Construction of Bovine Adenovirus Type 3 E1 and E3 Substitution Plasmids and the Sequencing Analysis of DNA pol and pTP.

By:

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### Abbreviations

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<th>Description</th>
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</thead>
<tbody>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>Ad</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>BAV</td>
<td>Bovine adenovirus</td>
</tr>
<tr>
<td>BAV3</td>
<td>Bovine adenovirus type 3</td>
</tr>
<tr>
<td>amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Ampicillin resistance</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>ddNTP</td>
<td>Dideoxynucleotide</td>
</tr>
<tr>
<td>DBP</td>
<td>DNA binding protein</td>
</tr>
<tr>
<td>DS</td>
<td>Donor serum</td>
</tr>
<tr>
<td>cpe</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>CR</td>
<td>Conserved region</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>E</td>
<td>Early region</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>E2F</td>
<td>Transcription factor activates early region 2</td>
</tr>
<tr>
<td>gp</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HAd</td>
<td>Human adenovirus</td>
</tr>
<tr>
<td>HS</td>
<td>Hours serum</td>
</tr>
<tr>
<td>kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Kanamycin resistance</td>
</tr>
<tr>
<td>ITR</td>
<td>Inverted terminal repeat</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>MDBK</td>
<td>Madin-Darby bovine kidney</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MLP</td>
<td>Major late promoter</td>
</tr>
<tr>
<td>MLTF</td>
<td>Major late transcription factor</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>mu</td>
<td>Map unit</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>pfu</td>
<td>Plaque forming unit</td>
</tr>
<tr>
<td>pi</td>
<td>Postinfection</td>
</tr>
<tr>
<td>pol</td>
<td>Polymerase</td>
</tr>
<tr>
<td>pRB</td>
<td>Cellular retinoblastoma tumor suppressor</td>
</tr>
<tr>
<td>pTP</td>
<td>Precursor to terminal protein</td>
</tr>
<tr>
<td>p53</td>
<td>Tumor suppressor factor</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>TAF</td>
<td>TBP-associated factor</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-binding protein</td>
</tr>
<tr>
<td>TFIID</td>
<td>Cotranscription factor D II</td>
</tr>
<tr>
<td>tk</td>
<td>Thymidine kinase</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TP</td>
<td>Terminal protein</td>
</tr>
<tr>
<td>VA</td>
<td>Virus associated</td>
</tr>
</tbody>
</table>
Abstract

The relative ease to concentrate and purify adenoviruses, their well characterized mid-sized genome, and the ability to delete non-essential regions from their genome to accommodate foreign gene, made adenoviruses a suitable candidate for the construction of vectors. The use of adenoviral vectors in gene therapy, vaccination, and as a general vector system for expressing foreign genes have been documented for some time.

In this study, the objective was to rescue a BAV3 E1 or E3 recombinant vector carrying the kanamycin resistant gene, a dominant selectable marker with useful applications in studying vectored gene expression in mammalian cells. To accomplish the objective of this study, more information about BAV3 DNA sequences was required in order to make the manipulation of the virus genome accessible. Therefore, sequencing of the BAV3 genome from 11.7% to 30.8% was carried out. Analysis of the determined sequences revealed the primary structure of important viral gene products coded by E2 including BAV3 DNA pol and precursor to terminal protein. Comparative analysis of these proteins with their counterparts from human and non human adenoviruses revealed important insights as to the evolutionary lineage of BAV3.

In order to insert the kanamycin resistance gene in either E1 or E3, it was necessary to delete BAV3 sequences to accommodate the foreign gene so as not to exceed the limit of the packaging capacity of the virus. To construct a recombinant BAV3 in which a foreign gene was inserted in the deleted E1 region, an E1 shuttle vector was constructed. This involved the deletion from the viral sequences a region
between 1.3% to 9% and inserting the kanamycin resistance gene to replace the deletion. The E1 shuttle vector contained the left (0%- 53.9%) segment of the genome and was expected to generate BAV3 recombinants that can be grown and propagated in cells that can complement the missing E1 functions.

To construct a similar shuttle vector for E3 deletion, DNA sequences extending from 78.9% to 82.5% (1281 bp) were deleted from within the E3 region that had been cloned into a plasmid vector. The deleted region corresponds to those that have been shown to be non-essential for viral replication in cell culture. The resulting plasmid was used to construct another recombinant plasmid with BAV3 DNA sequences extending from 37.1% to 100% and with a deletion of E3 sequences that were replaced by kanamycin resistance gene. This shuttle plasmid was used in cotransfections with digested viral DNA in an attempt to rescue a recombinant BAV3 carrying the kanamycin resistance gene to replace the deleted E3.

