTTATAGTGCTTATTTGCCTGCTGAGCAATTTAACTGAGATTCAGGCA 2040

AATTTGCGTAGGACTAGAAAGTTTCTTTTAAAAGCATTAAAGTGTTGTCTAGTCGGGCCA 2100

AAAGTTGCTAGTAAAAAAGAAGCCCAGCGACAAAAAGATTTAATTAGGTTGTTTAAGTTT 2160

ORF4 start
GGGTCAGCATTTGACTCAAAAGTATGTAAAATATCTTTAATTTCAGATGTTGTGCCTGTC 2220

ORF3 end
CAGGCCTACATATCAGCATAAAATCATTGGATATGTAGCTGCTCCGTTGTCTTGTTTACC 2280

TTTAGCTATGAATCAAGAATTTCCTATTCCGTGGAAGCATATATTGCCATCGTGGGAGTA 2340

CAGCCTGTTAAAAAATTATTTGTGTATCGCCTCTAGGTTTAAAATAGAGTGGATGGGGA 2400

ORF5 start
TCTAGCTTTAATGGTTAGACACAATCAGCCTTTATTGTGGAGCGTGTGCTGTCATGATCA 2460

ORF4 end ORF5 end
ACATGAAAATACGTTGCAAGCTTTTGCATGAGGTGTGTACCATGTAAAAGAGCTTGTGGG 2520

TCGGGTTTGAGTATAATTTAAAGTTTCCATTTTATAGAAAAATTGTAAACAAAAATTTAA 2580

GTTTTAGAATCCTGTATGAAGGAAGTTAGTGTTAAGCGAACGATTTAATATTTGTGAAAA 2640

ORF6 start
CTAAATGGGAGATATAAGCAGGATTAGAAAGTTAAAGTTTAATAATTCTGTTATTTGTAG 2700

AGGTTTTACTGGGGTGTACTTAGTGTTATTTTGTGATAGTTGCAATAATATGACAGAATA 2760

ORF6 end
TAGTGCTAGATTGTGTGCTGCAAACACTAGGGTTAAGTTAGTATTAGTAGCCCAATAATT 2820

GTGATACATTTCGTTGAAACTAGTGAATATGAAAGAAGAACAAATTGATATTCAGAAGTT 2880

AACTTATAAGCCGTGTGTATGTTTATAGATGCAGCTGAAGCTGTTTGTGCACACTGGACC 2940

AAAAGGCTCAGCACCATGTTAAATCTAGAGAAGAAGTATGGCTAGTAACTAGTGAAGACA 3000

TGATGTTTTACCCTTATGAAGATAATGAATATGAACTGGATCATGAAGTGTATATTCCCG 3060

polyA
ATGGTTATGATGTTTCAGTAGTTAGCTTTTTTTTTAATTTGTATATAAAAATGAATTACA 3120

GAGATAATGTAATTTCTAAAATTAGTCTAAAAATGTACGAGTGGTCTCCAGGTATGATTT 3180

TTATTCGACGCTGGAGAGCCAATTGTTCAAATTAAACTTACGTCTCTTTCAGCATGACGG 3240

CAAAAAAAATTCAGCTTGAGCCCGAAATTGAAATTGAAGTTGGACCACGAACATTTCAAA 3300

CCCCTCATTTTTAATGAAAATCTCACAGAAGTGCTTTGTGTTTAGAGAAGCTATTGAATT 3360

AAGAAAGTCGAGTGTCTTAAGTTTGATGTAAAATTAGAATCCAGATGGATATTATGGCAT 3420

GGGGTCTGTTTCTAGTTTGATTGGTCTGCCGTATGATATATGGGGAAGAATATTCACTGC 3480

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GGTCCTGTAA 3550

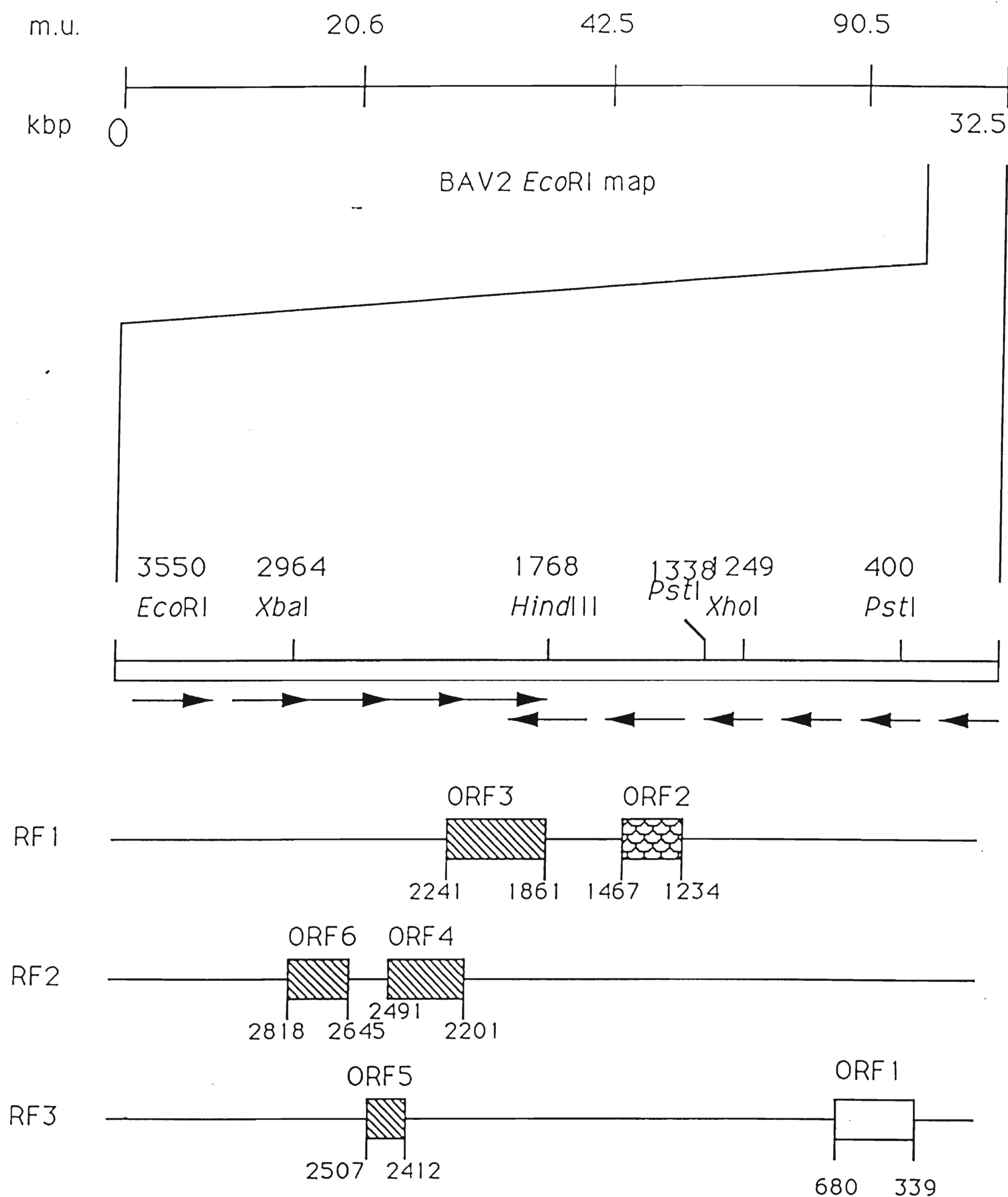


Figure 30 : The genome of BAV2 showing the location of the putative E4 region located within the EcoRI D fragment. Open reading frames that were found to resemble the Ad E4 proteins are represented by the shaded boxes. The arrows represent the primers used for sequencing.