In spite of repeated attempts of transfection, E1 or E3 recombinant BAV3 were not isolated. It seems that other approaches should be applied to make a final conclusion on BAV3 infectivity.
Chapter 1: Literature Review

1.1 Adenoviruses

1.1.1 Classification and Taxonomy

Adenoviruses (Ads) belong to the Adenoviridae family, which is divided into two genera: the Mastadenovirus genus includes those that infect humans, bovine, equine, simian, canine, and ovine species, and the Aviadenovirus includes all that infect birds (Figure 1-1). The members of each genus share a common cross-reactivity antigen; however, there is no common antigen that is shared by the whole family.

Bovine adenoviruses (BAVs) belong to Mastadenovirus subfamily. There are at least nine known BAV serotypes and are classified into two subgroups on the basis of several criteria including (i) antigenic properties (Adam et al., 1988; Bartha, 1969), (ii) DNA sequence homology with other Ads (Benko et al., 1990: Hu et al., 1984), (iii) replication in different established cell lines (Bartha, 1969), and (iv) restriction endonucleases analysis (Benko et al., 1988). Subgroup I includes serotypes 1, 2, 3, and 9 while subgroup II includes serotypes 4, 5, 6, 7, and 8 (Bartha, 1969). Recently, the existence of BAV10 has been recognized; however, this virus has not been classified (Smyth et al., 1996).
...
Figure 1-1: Classification of adenoviruses.
The best known Ads are those of human origin. Currently, there are 49 different human adenovirus (HAds) serotypes which have been recognized (Hierholzer et al., 1988 and 1991; Schnurr and Dondero, 1993). HAds are further subdivided into six groups (A to F) according to biochemical, immunological, and structural composition. This classification is based on the ability to agglutinate rat erythrocytes (Hierholzer et al, 1980), to produce tumor in animals (Green, 1970), and DNA homology and restriction endonuclease analysis (Wadell et al., 1980). Table 1-1 shows the classification of HAds and some of their properties. For example, group A shares several properties such as oncogenicity in newborn mice, similar hemagglutination pattern and base composition (low G + C content). Human adenovirus types 2 and 5 (HAds 2 and 5), which are the most characterized, belong to group C and are nononcogenic.

1.1.2 Particle Structure

Ads are nonenveloped icosahedral particles approximately 70 to 100 nm in diameter (Figure 1-2). The virus particle is composed of protein and DNA; 13% of the viral total mass is DNA and the other 87% is proteins. Electrophoretic analysis of viral proteins reveals that the virion contains at least 11 structural proteins that are designated by Roman numbers. The virus capsid is composed of at least seven structural proteins which surround the linear double-stranded DNA. The capsid consists of 240 hexons and 12 pentons (Khilko et al., 1990). A fiber protein is projected from each corner of the capsid.
Table 1-1: Classification of human adenoviruses and some of their properties (reproduced from Baum, 1984).

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Hemagglutination groups</th>
<th>Serotype</th>
<th>Oncogenic potential</th>
<th>Percentage of G-C</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tumors in animals</td>
<td>in tissue culture</td>
</tr>
<tr>
<td>A</td>
<td>Little or no agglutination</td>
<td>12, 18, 31</td>
<td>High</td>
<td>+</td>
</tr>
<tr>
<td>B</td>
<td>Complete agglutination of monkey erythrocytes</td>
<td>3, 7, 11, 14, 16, 21, 34, 35</td>
<td>Moderate</td>
<td>+</td>
</tr>
<tr>
<td>C</td>
<td>Partial agglutination of rat erythrocytes</td>
<td>1, 2, 5, 6</td>
<td>Low or non</td>
<td>+</td>
</tr>
<tr>
<td>D</td>
<td>Complete agglutination of rat erythrocytes</td>
<td>8, 9, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-47</td>
<td>Low or non (mammary tumors)</td>
<td>+</td>
</tr>
<tr>
<td>E</td>
<td></td>
<td>4</td>
<td>Low or non</td>
<td>+</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td>40, 41</td>
<td>Unknown</td>
<td></td>
</tr>
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<td>Column 2</td>
<td>Column 3</td>
<td>Column 4</td>
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<td>10</td>
<td>37</td>
<td>38</td>
<td>39</td>
<td>40</td>
</tr>
</tbody>
</table>
**Figure 1-2:** Schematic illustration of an adenovirus particle. Virion polypeptides are indicated by Roman numerals with the exception of TP (reproduced from Stewart et al., 1991).

**Table 1-2:** The table shows the viral polypeptides and their functions (modified from Shenk, 1996).

<table>
<thead>
<tr>
<th>Designation</th>
<th>Protein</th>
<th>Known Functions</th>
<th>MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>hexon</td>
<td>structural</td>
<td>120Kd</td>
</tr>
<tr>
<td>III</td>
<td>penton base</td>
<td>penetration</td>
<td>85Kd</td>
</tr>
<tr>
<td>IIIa</td>
<td>penton base-associated</td>
<td>penetration</td>
<td>66Kd</td>
</tr>
<tr>
<td>IV</td>
<td>fiber</td>
<td>receptor-binding, haemagglutination</td>
<td>62Kd</td>
</tr>
<tr>
<td>V</td>
<td>core protein</td>
<td>histone-like-packaging</td>
<td>48.5Kd</td>
</tr>
<tr>
<td>VI</td>
<td>hexon-associated</td>
<td>stabilization/assembly of particle</td>
<td>24Kd</td>
</tr>
<tr>
<td>VII</td>
<td>core protein</td>
<td>histone-like</td>
<td>18.5Kd</td>
</tr>
<tr>
<td>VIII</td>
<td>hexon-associated</td>
<td>stabilization/assembly of particle</td>
<td>13Kd</td>
</tr>
<tr>
<td>IX</td>
<td>hexon-associated</td>
<td>stabilization/assembly of particle</td>
<td>12Kd</td>
</tr>
<tr>
<td>TP</td>
<td>genome</td>
<td>replication</td>
<td>55Kd</td>
</tr>
</tbody>
</table>
The fiber, which is composed of three molecules of polypeptide IV, is located at the 12 vertices of the icosahedron and has a different length depending on the serotype. For example, bovine adenovirus type 3 (BAV3) fiber is longer than all other fibers due to the presence of 15-residue repeats in the fiber shaft (Ruigrok et al., 1994). The amino terminal (the tail) of the fiber is embedded in the penton base; however, the carboxy terminal (the knob) gives the fiber its terminal bulb, which is responsible for interaction with the host receptor (Van Oostrum and Burnett, 1985). With the exception of Ad40 and Ad41 all HAds encode a single fiber protein. The presence of more than one fiber protein, in case of Ad40 and Ad41, suggests that these viruses might recognize different cellular receptors.

The penton base consists of five molecules of polypeptide III and is surrounded by five hexons. Three molecules of polypeptide II, the most abundant among the viral polypeptides, form the trimeric structure of the hexon capsomer. The hexon capsomer is found associated with four different polypeptides (VI, VIII, IX, and IIIa), which play a role in stabilizing the viral capsid; polypeptide IX stabilizes hexon-hexon contacts within the triangular structure; polypeptide IIIa joins hexon from adjacent facets, and polypeptides VI and IIIa seem to connect the core to the viral capsid (Everitt et al., 1973).

The remaining four viral proteins are located within the core. Proteins V, VII, and μ are directly attached to the viral DNA. The last protein, which is the 55Kd terminal protein (TP), is covalently attached to the 5' end of each viral DNA strand and plays an important role in viral DNA replication (Rekosh et al., 1977).
1.1.3 Genome Organization

The Ad genome is a single, linear, double-stranded DNA molecule of approximately 30,000 to 38,000 base pairs (bp) in size. The viral genome is approximately 11μm in length and is divided into 100 map unit (mu) by convention. The viral DNA molecule is located within a core that consists of two major proteins: polypeptide V and VII. Polypeptide VII is tightly associated with the viral DNA.

The viral DNA contains inverted terminal repeats (ITRs) of 100 to 200 bp in length (depending on the serotype) that are arranged in inverted fashion. Denaturation of the double-stranded DNA molecule under experimental conditions could lead to the formation of a single-stranded circular DNA molecule by annealing the two termini by base pairing. Within the two ITRs, two identical origins of replication can be found. A cis-acting packaging sequence has been localized within several hundreds base pairs at the left end of the viral genome, and has been shown to be involved in the encapsidation process of the viral DNA (Hammarskjold et al., 1980; Hearing and Shenk, 1983; Hearing et al., 1987; Grable and Hearing, 1992).

The genome of Ads has been grouped into four early transcription regions and one late transcription unit (Figure 1-3). The early transcription regions, which include early regions (E)1, 2, 3, and 4, are transcribed before the onset of viral DNA replication. The late transcription region is activated by the major late promoter (MLP), located at 16.5 mu, and generates five families of late mRNAs (L1 to L5) that encode for viral structural proteins.
Figure 1-3: A schematic illustration of adenovirus type 2 genome and its division into 100 mu. Early transcripts are indicated by (E), late transcripts are indicated by heavy arrows and (L). The structural polypeptides are indicated by Roman numeral, non structural polypeptides are shown by (Kd) (from Broker, 1984).
1.2 Adenovirus Infection and Replication Cycle

The replication cycle of Ads, which lasts from 20 to 24 hours (h), is characterized by two phases: early and late. The early phase includes all events that occur before the onset of viral DNA replication: adsorption and entry of the viral particles into the cell, and transcription and translation of early genes. When the viral DNA starts replication, it is a signal for the late phase to begin. Figure 1-4 showing the relative timing of the major events that occur during viral replication cycle.