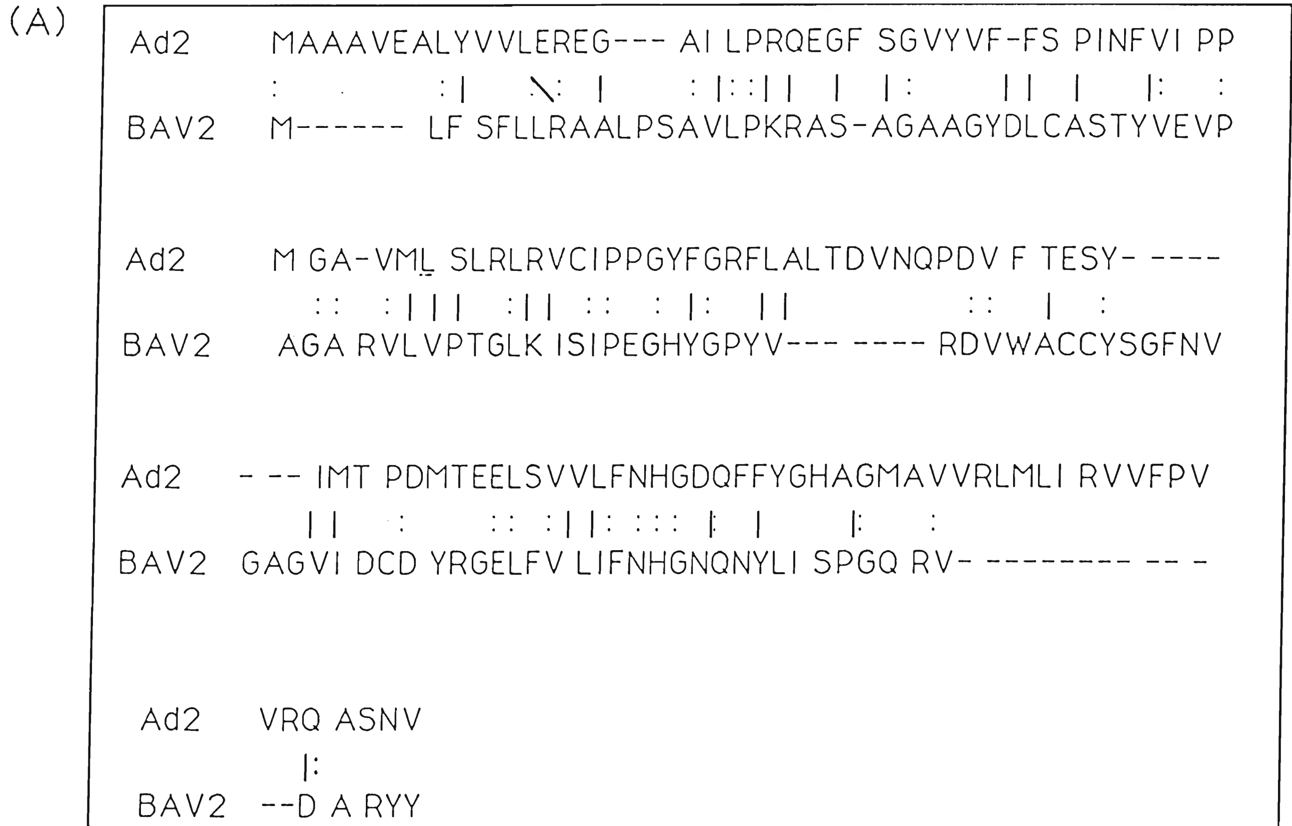
the FASTA program (Pearson and Lipman, 1988), revealed that six of these ORFs share homology to the well characterized E4 proteins of human Ads (Figure 30).

In Ad2, E4 mRNAs are produced through a series of differential splicing of a primary transcript. It has been reported that the cap site for Ad E4 mRNAs (5'-TTTTTA-3') is located between 324-329 bp, whereas the poly(A) addition site follows the second A in the sequence 5'-CATAA-3' at position 3136. The only potential cap site (TTTTA) in the putative E4 sequence of BAV2 is located at position 309 (30 bp upstream from the first ATG of ORF1). A possible poly(A) site (ATAAAAA) is located at position 3104 (288 bp downstream from the stop codon of ORF6).

ORF1 encodes a 114 amino acid long protein which has 28.9% identity and 54.4% homology with the E4 14kDa protein encoded by ORF1 of Ad2 (Figure 31A). The location and size of this ORF is remarkably similiar to the first ORF of the E4 region of Ad2 (Virtanen *et al.,* 1984) suggesting that this ORF is the first exon of the E4 transcription unit. Therefore, the only potential TATA box (TAATA) is located at 256 bp, which is 83 bp upstream from the first ATG in ORF1 (position 339 bp). The 78 amino acid long protein encoded by ORF2 shares 11.3% identity and 40.2% homology (Figure 31B) to the known Ad2 E4 13kDa protein of ORF3 (Virtanen *et al.,* 1984). Although this ORF appears in a similiar position, the protein encoded is slightly smaller than the 13kDa which may be accounted for by the fact that BAV2 is slightly smaller than Ad2.

ORF3 has the potential to encode a 128 amino acid long polypeptide which shares 11.7% identity and 59% homology with the known Ad2 E4 34kDa protein (Virtanen *et al.*,1984). The 96 amino acid long polypeptide encoded by ORF4 has 13.5% identity and 61% homology to the E4 34kDa

(A)



(B)

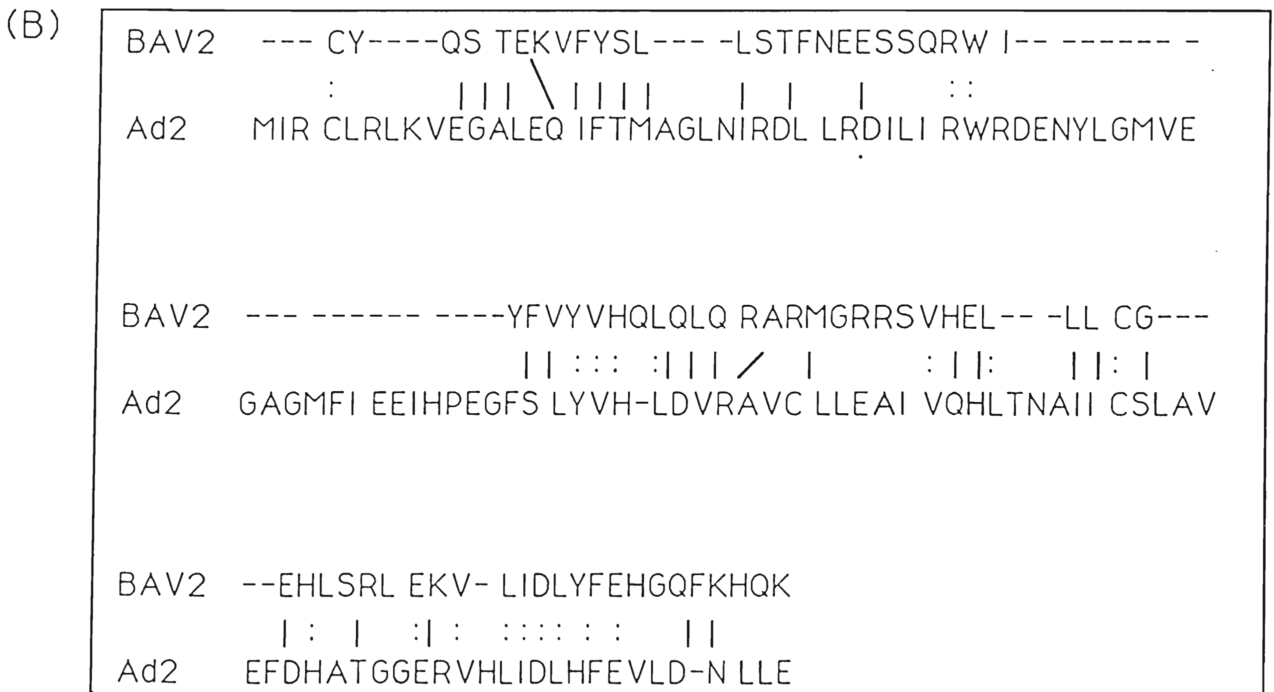


Figure 31: Alignment of the amino acid sequence predicted from the BAV2 ORF1 and the known E4 14kDa (ORF1) protein of Ad2 (A), and ORF2 with the E4 13kDa (ORF3) protein of Ad2 (B).