In the early phase, four separate promoters are active, producing transcripts from E1 (E1A and E1B), E2 (E2A and E2B), E3, and E4. About 25 early proteins function to take over the control of the host cellular machinery, convert it into an efficient factory for virus replication and counteract host antiviral defense. Approximately 8-9 h postinfection (pi) and along with the beginning of viral DNA replication, the MLP is activated to produce most of the viral transcripts that code for viral structural proteins. The division of the replication cycle of Ads into two phases may not be accurate because some late genes are expressed early (eg., L1), and some early genes continue to be expressed during the late phase. Genes which give rise to IVa2 and IX proteins are expressed intermediately, so they are classified as delayed early genes (Pettersson and Roberts, 1986). With the exception of the virus associated (VA) RNA I and II which are transcribed by RNA polymerase (pol) III, all viral genes are transcribed by RNA pol II (reviewed in Mathews and Shenk, 1991).

Both viral DNA replication and transcription take place in the host cell nucleus, and all transcripts are transported to the cytoplasm to be translated to form specific viral proteins. After polyribosome translation in the host cell’s cytoplasm, viral polypeptides are transported to the nucleus where the virus particle is assembled about one day pi.
Figure 1-4: Diagram showing the major events which take place during the adenovirus replication cycle. The genes responsible for every event are shown. The lower scale displaying the time corresponding of each event (from Fields et al., 1996).
Cellular DNA replication and protein synthesis are greatly enhanced by adenovirus infection. Some Ads such as Ad12 enhance cellular DNA replication and stimulate cell cycle growth, suggesting that these Ads are able to transform cells (Van Oostrum et al., 1985). During the late phase of infection, inhibition of cellular protein synthesis takes place by inhibiting the transport of cellular mRNAs to the cytoplasm.

Ads have restricted host range: permissive, semipermissive, and nonpermissive. Host range is determined by the presence or absence of cellular factors required for transcription and translation of viral genes as well as presence of receptors (Katze et al., 1983). In a permissive infection, approximately 10,000 viral particles are being produced per cell and excess of viral DNA and proteins that are not involved in the assembly process (Green and Daesch, 1961). In a nonpermissive infection no virus replication nor production can be detected.

1.2.1 Attachment and Entry

Compared to other viruses, Ads are found to efficiently enter their target cells, approximately 85% of virus bound to susceptible cell can enter the cell and in a very short period of time. Ads bind to specific receptors on the cell surface through the fiber protein. There are at least $10^4$ fiber receptors per cell (HeLa cells) (Fields and Knipe, 1990).

The N-terminal of the subunits of the fiber protein (polypeptide IV), each of which is 40 amino acid (aa) residues in length in the case of HAd2, are embedded in the penton base. The C-terminal (180 residues segment) gives the fiber its terminal knob, and it is believed to be involved in binding to the receptors on the cell (Devaux et al., 1987). Since different serotypes posses different fiber proteins specifically because of
sequence variation at the carboxy-terminal (knob), Ads bind to a variety of receptors on the susceptible cells. It has been shown that the penton base of Ads is involved in binding of the viral particle to the cell by interacting with a specific receptor on the cell surface named integrins; moreover, the penton base plays a very important role in internalizing viral particle into the cell (Huang et al., 1996).

The virus receptor complex migrates through the plasma membrane to clathrin-coated pits and is endocytosed; however, the mechanism by which these pits are induced is not understood (Chardonnet and Dales, 1970; FitzGerald et al., 1983; Varga et al., 1991). Figure 1-5 shows the steps involved in internalization of Ads into the target cell. Fifteen minutes (min) pi and before the formation of lysosomes within the endosomes, a great number of Ad particles are released to the cytoplasm following a drop in pH (pH 5.5), which is believed to be induced by the viral penton base (Seth et al., 1984; Svensson, 1985; Pasten et al., 1986).

During the escape of the viral particles from the endosome, polypeptides IV and IIIa, then polypeptide III, are degraded and dissociated from the viral particle (Philipson et al., 1968). Following the degradation of polypeptides IV and IIIa, which are subunits of penton capsomer, polypeptide VI and VIII are proteolyzed, a process thought to be mediated by a virally encoded protease (Mangel et al., 1993; Tihanyi et al., 1993; Webster et al., 1993; Greber et al., 1996).