protein of Ad12. ORFs 5 and 6 have the potential to encode for 31 and 57 amino acid long polypeptides, respectively. The 31 amino acid long polypeptide has 24.8% identity and 70% homology to the E4 34kDa protein of Ad2, whereas the 57 amino acid long polypeptide encoded by ORF6 shares 19.4% identity and 67% homology to the E4 34kDa protein of Ad2 and 16.5% identity and 50% homology to the E4 34kDa protein of Ad12.

ORFs 3-6 encode polypeptides which all share homology to known E4 34kDa protein. In addition to this, the nucleotide sequence of these four ORFs (1861-2818bp) share 54.9% identity with the nucleotide sequence of ORF6 (34kDa protein) of Ad2 (data not shown). The first ATG of ORF3 is found in a corresponding region (1861bp) as the first ATG of ORF6 of Ad2. However, the ORF6 of Ad2 appears as one continuous ORF (Virtanen *et al.,*1984). In contrast, there are 4 non-continuous and partially overlapping ORFs present in this sequence which share homology to the E4 34kDa protein of Ad2 (Figure 30).

CHAPTER 5: DISCUSSION

The ultimate goal of our research group is to engineer a BAV2 gene transfer and expression vector analogous to the human Ad based vectors. Although human Ads have proven to be very effective as gene transfer and expression vectors in cell culture and various animal models (Haj-Ahmad and Graham, 1986; Rosenfeld, 1991; Jaffe *et al.,* 1992; Lemarchand *et al.,* 1993; Engelhardt *et al.,* 1993; Rich *et al.,* 1993; Li *et al.,* 1993; Bout *et al.,* 1994; Brody *et al.,* 1994), immunological memory poses a limitation on the use of these vectors in humans. A BAV2 based vector could overcome this limitation.

The objectives of this study were to clone and reconstitute a BAV2 E3 deletion mutant and to sequence the neighboring E4 region. The need for such work is, at least, fourfold. First, there are only two restriction enzyme maps (Belak *et al.,* 1986 and Salmon *et al.,* 1993) available for BAV2 for a limited number of enzymes. The sequence and restriction enzyme map for the E4 region would aid in designing strategies for molecular manipulation of the viral genome. Second, the availability of well characterized recombinant plasmids offer an inexpensive and inexhaustible source of DNA. Third, it is easier to carry out molecular manipulation, such as the deletion of the E3 region, on smaller plasmids rather than on an intact viral genome. Finally, a BAV2 E3 deletion mutant could be used as a recombinant vaccine analogous to the systems

established for human Ads (Johnson *et al.*, 1988, Morin *et al.*, 1987, Dewar *et al.*, 1989, Schneider *et al.*, 1989, Prevec *et al.*, 1990, Collins *et al.*, 1990, and Hsu *et al.*, 1991). A vaccine of this type could offer several advantages: it would be inexpensive, easy to administer and ought to provide long lasting immunity.

The E3 region was deleted for three reasons: One, the E3 region is not required for the *in vitro* and *in vivo* growth of adenoviruses (Morin *et al.*, 1987; Ginsberg *et al.*, 1989), however its gene products are responsible for counteracting host immunosurveillance. This has implications for the development of BAV2 as a viral vector. The maximum amount of DNA that can be packaged in the virions is approximately 105% of the wildtype genome (Ghosh-Choudhury *et al.*, 1986). Recombinant Ads with genomes exceeding 105% undergo rapid rearrangement of the genome to lose the insert (Bett *et al.*, 1993). To incorporate larger DNA segments, it is therefore necessary to delete appropriate areas of the genome. The 2.3Kbp putative E3 region in BAV2 is larger than the E3 region in any other non-human Ad studied (Esford and Haj-Ahmad, 1994). Therefore, if this region is deleted in BAV2, the capacity of a DNA insert would increase to 4.5Kbp. Secondly, the location and sequence of the E3 region of BAV2 was available (Esford and Haj-Ahmad, 1994), making the molecular manipulation of this area feasible. Finally, human Ads containing an E3 deletion and a cloned foreign gene have been shown to raise neutralizing antibodies in a variety of animal species and protect them against subsequent challenge with the antigen (Johnson *et al.*, 1988; Morin *et al.*, 1987; Dewar *et al.*, 1989; Schneider *et al.*, 1989; Prevec *et al.*, 1990; Collins *et al.*, 1990; Hsu *et al.*, 1991). An analogous system for BAV2 could also be used as a recombinant vaccine.

During this study, the subcloning of pEA48 resulted in the construction of two recombinant plasmids with inserts spanning 65.2% to 90.5% and 77.1% to 90.5% of the the viral genome. The plasmid pPE1 has unique *Pst*I and *Sca*I restriction enzyme sites which flank the E3 region, making the deletion of this region feasible. The precise deletion was confirmed by nucleotide sequencing of the multiple cloning site-BAV2 junction. The determined nucleotide sequence of this junction shows that the deletion was precisely made between *Pst*I at 77.1 map units and *Sca*I at 82.5 map units (removing a total of 1800bp), and didn't delete the E3 promoter sequences or extend into the important sequences encoding structural proteins which exist on either side of the E3 region (Esford and Haj-Ahmad, 1994). Subsequent to this, five recombinant plasmids of various sizes carrying a deletion in the E3 region were constructed spanning from 29.5 to 90.5 map units or 61% of the entire genome. Of these 5 recombinant plasmids, two have a reporter gene (tk) cloned into the unique *Bam*HI site at the MCS. The tk gene is used by cells in the salvage pathway of DNA synthesis and catalyses the formation of thymidine deoxyribose monophosphate (dTMP) from thymidine deoxyribose (TdR) (Cheng and Prusoff, 1974). The insertion of the tk gene served two purposes: One, the presence of tk sequences would enable the differentiation between a recombinant BAV2 vector and wtBAV2 using hybridization techniques with a tk labelled probe. Secondly, future experiments involving transient expression assays could be done to assess both tk expression and E3 promoter strength, both of which have been done before using human adenoviral systems (Haj-Ahmad and Graham, 1986; Nagpal and Ostrove, 1991).

During this investigation, the recombinant plasmid pdlE3-4tk1, spanning from 29.5 to 90.5 map units of the BAV2 viral genome with the tk gene cloned in the deleted E3 region was constructed. This plasmid was then used in the attempt to rescue a BAV2 E3 deletion mutant. First, MDBK cells were infected with wtBAV2, followed by transfection with banded pdlE3-4tk1, using the $CaPO_4$ precipitation method (Graham and van der Eb, 1973). Theoretically, a recombinant BAV2 vector would result from homologous recombination between the wtBAV2 genome and the recombinant plasmid. Hybridization results using Hirts extracted pooled viral DNA, digested with *Bam*HI and *Bam*HI+*Eco*RI and probed with ^{32}P labelled tk173 show the expected 2Kbp band which co-migrated with the tk gene (See Figure 27). To further investigate this observation, 70 plaque purified virus were examined using restriction enzyme analysis and hybridization techniques. The *Bam*HI digested DNA of 4 plaque-purified virus showed three bands when hybridized to ^{32}P labelled tk173 and BAV2 DNA. Of the two larger bands observed, one was approximately 20Kbp, of which an accurate size could not be determined and the other band observed was approximately 5.8Kbp. The size and position of these two larger bands correspond closely to the banding pattern of *Bam*HI digested BAV2 DNA: 26Kbp and 5.8Kbp (Salmon *et al.,* 1993). Finally, the third band migrated slightly higher than the tk gene (2Kbp). There are two possible explanations for the tk fragment appearing larger than expected. One, is recombinantion events occurring *in vivo* and the second is gel shifting. Nonetheless, these hybridization results indicate the presence of tk sequences and a BAV2 E3 deletion mutant, however attempts to purify this mutant have been unsuccessful.

Overall, 9 plasmids were constructed during this study. These

recombinant plasmids not only offer an inexhaustible and inexpensive source of DNA, they can be used as templates for future manipulation of the BAV2 genome and the construction of larger plasmids. The molecular manipulation of recombinant plasmids is much more practical than an intact viral genome. Two plasmids of particular interest are pdlE1-E and pdlE3-5 which span from 0 to 42.5 map units and 65.2 to 100 map units of the BAV2 genome, and contain a deletion within the E1 and E3 region, respectively. These two plasmids could serve two purposes: one, they could be used individually in future experiments to co-infect a permissive cell line with BAV2 DNA to yield a recombinant BAV2 vector through homologous recombination, as described for human adenoviral systems (Graham and Prevec, 1991; 1992). However, the propagation of a BAV2 E1 deletion mutant would require the establishment of a cell line which could constitutively express the E1 genes of BAV2 because the gene products of this region are required for viral growth. Secondly, pdlE3-5 and pdlE1-E could be used as templates for the construction of larger plasmids

An essential step for deletion studies involving the BAV2 genome for the purpose of engineering a BAV2 viral vector is the sequencing and analysis of the early region genes. The E1 and E3 regions of BAV2 have been sequenced and analyzed (Esford and Haj-Ahmad, 1994; Salmon and Haj-Ahmad, 1994). However the E4 region of BAV2 has not been studied. The sequence and restriction enzyme map of this region would be a valuable tool for future studies involving BAV2 E4 deletions. Another reason for sequencing and analyzing the E4 region was to investigate the slow growth rate of BAV2 observed in MDBK cell line. Studies investigating the function of the E4 gene products of Ad2 and Ad5 show that mutants with a large deletion within the E4 region of their genome

display profound defects in the accumulation of late mRNAs both in the nucleus and the cytoplasm of infected cells, inefficient shutoff of host cell protein synthesis and reduced levels of viral DNA unless propagated in W162 cells which provide the missing functions of the E4 region *in trans* (Weinberg and Ketner, 1986; Sandler and Ketner, 1989). More specifically, the gene products encoded by ORF6 (34kDa) and ORF3 (13kDa) have redundant effects on viral infection. Both gene products augment viral DNA replication, viral late protein synthesis, the shutoff of host cell protein synthesis, and the production of infectious virus (Bridge and Ketner, 1989; Huang and Hearing, 1989). However, the product of ORF6 (34kDa) is more efficient in the regulation of these processes than the product of ORF3 (13kDa).

The nucleotide sequence of the extreme right end (90.5 to 100 map units) of the BAV2 genome containing the putative E4 region and the right ITR contains some conserved adenovirus landmarks such as the origin of replication (position 1-17) and the nuclear factor I binding site (position 29-41). The right ITR sequence of BAV2 has 100% identity with the left ITR sequence (Salmon and Haj-Ahmad, 1994). Six ORFs encode polypeptides that have homology to the well characterized E4 proteins of human Ads..

In Ad2, E4 mRNAs are produced through a series of differential splicing of a primary transcript. It has been reported that the cap site for E4 mRNAs (5'-TTTTTA-3') is located between 324-329 bp (Virtanen *et al.,* 1984), whereas the poly(A) addition site follows the second A in the sequence 5'-CATAA-3' at position 3136 (Virtanen *et al.,* 1984). The only potential cap site (TTTTA) in the putative E4 sequence of BAV2 is located at position 309 (30 bp upstream from the first ATG of ORF1). A possible

poly(A) site (ATAAAAA) is located at position 3104 (288 bp downstream from the stop codon for ORF6).

ORF1 encodes a 114 amino acid long protein which has strong homology with the E4 14kDa protein encoded by ORF1 of Ad2. The location and size of this ORF is remarkably similiar to the first ORF of the E4 region of Ad2 (Virtanen *et al.*, 1984) suggesting that this ORF is the first exon of the E4 transcription unit. The function of the 14kDa protein is not known.

The 78 amino acid long protein encoded by ORF2 shares homology to the known Ad2 E4 13kDa protein of ORF3 (Virtanen *et al.*, 1984) Although this ORF appears in a similar position, the protein encoded is slightly smaller than the 13kDa which may be accounted for by the fact that BAV2 is slightly smaller than Ad2. Studies involving Ad2 and Ad5 mutants with deletions in both ORF3 and ORF6 (34kDa) were found to be severely defective for growth. However, mutants with a deletion only in ORF6 were partially defective for growth in HeLa cells suffering a 100 fold reduction in final virus yield when compared to wild-type virus. This suggested that the ORF3 gene product could compensate for the loss of the ORF6 gene product suffering only a moderately defective phenotype.

ORFs 3-6 encode polypeptides which share homology to known E4 34kDa protein. In addition to this, over half of the nucleotide sequence of these four ORFs is identitical to that of ORF6 (34kDa protein) of Ad2. The first ATG of ORF3 is found in a corresponding region (1861bp) to the first ATG of ORF6 of Ad2. However, the ORF6 of Ad2 appears as one continuous ORF (Virtanen *et al.*, 1984). In contrast, there are 4 non-continuous and partially overlapping ORFs present in this sequence which share homology to the E4 34kDa protein of Ad2. There are three possible explanations for

this observation. First, this may be simply due to error in the sequencing. However, repeated sequencing and analysis gave the same results. Second, it is reasonable to assume that the E4 34kDa protein in BAV2 is truncated. If a fully functional 34kDa protein is not being produced then other gene products of the E4 region could be functionally compensating for this loss, resulting in a moderately defective phenotype as observed in human Ad E4 deletion mutants (Huang and Hearing, 1989). This could account for the slow growth rate observed for BAV2 in MDBK cells. However, further analysis involving the E4 gene products would be required in order to resolve this uncertainty. A third explanation could be complex splicing, which appears unlikely because the ORFs are partially overlapping. Future studies designed to map the E4 mRNAs of BAV2 will need to be completed. These will include Northern blot analysis, cDNA sequencing, and nuclease protection and primer extension assays. Other future experiments involving SDS-PAGE analysis or ELISA tests may be conducted to determine whether or not the 34kDa protein is present.

SUMMARY

Based on the results obtained throughout this project the following conclusions can be made:

(1) Several plasmids were constructed when subcloning the BAV2 genome in pUC19: pSE1 and pPE1

(2) A deletion of 1800 bp was made within the E3 region of BAV2 and a linker containing a multiple cloning site was subsequently inserted.

(3) The BAV2 genome was reconstructed to contain from 29.5%-90.5% in pUC19 in a plasmid pdlE3-4.

(4) The thymidine kinase gene along with its endogenous promoter was inserted in the E3 region in both orientations.