The transport of the viral particle to the nucleus is mediated by microtubules, and some studies have shown that the hexon protein is involved in the interaction between the viral particle and these microtubules (Dales and Chardonnet, 1973; Luftig and Weihting, 1975). The viral particles reach the nuclear pores 40 min pi and start releasing their DNA molecules inside the nucleus. There is speculation that the released viral
Figure 1-5: Diagram representing the attachment and entry of adenoviruses to the host cell.
DNA into the nucleus is associated with basic viral proteins, and a controversy has been raised that the viral DNA might be associated with cellular histones instead of viral proteins since the viral DNA becomes sensitive to DNase (Tate and Philipson, 1979; Vayda and Flint, 1987). When the viral DNA enters the nucleus, transcription and replication of the viral genome start.

1.2.2 Early Gene Expression

Transcription of early genes starts shortly after the entry of viral DNA into nucleus and before the beginning of viral DNA replication. The early transcription units are located on both strands of the viral genome: E1A, E1B, pIX, and E3 are mapped on the r-strand; however, E2, IVa2, and E4 are found on the l-strand. The transcripts of all these regions with the exception of pIX undergo alternative splicing, and regions E2 and E3 are characterized by polyadenylation.

The products of the early genes are considered to have important functions during the replication cycle of Ads. Products of the E1 region (E1A and E1B) play an important role in the activation of other early genes transpiration and in enhancing cell cycle growth to achieve an optimal condition for viral DNA replication. E2 proteins are necessary for viral DNA replication in terms of starting the DNA replication and elongation of viral strands. E3 codes for several proteins that have a defense function against antiviral products produced by the host. E4 genes are involved in viral DNA replication and shutoff of cellular macromolecules biosynthesis. Because E1A and E1B proteins are the key for activating the transcription of the viral DNA and inducing cell cycle progression, they are the most studied and characterized regions within the Ads genome.
1.2.3 Adenovirus Early Transcription Units and Protein Products

1.2.3.1 Early Region 1

In Ad5, E1 is located between 1.3-11.2 μu and comprises two subunits: E1A and E1B (Figure 1-6). E1A is the first unit to be transcribed on the viral genome. This unit produces five proteins termed according to the mRNAs encoding them: 13S, 12S, 11S, 10S, and 9S. The 13S and 12S transcripts are synthesized and translated early, while the other three transcripts (unknown function) are translated late in infection (Stephens and Harlow, 1987).

The 13S protein product of E1A consists of three conserved regions (CRs) among Ads and are designated: CR1, CR2, and CR3 and are separated by two nonconserved domains. The 12S protein product possesses two conserved and two nonconserved regions (Dyson et al., 1992; Van Ormondt et al., 1980). E1A proteins are involved in several viral and cellular functions, including transcription activation and repression, induction of cellular DNA synthesis, cell immortalization and cell transformation. It has been shown that E1A proteins can transactivate and transrepress both viral and cellular promoters through different mechanisms. The E1A-13S protein product can activate transcription of viral DNA by binding (through CR3) to TATA-binding protein (TBP) which is a subunit of cotranscription factor DII (TFIID) a multisubunit complex that consists of TBP and at least seven TBP-associated factors (TAFs) (Horikoshi et al., 1991; Lee et al., 1991). TFIID, in turn, binds to the TATA elements of RNA pol II promoters as a first step in the formation of the transcription preinitiation complex that includes at least six other components (TFIIA, -B, -E, -F, -H, and -J) and pol II. At the same time, when the 13S protein product interacts with TBP, it
Figure 1-6: Early region 1 of adenovirus type 5.

E1 region is located at 1.3-11.2 μu of the virus genome. E1A encodes for 13S, 12S, 11S, 10S, and 9S mRNA species. Conserved region 1 (CR1), CR2, and CR3 are indicated by bars. E1B encodes for 19Kd and 55Kd phosphoproteins. The coding region for pIX is shown. Dashed lines are introns, and solid lines are exons.
serves as a connection between TBP and other transcription activating proteins. Moreover, binding of the 13S protein to TBP prevents p53 (tumor suppresser protein) from interacting with TBP. If p53 competes and replaces the 13S protein from its binding site on TBP, inhibition of transcription process would take place (Seto et al., 1992; Horikoshi et al., 1995).

In addition to p53, the E1A proteins can provoke other transcription suppresser proteins: E1A proteins interact (through CR2) with the 105Kd cellular retinoblastoma tumor suppresser (pRB) or other members of pRB family such as p107 and p130 (Whyte et al., 1989; Hermann et al., 1991; Wang et al., 1991; Howe and Bayley, 1992) preventing their association with E2F (transcription factor which activates E2 expression) (Kovesdi et al., 1987; Yee et al., 1989; Zamanian and Thangue, 1992). The association of pRB with E2F prevents the latter from interacting with E2 promoters and activating E2 transcription (Chellappan et al., 1992; Dalton, 1992; Hamel et al., 1992; Hiebert et al., 1992).

Binding of E1A proteins to p53 and pRB not only enhances viral DNA transcription, but also induces the host cell growth cycle by inhibiting their regulation of cell growth in order to create an optimal environment for viral DNA replication (Harlow et al., 1986; Giordano et al., 1991; Hiebert et al., 1992). E1A proteins induce indirectly the cell cycle progression. As mentioned previously, E1A proteins block the formation of E2F-pRB complex by interacting with pRB or other pRB family members (p107 and p130) (Wang et al., 1993). The released E2F is able to interact with genes containing its recognition site, like thymidine kinase (tk) and DNA pol α; which play a major role in inducing quiescent cells to enter S phase (Goodrich et al., 1991; Johnson et al., 1993). The ability of E1A proteins to bind to different tumor suppresser factors and inhibit their
function suggests that E1A proteins are oncoproteins and are able to transform cultured cells.

Similar to the E1A proteins, the E1B-19Kd and 55Kd phosphoproteins are oncoproteins, in reference to the ability of E1B-55Kd to bind to p53 blocking its function of regulating cell cycle growth. High levels of p53, which is induced by E1A, might induce apoptosis (programmed cell death) (Moran, 1993; White et al., 1994; Grand et al., 1996). The E1B-19Kd protein can block apoptosis through its interaction with p53; however, the mechanism by which E1B-19Kd protein blocks apoptosis is not well understood (Rao et al., 1992; White, 1994). Some studies suggested that E1B-19Kd may prevent apoptosis by alleviating repression by p53 on cellular survival proteins (White et al., 1991). The E1B-55Kd protein functions in transporting viral mRNAs, shutting off host cell biosynthesis and inhibiting E1A-induced apoptosis by binding to and inactivating p53 (Babiss et al., 1985; Lowe and Ruley, 1993).

In general, E1A and E1B proteins play a very critical function in activation and regulation of viral DNA transcription and inducing cell cycle progression. All these mechanisms can be fulfilled through the ability of E1A and E1B proteins to interact with cotranscription factors and induce them to activate transcription. Transcription enhancement can be accomplished by the interactions between E1A and E1B and other tumor suppresser factors with known ability to inhibit transcription activation. Binding of E1A and E1B proteins with such factors makes infected cells to enter S phase of their growth cycle, creating an optimal condition for viral DNA replication.
1.2.3.2 Early Region 2

E2 of HAds comprises two subregions: E2A at 67-61.5 mu and E2B at 29-14.2 mu in Ad5. E2 can be transcribed off two promoters: early at 75 mu and is active throughout the infection, and late at 72 mu is active after initiation of replication (Nevins and Darnell, 1978). E2A codes for the 72Kd DNA binding protein (DBP) (Kruijer et al., 1981), and E2B codes for two proteins: 80Kd precursor to terminal protein (pTP) and 140Kd DNA pol. These three proteins are known to play critical roles in viral DNA replication.

The transcription of DNA pol as well as pTP is initiated at the E2A promoter at 72 mu and share leader segments at 75, 68, and 39 mu (Figure 1-7) (Stillman et al., 1981; Gingeras et al., 1982). The main body is at 22.8-14.1 mu for DNA pol and at 28.8-23.4 mu for pTP in case of HAd5 (Dekker and Van Ormondt, 1984). The 72 Kd DBP is required for viral DNA elongation, host range determination and assembly of the viral particle, and it binds to the ends of single-and double-stranded DNA (Fowlkes et al., 1979; Klessig and Quinlan, 1982). It has been determined that in the presence of DBP, the DNA pol functions more efficiently and is able to travel the entire length of the viral chromosome (Field et al., 1984; Lindenbaum et al., 1986). Recombinant virus with a mutation in the coding region for DBP fail to replicate at permissive temperature even in the presence of exogenous DBP (Enomoto et al., 1981; Challberg et al., 1982; Friefeld, 1983).
Figure 1-7: Early Region 2B of adenovirus type 2.
mRNAs are derived from the leader at map unit 39. Regions coding for Ad DNA pol and TP are shown. Black arrows indicate the open reading frames starting from the first ATG.
1.2.3.2.1 Viral DNA Polymerase

Ad DNA pol is an essential enzyme for both the initiation and elongation steps of DNA replication. Recombinant Ad with mutation in the coding region for DNA pol fail to replicate (Friefeld et al., 1983; Lichy et al., 1983). Ad DNA pol is purified in association with pTP from Ad-infected cytoplasmic extract; however, it can be separated by glycerol gradient sedimentation in the presence of urea (Lichy et al., 1982).