(5) Southern Blotting and subsequent Hybridization with tk173 reveal that tk sequences are present within the viral DNA from purified plaque. This suggests the presence of a recombinant BAV2 vector carrying the tk gene in place of the E3 region.

(6) The DNA sequence of the putative E4 region of BAV2 has been determined and the amino acid sequences of the 6 ORFs were compared to the amino acid sequences of proteins encoded by the E4 region of Ad 2.

-ORF1 was found to share 28.9% identity and 54.4% homology to the E4 14kDa protein of Ad2
-ORF2 was found to share 11.3% identity and 40.2% homology to the 13KDa protein encoded by the E4 region of Ad2
-ORFs3-6 were found to share 11.7-24.8% identity and 59-70% similiarity to the 34KDa protein encoded by the E4 regions of Ad2 and Ad12.

REFERENCES

Adami, G.R. and L.E. Babiss. 1990. The efficiency of adenovirus transformation of rodent cells is inversely related to the rate of viral E1A gene expression. Journal of Virology **64(7)**: 3427-3436.

Ausubel, F.M., B. Roger, R. E. Kingston, D.D. Moore, J.G. Serdman, J.A. Smith and K. Struhl (eds).1992. Analysis of DNA sequences by blotting and hybridization: Short protocols in molecular biology. 2nd ed. Greene Publishing Association and John Wiley and Sons, USA.

Baber, D.J. and J. B. Condy. 1981. Isolation and characterization of bovine adenoviruses types 3, 4, and 8 from free-living African buffaloes. Res. Vet. Sci. **31**:69-75.

Bajocchi, G., S.H. Geldman, R.G. Crystal, and A. Mastrangeli. 1993. Direct *In vivo* gene transfer to ependymal cells in the central nervous system using recombinant adenovirus vectors. Nature Genetics **3**: 229-234.

Bartha, A. 1969. Proposal for subgrouping of bovine adenoviruses. Acta.Vet. **19**: 319-321.

Belak, S., G. Berencsi, M. Rusvay, K Lukacs, and I. Nasz. 1983. DNA structure, and hemagglutination properties of bovine adenovirus type 194.

Belak, S. and V., Palfi. 1974a. An adenovirus isolated from sheep and its relationship to type 2 bovine adenovirus. Arch. ges. Virusforsch. **46**:366-369.

Belak, S. and V. Palfi. 1974b. Pneumonenteritis of lambs caused by adenovirus. Acta Vet. Hung. **24**:327-328.

Belak, S., A. Virtanen, J. Zabielski, M. Rusvai, G. Berencsi, and U. Pettersson. 1986. Subtypes of bovine adenovirus type 2 exhibit major differences in region E3. Virology **153**:262-271.

Berget, S.M., C. Moore and P.A. Sharp. 1977. Spliced segments at the 5' terminus of adenovirus 2 mRNA. Proc. Natl. Acad. Sci. USA. **74**: 3171-3175.

Berk, A.J. 1986. Adenovirus promoters and E1A transactivation. Ann. Rev. Genet. **20:** 45-79

Bett, A.J., L. Prevec, and F.L. Graham. 1993. Packaging capacity and stability of human adenovirus type 5 vectors. Journal of Virology **67(10)**:5911-5921.

Beutler, B. and A. Cerami. 1989. The biology of cachectin/TNF-a primary mediator of the host response. Annu. Rev. Immunol. **7**:625-711

Boros, B., Z. Graf, M. Benko and A. Bartha. 1985. Isolation of bovine adenovirus from fallow deer (*Dama dama*). Acta Vet. Hung. **33**: 119-123.

Bos, J.L., L.J. Polder, R. Bernards, P.I. Schrier, P.J. van den Elsen, A.J. van der Eb, and H. van Ormondt. 1981 The 2.2 kb E1b mRNA of human Ad12 and Ad5 codes for two tumor antigens starting at different AUG triplets. Cell. **27:** 121-131.

Bout, A., M. Perricaudet, G. Baskin, J. Imler, B.J. Scholte, A. Pavirani, and D. Valerio. 1994. Lung gene therapy: *In vivo* adenovirus-mediated gene transfer to rhesus monkey airway epithelium. Human Gene Therapy **5**:3-10.

Brody, S.L., H.A. Jaffe, S.K. Han, R.P. Wersto, and R.G. Crystal. 1994. Direct *In vivo* gene transfer and expression in malignant cells using adenovirus vectors. Human Gene Therapy **5**: 437-447.

Burgert, H. and S. Kvist. 1985. An adenovirus type 2 glycoprotein blocks cell surface expression of human histocompatibility class I antigens. Cell **41**:987-997.

Chanda, P., R. Natuk, B. Mason, R. Bhat, L. Greenberg, S. Dheer, M. Morin, K. Molnar-Kimber, S. Mizutani, S. Lubeck, A. Davis and P. Humg. 1990. High level expression of the envelope glycoproteins of the human immunodeficiency virus type 1 in presence of *rev* gene using helper-independent adenovirus type 7 recombinants. Virology **165**: 535-547.

Cheng, S., Lee, S., Ronchett-blume, M., Hum, W., Politis-Virk K., Mizutani, S., Davis, A., Hung, P.P., Hirsch, V., Chanock, R.M., Purcell, R.H. and Hohnson, P.R. 1991. Robust expression of the SIV envelope protein by a recombinant Human Adenovirus Host-range mutant. Vaccines **91**: 145-147.

Cheng, Y., and W. Prusoff. 1974. Mouse Ascites sarcoma 18-Deoxythymidine Kinase. General Properties an Inhibition Studies. Biochemistry. **13(6)**: 1179-1185.

Chengalvals, M.V., B.M. Bhat, R. Bhat, M.D. Lubeck, S. Mizutani, A.R. Davis and P.P. Humg. 1994. Immunogenicity of high expression adenovirus-hepatitis B virus recombinant vaccines in dogs.Journal of General Virology **75**: 125-131.]

Chow, L.T., and T.R. Broker. The elucidation of RNA splicing in the adenoviral system. In: Johnson, L.E., Kimball, P.C., Perlman,P.S., eds. Gene Structure and Expression. Columbus, Ohio: Ohiostate University Press, 1980: 175-209.

Chow, L.T., T.R. Broker, and J.B. Lewis. 1979. Coomplex splicing patterns of RNAs from early regions of adenovirus. J. Mol. Biol. **134:** 265-303.

Chow,L.T., R.E. Gelinas, T.R. Broker and R.J. Roberts. 1977. An amazing sequence arrangement at the 5' ends of Ad2 messenger RNA. Cell **12**: 1-8.

Cladaras, C. and W.S.M. Wold. 1985. DNA sequence of the E3 transcription unit of adenovirus 5.Virology **140**: 28-43.

Cutt, J. R., T. Shenk, and Pl Hearing. 1987. Analysis of adenovirus early region 4-encoded polypeptides synthesized in productively infected cells. J. Virol. **61**: 543-552.

Davis, H.L., B.A. Demeneix, B.Quantin, J. Coulombe, and R.G. Whalen. 1993. Plasmid DNA is superior to viral vectors for direct gene transfer into adult mouse skeletal muscle. Human Gene Therapy **4**: 733-740.

Dewer, R.L., V. Natarajan, M.B. Vasudevachari, N.P. Salzman. 1989. Synthesis and processing of Human Immunodeficiency type-1 envelope proteins encoded by a recombinant human adenovirus. J. Virol. **71** : 129-136.

Dix, I. and D.N. Leppard. 1992. Open reading frames 1 and 2 of adenovirus region E4 are conserved between human serotypes 2 and 5.Journal of General Virology **73**: 2975-2976.