Ad DNA pol has different properties from eukaryotic DNA pols α, β, and γ with respect to template preferences and sensitivity to inhibitors. Ad DNA pol is characterized by many activities such as enhancing the formation of pTP-dCMP (Lichy et al., 1981; Challberg et al., 1982), and DNA elongation activity on Ad DNA duplex (Lichy et al., 1981). Ad DNA pol contains 5' to 3' polymerase activity and 3' to 5' exonuclease activity that probably serves as proofreading function during polymerization (Field et al., 1984). Comparison of deduced sequences between Ad DNA pol and other DNA pols such as human DNA pol α, DNA pol of herpes simplex and from vaccinia has shown that at least six regions that show a great homology among them. The presence of these conserved sequences suggests that they might be derived from the same origin and these domains might serve a common important biological function (Earl et al., 1986; Wong et al., 1988).

1.2.3.2.2 Terminal Protein and Its Precursor

The mature virion TP was first recognized as enabling virus DNA to circularize upon viral DNA denaturation (Robinson et al., 1973). TP is subsequently identified as a 55Kd protein covalently linked to each 5' end of the linear virus DNA (Rekosh et al., 1977). The TP is derived from 80Kd pTP which is larger than TP and structurally related
to it. It has been shown that pTP is cleaved by a virally coded 23Kd protease to produce TP (Stillman et al., 1981). The conversion of pTP to TP has no effect on viral DNA replication. A mutant virus that failed to cleave pTP to its mature counterpart TP because of mutation in the gene coding for 23Kd protease did not show any effect on the efficiency of viral DNA replication (Bhatti and Weber, 1979).

The formation of pTP-dCMP complex is enhanced by Ad DNA pol but independent of DBP (Challberg, 1982; Friefeld et al., 1983). It has been shown that the formation of the initiation complex is sequence dependent. This complex cannot be formed with heterologous DNA sequence, which led to speculation that specific sequences within ITR in homologous DNA might enhance and support the formation of pTP-dCMP complex. In addition to its function in viral DNA replication, TP plays a role in enhancing the transcription of the viral DNA during Ads infection (Vayda and Flint, 1987). When viral DNA reaches the host nucleus it becomes tightly associated to the nuclear matrix leading to efficient transcription (Bodnar et al., 1989; Schack et al., 1990; Fredman and Engler, 1993; Angeletti and Engler, 1996). The TP, which arrives with the infecting genome, is the first viral product that functions within the nucleus to initiate viral gene expression.

### 1.2.3.3 Early Region 3

The E3 promoter is activated in early infection; however, late in infection E3 region can be transcribed off the MLP (Chow et al., 1979). E3 of Ads can be deleted and does not affect viral replication in cell culture (Kvist et al., 1978; Pääbo et al., 1983), but an E3 mutant failed to replicate efficiently in an organism, suggesting that E3 products are essential for viral interaction with the defense system of an organism.
Chapter 2

The current state of the art, in terms of system design, is not sufficient to address the challenges of scalable and efficient data management in large-scale distributed systems. The need for robust and scalable data management systems has led to the development of new approaches and technologies. One such approach is the use of distributed hash tables (DHTs) for efficient key-value storage.

DHTs provide a flexible and scalable way to store and retrieve data in a decentralized network. They are used in various applications, including peer-to-peer file sharing, content delivery networks, and distributed databases. The key feature of DHTs is their ability to distribute data across a network of nodes, allowing for high availability and fault tolerance.

In addition to the traditional DHTs like Chord and Kademlia, there has been a recent trend towards using machine learning techniques to improve the performance and efficiency of DHTs. This involves using algorithms that can learn from historical data and adapt to changing network conditions.

Overall, the development of new technologies and approaches for efficient data management in distributed systems is an active area of research, and it continues to evolve as new challenges arise.
When Ads infect a host, two types of immune response are induced: nonspecific and specific responses. The specific response is mediated by cytotoxic T lymphocytes (CTL) which can recognize the virally-infected cells by the presence of a viral polypeptide antigen in a complex with major histocompatibility complex (MHC class I) on the surface of the cell. E3 proteins have been found to protect the infected cell from cytolysis by CTL and tumor necrosis factor (TNF α), a cytokine produced by activated macrophages (Ginsberg et al., 1991; Prince et al., 1993).

E3 codes for nine proteins (Figure 1-8), so far seven of them are identified: E3-6.7Kd (Wilson-Rawls et al., 1990), 10.4Kd (Tollefson et al., 1990b), 11.6Kd (Wold et al., 1984), 12.5Kd (Hawkins and Wold, 1992), 14.5Kd (Tollesfson et al., 1990a), 14.7Kd (Tollefson and Wold, 1988; Wang et al., 1988), and 19Kd (Persson et al., 1980a, 1980b). Four of these proteins are involved in protecting the Ad-infected cells from recognition and destruction by the host's immune system (Mullbacher, 1992; Wold, 1992).