Doefler,W. 1991. Abortive infection and malignant transformation by adenoviruses: integration of viral DNA and control of viral gene expression by specific patterns of DNA methylation. Advances in Virus Research **39** :89-128.

Doefler, W. and P. Bohm (eds). 1992. The malignant transformation by DNA viruses: Molecular Mechanisms. Weinheim. Verlag, Chemie.

Doefler, W. 1994. Adenoviruses: Molecular Biology. *In*: Encyclopedia of Virology. eds. R.G. Webster and A. Granoff. Vol. 1. Academic Press, pp. 8-14.

Elgadi, M. and Y. Haj-Ahmad. 1992. Molecular cloning and restriction enzyme analysis of bovine adenovirus type 3. Intervirology **34**:124-132.

Elgadi, M., N. Rghei, and Y. Haj-Ahmad. 1993. Sequence and sequence analysis of E1 and pIX regions of the BAV3 genome. Intervirology **36**:113-120.

Engelhardt, J.F., Y. Yang, L.D. Stratford-Perricaudet, E.D. Allen K. Kozarsky, M. Perricaudet, J.R. Yankaskas, and J.M. Wilson. 1993. Direct gene transfer of human CFTR into human bronchial epithelia of xenografts with E1-deleted adenoviruses. Nature Genetics **4**: 27-34.

Erzurum, S.C., P. Lemarchand, M.A. Rosenfeld, J. Yoo, and R. G. Crystal. 1993. Protection of human endothelial cells from oxidant injury by adenovirus-mediated transfer of the human catalase cDNA. Nucleic Acids Research **21(7)**:1607-1612.

Esford, L. and Y. Haj-Ahmad. 1994. Sequence analysis of the putative E3 region of bovine adenovirus type 2. Intervirology **37**:277-286.

Fisher, K.J. and J.M. Wilson. 1994. Biochemical and functional analysis of an adenovirus-based ligand complex for gene transfer. Biochem. J. **299**: 49-58.

Flomenberg, P.R., M. Chen and M.S. Horwitz. 1988. Sequence and genetic organization of adenovirus type 35 early region 3. J. Virol. **62** : 4431-4437.

Gallimore. P.H. P.A Sharp and J. Sambrook 1974. Viral DNA in transformed cells.II A study of the sequences of adenovirus 2 DNA in nine lines of transformed rat cells using specific fragments of the viral genome. J. Mol. Biol.**89**: 49-72.

Gaynor, R.B., A. Tsukamoto, C. Montell and A.J. Berk. 1982. Enhanced expression of adenovirus transforming proteins.J. Virol. **44:** 276-285.

Ghosh-Choudhury, G., Y. Haj-Ahmad, and F.L. Graham. 1986. Protein IX, a minor component of the human adenovirus capsid is essential for the paclaging of full length gemomes. EMBO J. **6:** 1733-1739.

Ginsberg, H,. H. Pereira, R. Balentine and W. Wilcox. 1966. A Proposed Terminology for the Adenovirus Antigens and Virion Morphological Subunits. Virol. **28**: 782-783.

Ginsberg, H.S., U. Lundholm-Beauchamp, R.L. Horswood, B. Pernis, W.S.M. Wold, R.M. Chanock, and G.A. Prince. 1989. Role of early region 3 (E3) in pathogenesis of adenovirus disease. Proc. Natl. Acad. Sci. U.S.A. **86:** 3823-3827.

Gooding, L.R., L.W. Elmore, A.E. Tollefson, H.A. Brady and W.S.M. Wold. 1988. A 14,700 MW protein from the E3 region of adenovirus inhibits cytolysis by tumor necrosis factor. Cell **53**: 341-346.

Gooding, L.R., L. Aquino, P.J. Duerksen-Hughes, D. Day, T. H. Horton, S. Yei, and W.S.M. Wold. 1991a. The E1B 19,000-molecular-weight protein of gropu C adenoviruses prevents tumor necrosis factor cytolysis of human cells but not mouse cells. J. Virol. **65(6):** 3083-3094.

Gooding, L.R., T.S. Ranheim, A.E. Tollefson, L. Aquino, P. Duerksen-Hughs, T. M. Horton, and W.S.M. Wold. 1991b. The 10,400-and 14,500-dalton proteins encoded by region E3 of adenovirus function together to protect many but not all moude cell lines against lysis by tumor necrosis factor. J. Virol. **65(8):** 4114-4123.

Graham, F., and A. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA.Virol.**52**: 456-467.

Graham, F.L., J. Smiley, W.C. Russell and R. Nadfirn. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen.Virol. **36**: 59-72.

Graham, F.L., P.J. Abrams, C. Mulder, H.L. Heyreck, S.O. Warnaar, F.A.J.de Vries, W. Fiers and A.J.van der Eb. 1974. Studies on *in vitro* transformation by DNA and DNA fragments of human Adenovirus and simian virus 40. Cold Spring Harbor Symp. Quant. Biol. **39**: 637-650.

Graham, F.L. and L. Prevec. 1991. Manipulation of adenovirus vectors. In: Methods in Molecular Biology. The Humana Press Inc. 109-124.

Graham, F.L. and L. Prevec. 1992. Adenovirus-based expression vectors and recombinant vaccines. pp 363-390.

Grand, R.J.A. 1987. The structure and functions of the adenovirus early region 1 proteins. Biochem. J.**241**: 25-38.

Green, M., and G. E. Daesch. 1961. Biochemical studies on adenovirus multiplication. I. Kinetics of nucleic acid and protein synthesis in suspension cultures. Virology **13**: 169-176.

Green, M. J.K. Mackey, W.S.M. Wold and P. Rigden. 1979. Virology **93**: 481.

Haj-Ahmad, Y. and F.L. Graham. 1986. Development of a helper-independent human adenovirus vector and its use in the transfer of the herpes simplex virus thymidine kinase gene. Journal of Virology **57(1)**: 267-274.

Halbert, D.N., J.R. Cutt and T. Shenk. 1985. Adenovirus Early Region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. Journal of Virology**56(1)**: 250-257.

Hierholzer, J. C. 1973. Further subgrouping of the human adenoviruses by differential hemagluttination. J. Infect. Dis. **128**:541-550.

Herisse, J., and F. Galibert. 1981. Nuceotide sequence of the *Eco*RI fragment of adenovirus 2 genome. Nucleic Acid Res. **9**: 1229-1240.

Herisse, J., G, Courtois and F, Galibert. 1980. Nucleotide sequence of the *Eco*RI D fragment of adenovirus 2 genome. Nucleic Acids Res.**8**: 2173-2192.

Hong, J.S., K.G. Mullis and J.A. Engler. (1988). Characterization of the early region 3 and fiber- genes of Ad7. Virology **167**: 545-553.

Horwitz, N.S. (1990) in Virology, Second Edition. Fields, B.N., and D.M. Knipe, eds. Raven Press, New York. 1679-1721.

Hsu,K., M.D. Lubeck, A.R. Davis, R.A. Bhat, B.H. Selling, B.M. Bhat, S. Mizutani and P.P. Hung.1991. Immunogenecity and protective efficacy of adenovirus vectored respiratory syncytial virus vaccine. In: Modern approaches to new vqccines including prevention of Aids. The Cold Spring Harbor Laboratory Press. 293-297.

Hu. S., W.W. Hays and D.E. Potts. 1984. Sequence homology between bovine and human adenoviruses. Journal of Virol. **49(2)**: 604-608.

Huang, M-M., and P. Hearing. 1989a. Adenovirus early region 4 encodes two gene products with redundant effects in lytic infection. J. Virol. **63**: 2605-2615.

Huang, M-M., and P. Hearing. 1989b. The adenovirus early region 4 open reading frame 6/7 protein regulates the DNA binding activity of the cellular transcription factor, E2F through a direct complex. Genes Dev. **3**: 1699-1710.

Ishibasi, M. and H. Yasue. 1984. Adenoviruses of animals. In Adenoviruses, (H.S. Ginsberg ed.) Plenum Press, New York.

Jaffe, H.A., C. Danel, G. Longenecker, M. Metzger, Y. Setoguchi, M.A. Rosenfeld, T.W. Gant, S. S. Thorgeirsson, L. D. Stratford-Perricaudet, M. Perricaudet, A. Pavirani, J.P. Lecocq and R.G. Crystal. 1992. Adenovirus-mediated *in vivo* gene transfer and expression in normal rat liver. Nature Genetics **1**:372-378.

Johnson, D.C., Chosh-Choudhury, J.R. Smiley, L. Fallis, F.L. Graham and L. Prevec. 1988. Abundant expression of herpes simplex virus glycoprotein in adenovirus vector Virology **164**:1-14.

Joklik, Wolfgang. 1988. Adenoviruses and Adenovirus-associated Viruses, In Virology. 3rd ed., Appleton & Lange, Norwalk, CT.

Jones, N., and T. Shenk. 1979. Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. Cell. **17:** 683-689.

Joyce ,C. 1992. Pioneers push back the limits of gene therapy. New Scientist. **131**: 13.

Kitchingman, G. R. 1994. Malignant Transformation and Oncology. *In*: Encyclopedia of Virology. eds. R. G. Webster and A. Granoff. Vol 1. Academic Press.

Klein,M., E. Early, and J. Zellat. 1959. Isolation from cattle of a virus related to human adenovirus. Proc. Soc. Exp. Bio. Med. **102(1)**:1-4.

Klein, M., J.Zellat and T.C. Michailson. 1960. A new bovine adenovirus related to human adenovirus. Proc. Soc. Exp. Bio. Med. **165:** 340-342.

Kovesdi, I, R. Reichel, and J. Nevins. 1986. Identification of a cellular transcription factor involved in E1A trans-activation . Cell. **45**:219-228.

Kvist, S., L. Ostberg, H. Persson, L. Philipson and P.A. Peterson. 1978. Molecular association between transplantation antigens and cell surface antigen in adenovirus-transformed cell line. Proc. Matl. Acad. Sci. USA **75**: 5674-5678.

Le Gal La Salle, G., J.J. Robert, S. Berrard, V. Ridoux, L.D. Stratford-Perricaudet, M. Perricaudet, and J. Mallet. 1993. An adenovirus vector for gene transfer into neurons and glia in the brain. Science **259**: 988-990.

Lemarchand, P., M. Jones, E. Yamada, and R.G. Crystal. 1993. *In vivo* gene transfer and expression in normal uninjured blood vessels using replicaiton-deficient recombinant adenovirus vectors. Circulation Research **72(5)**: 1132-1138.

Li,Q.,M.A. Kay, M. Finegold, L.D. Stratford-Perricaudet, and S.L.C. Woo. 1993. Assessment of recombinant adenoviral vectors for hepatic gene therapy. Human Gene Therapy **4**: 403-409.

Loyter, A., G.A. Scangos and F.H. Ruddle. 1982. Mechanisms of DNA uptake by mammalian cells: Fate of exogenously added DNA monitored by the use of fluorescent dyes. Proc. Natl. Acad. Sci.USA.**79**: 422-426.

Marton, M.J., S.B. Baim, D.A. Ornelles, and T. Shenk. 1990. The adenovirus E4 17-kilodalton protein complexes with the cellular transcription factor E2F, altering its DNA-binding properties and stimulating E1A-independent accumulation of E2 mRna. J. Virol. **64:** 5657-5662.

Mason, B., A. Davis, B. Bhat, M. Chengalvala, M. Lubeck, G. Zandle, B. Kostek, S. Cholodofsky, S. Dheer, K. Molnar-Kimber, S. Mizutani, and P. Hung. 1990. Adenovirus vaccine vectors expressing hepatitis B surfaace antigen: importance of regulatory elements in the adenovirus major late intron. Virology. **177**:452-461.

Mattson, D.E. 1973. Adenovirus infection in cattle. JAVMA **163**:894-896.

McGlade, C., M. Tremblay, S. Yee, R. Ross, and P.Branton. 1987. Acylation of the 176R (19-kilodalton) early region 1B protein of human adenovirus type 5. Journal of Virology. **61**: 3227-3234.

Mohanty, S.B. 1971. Comparative study of bovine adenoviruses. Am. J. Vet. Res. **32(12)**: 1899-1905.

Moran, E., and M.B. Mathews. 1987. Multiple functional domains in the adenovirus E1A gene. Cell. **48:** 177-178.

Morin, J.E., M.D. Lubeck, J.E. Barton, A. J. Conley, A.R. Davis, and P.P. Hung. 1987. Recombinant adenovirus induces antibody response to hepatitis B virus surface antigen in hamster. Proc. Natl. Acad. Sci. U.S.A. **84:** 4626-4630.

Nagpal, S., and J. Ostrove. 1991. Characterization of a Potent Varicella-Zoster Virus-Encoded *trans* -Repressor. Journal of Virology. **65(10)**:5289-5296.

Nevins, J.R. 1981. Mechanism of activationof early viral transcription by adenovirus E1a gene product. Cell **26**: 213-220.

Nevins, J.R. 1986. Control of cellular and viral transcription during adenovirus infection. Crit. Rev. Biochem. **19**: 307-322.

Nevins, J.R. 1987. Regulation of early adenovirus gene expression. Microbiol. Rev. **51(4):** 419-430

Norrby, E. 1966. The relationship between soluble antigens and the virion of adenovirus type 3. III. Immunologic characteristics. Virology**28**:565-576.

Norrby, E. 1969. The relationship between the soluble antigens and the viron of adnovirus- type 3. IV. Immunological characteristics. Virology. **37:** 565-576

Norrby, E. 1969. The structural and functional diversity of adenovirus capsid components. J. Gen. Virol. **5:** 221-236.

Perricaudet, M., G. Akusjarvi, A. Virtanen and U. Petterson. 1979. Structure of two spliced mRNAs from transforming region of human subgroup C adenoviruses. Nature **281**: 694-696.

Persson, H., H. Jornvall, and J. Zabielski. 1980. Multiple mRNA species for the precursor to an adenovirus-encoded glycoprotein: Identification and structure of the signal sequence . Proc. Natl.Acad. Sci. USA **77**: 6349-6353.

Prevec L., J.B. Campbell, B.S. Christie, L. Belbeck and F. L. Graham. 1990. A recombinant human adenovirus vaccine against rabies. The Journal of Infectious Diseases **161**:27-30.

Prevec,L., M. Schneider, K.L. Rosenthal, L.W. Belbeck, J.B. Derbyshire and F.L Graham. 1989. Use of human adenovirus based vectors for antigen expression in animal J. Gen. Virol. **70**: 429-434.

Ragot, T., N. Vincent, P. Chafey, E. Wigne, H. Gilgenkrantzs, D. Couton, J. Cartaud, P. Briand, J. Kaplan, M. Perricaudet and A. Kahn. 1993. Efficient adenovirus-mediated transfer of a human minidystrophin gene to skeletal muscle of mdx mice. Nature **361**:647-650.

Ricciardi, R., R. Jones, C. Cepko, P. Sharp and B. Roberts. 1981. Expression of early adenovirus genes requires viral encoded acidic polypeptide. Proc. Natl. Acad. Sci. USA. **78**: 6121-6125.

Rich, D.P., L.A. Couture, L.M. Cardoza, V.M. Gioggio, D. Armento, P.C. Espino, K. Hehir, A.F. Smith. 1993. Development and analysis of recombinant adenoviruses for gene therapy for cystic fibrosis. Human Gene Therapy **4(4)**: 461-476.

Rosen, L.1958. Hemaglutination by adenoviruses. Virology **5(3)**: 574-577.

Rosenfeld, M. A., K. Yoshimura. 1992. *In vivo* transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. Cell **68**: 143-155.

Rosenfeld M.A., W. Siegfried, K. Yoshimura, K. Yoneyama, M. Fukayama, L.E. Stier, P.K. Paakko, P. Gilardi, L.D. Stratford-Perricaudet, M. Perricaudet, S. Jallat, A. Pavirani, J. Lecocq, and R.G. Crystal. 1991.Adenovirus-mediated transfer of a recombinant alpha1-antitrypsin gene to the lung epithelium *in vivo*. Science **252**: 431-434.

Rowe, W.P., R.J. Huebne, L.K. Gilmore, R.H. Parrott and T.G. Ward. 1953. Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. Proc. Soc. Exp. Biol. Med. **84**: 570-573.

Salmon, K. and Y. Haj-Ahmad. 1994. Sequence analysis of the bovine adenovirus type 2 early region 1 and pIX gene. Intervirology **37:**298-305.

Salmon, K., L. Esford, and Y. Haj-Ahmad. 1993. Subcloning and restriction enzyme mapping of bovine adenovirus type 2. Intervirology **36(2)**:72-78.

Sandler, A.B. and G. Ketner. 1989. Adenovirus early region 4 is essential for normal stability of late nuclear RNAs. Journal of Virology **63(2)**:624-630.

Sanger, F., S. Nicklen, A.R. Coulson. 1977. DNA sequencing with chain termination inhibitors. Proc. Natl. Acad. Sci. U.S.A. **74:** 5463-5467.

Sarnow,P., P. Hearing, C. W. Anderson, Reich, and A. J. Levine. 1982. Identification and characterization of an immunologically conserved adenovirus early region 11,000 M protein and its association with the nuclear matrix. J. Mol. Biol. **162**: 565-583.

Sarnow, P., P. Hearing, C.W. Anderson, D.N. Halbert, T. Shenk and A.J. Levine. (1984). J. Virol. **49** : 692.

Schneider, M., F.L. Graham, and L. Prevec. 1989. Expression of the glycoprotein of vesicular stomatitis virus by infectious adenovirus vectors. J.Gen. Virol.**70**: 417-427.

Shinagawa, M., I. Yoichi, A. Matsuda, T. Tsukiyama and G. Sato. 1987. Phylogenetic relationships between adenoviruses as inferred from nucleotide sequences of inverted terminal repeats. Gene **55**:85-93.

Sharp, P.A. 1984. *In.*: The Adenoviruses (Ginsberg, H.S. , ed.) pp.173-204., Plenum Press, New York.

Signas , C., G. Akusjarvi, U. Petersson. 1986. Region E3 of human adenoviruses: Differences between the oncogenic adenovirus 3 and the nononcogenic adenovirus 2. Gene **50**: 173-184.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments seperated by gel electrophoresis. J. Mol. Biol. **98**: 503-517.

Tollefson, A.E., P. Krajsci, M.H. Pursley, L.R. Gooding and W.S.M. Wold. 1990b. A 14,500 MW protein is coded by region E3 of group C human adenoviruses. Virology **175**: 19-29.

Tollefson,A.E.,P. Krajsci, S. Yei, C.R. Carlin and W.S.M. Wold. 1990a. A 10,400-molecular-weight membrane protein is coded by region E3 of adenovirus. J. Virol. **64**: 794-801.

Tollefson, A.E. and W.S.M. Wold. 1988. Identification and gene mapping of a 14,700-molecular-weight protein encoded by region E3 of group C adenoviruses. J. Virol. **62**: 33-39.

Tooze, 1981. DNA tumor viruses. The molecular biology of human viruses. Second Edition. (J. Tooze, Ed;) Cold Springs Harbor Labratory Press, Cold Springs Harbor, New York. pp. 383-576.

Trentin, J.J. and T.G. Yabey.1962.The quest for human cancer viruses. Science **137**: 835-849.

van der Eb, A.1973. Intermediates in type 5 adenovirus DNA replication. Virology. **51**:11-23.

Virtanen, A., P. Gilardi, A. Naslund, J.M. LeMoullec, U. Pettersson and M. Perricaudet. 1984. mRNAs from human adenovirus 2 early region 4. Journal of Virology **51**: 822-831.

Wadell,G., A. Allard, M. Evander and Li Quan-gen. 1985 Genetic Variability and Evolution of Adenoviruses. Chemica Scripta **26B**: 2-12.

Wadell, G. 1994. Adenoviruses. *In*: Encyclopedia of Virology. eds. R. G. Webster and A. Granoff. Vol. 1. Academic Press, pp 1-7.

Wang, E.W., M.O. Scott and R.P. Ricciardi. 1988. An adenovirus mRNA which encodes a- 14,700-*M* protein that maps to the last open reading frame in region E3 is expressed during infection. J. Virol. **62**: 1456-1459.

White, E., P. Sabbatini, M. Debbas, W.S.M. Wold, D.I. Kusher. and L.R. Godding. 1992. The 19 kilodalton adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by TNF Mol. Cell. Biol. **12(6):** 2570-2580.

Wilson-Rawls,J., S.K.Saha, P. Krajcsi, A.E. Tollefson, L.R. Gooding and W.S.M. Wold. (1990). A 6700MW membrane protein is encoded by region E3 of adenovirus type 2. Virology **178**: 204-212.

Weinberg, D.H. and Gary Ketner. 1983. A cell line that supports the growth of a defective early region 4 deletion mutant of human adenovirus type 2. Proc. Natl. Acad. Sci. USA **80**:5383-5386.

Weinberg, D.H. and Gary Ketner. 1986. Adenoviral Early Region 4 is required for efficient viral DNA replication and for late gene expression. Journal of Virology.**57(3)**: 833-838.

Wold, W.S.M., C. Cladaras, S.C. Magie and N. Yacoub. 1984. Mapping a new gene that encodes and 11,600-molecular-weight protein in the E3 transcription unit of adenovirus 2. J. Virol. **52** : 307-313.

Wold W.S.M and L.R. Gooding 1990. Molecular mechanisms by which adenovirus counteract antiviral immune defenses. Crit. Rev. Immunol. **10**: 53-71.

Wold, W.S.M and L.R. Gooding. 1991. Region E3 of adenovirus: A cassette of genes involved in host immunosurveillance and virus-cell interactions. Virology.**184**: 1-8.

Yoder, S.S. and S.M. Berget. 1986. Role of adenovirus type 2 early region 4 in the early-to-late swithch during productive infection. J. Virol. **60**:779-781.

Yoshida, K.,L. Venkatesh, M. Kuppuswamy, and G. Chinnadurai. 1987. Adenovirus transforming 19Kd T antigen has an enhancer-dependent trans-activation function and relieves enhancer repression mediated by viral and cellular genes.Genes Dev. **1**: 645-658.

Zabner, J., L.A. Couture, R.J. Gregory, S.M. Graham, A.E. Smith and M.J. Welch. 1993. Adenovirus-mediated gene transfer transiently corrects the chloride transport defect in nasal epithelia of patients with cystic fibrosis. Cell **75**: 207-216.

Zhang, S., S. Mak, and P.E. Branton. 1992. Overexpression of the E1B 55-kilodalton (482R) protein of human adenovirus type 12 appears to permit efficient transformation of primary baby rat kidney cells in the absence of the E2B 19-kilodalton protein. J. Virol. **66(4):** 2302-2309.