In the case of Ad infection, it has been found that the E3-19Kd glycoprotein (gp) is presented on the cell surface in a complex with MHC class I. However, E3-19Kd gp has low sensitivity to CTL mediated lysis (Anderson and Fennie, 1987; Burget and Kvist, 1987). The E3-19Kd protein is able to downregulate MHC and antigen expression. E3-19Kd noncovalently binds to MHC class I in infected cell endoplasmic reticulum (ER) inhibiting its transport to the cell surface (Kvist et al., 1978; Cox et al., 1991).

In addition to the E3-19Kd, the E3-14.7Kd, E3-10.4Kd, and E3-14.5Kd proteins are found to protect cells infected by Ads from the antiviral effect of TNF α (Gooding et al., 1991) either as a single protein (e.g. E3-14.7Kd protein) or in a complex (e.g. E3-
Figure 1-8: Early region 3 of adenovirus type 5.

E3 region is located at map units 76.6-86.0 of the virus genome. E3 proteins are indicated by size. The exons are shown by heavy arrows, introns by dashed lines.
14.5Kd/E3-10.4 proteins complex). In 1988, Gooding et al., have shown that cells infected with a mutant Ad lacking E3 region are susceptible to lysis by TNF α.

The E3-11.6Kd is synthesized in small amounts at early stages of infection, and is expressed abundantly during late phase by MLP. Under such circumstances, The E3-11.6Kd is considered as a late protein and represents the sixth family of major late proteins. This protein is required for efficient lysis of Ad-infected cells beginning at 2 or 3 days pi (Tollefson et al., 1996a, 1996b).

1.2.3.4 Early Region 4

E4 proteins are important in viral DNA replication, shutting off cellular macromolecules biosynthesis and inducing apoptosis (Marcellus et al., 1996). Out of seven open reading frames (ORFs) within E4, three proteins are identified: E4-14Kd (Sarnow et al., 1982; Downey et al., 1983), 17Kd (Cutt et al., 1987), and 34Kd (Sarnow et al., 1984). E4 mutant Ads are defective in viral DNA replication, fail to shut off host cell biosynthesis and fail to switch from early to late phase of viral expression cycle (Halbert et al., 1985; Falgout and Ketner, 1987).

The E4-17Kd polypeptide activates the transcription factor E2F protein by enhancing its affinity to E2 promoters, leading to the activation of E2 transcription (Hardy et al., 1989; Huang and Hearing, 1989; Raychaudhuri et al., 1990). When the replication of the viral DNA starts and enough late mRNAs have been accumulated, the transport of cellular mRNAs to the cytoplasm is blocked and accumulation of viral mRNAs will be enhanced. The blocking process is the function of the E4-34Kd protein when it forms a complex with the E1B-55Kd protein (Halbert et al., 1985; Weinberg and Ketner, 1986; Bridge and Ketner, 1990). The mechanism by which E1B-E4 complex
activates the accumulation of viral mRNA and blocks the accumulation of cellular mRNA is not clear. Some cellular genes are cloned within viral DNA are transcribed and their mRNAs are transported to the cytoplasm, suggesting that the E1B-E4 complex does not recognize the identity of mRNAs to be transported to the cytoplasm. E1B-E4 complex might control some factors which mediate the transport of cellular and viral mRNAs to the cytoplasm (Ornelles and Shenk, 1991; Swaminathan and Thimmapaya, 1996).

1.2.4 Viral DNA Replication

When enough E2 products, which are involved in viral DNA replication, are available, and the host cell enters S phase, the condition is set for viral DNA replication to start (reviewed in Challberg and Kelly, 1989).

Ad DNA replication is characterized by a protein-primed strand displacement mechanism and occurs at either end of the viral DNA duplex at the ITR (Figure 1-9). The 80Kd pTP, which is linked to each 5' end of the viral DNA, serves as a primer for initiation of DNA synthesis. In this mechanism, the β-hydroxyl group of a serine residue in pTP is covalently linked to the 5'-hydroxyl of the terminal deoxycytosine residue of viral DNA via a phosphodiester bond to form the pTP-dCMP complex (Challberg et al., 1980). The 3'-hydroxyl group of pTP-dCMP serves as a primer for the elongation of the nascent strand. The elongation of the complementary strand proceeds unidirectionally from either origins of replication toward the other end (5' to 3') of the template, displacing the non-template DNA strand. Thus, the result of this step is a duplex consisting of a parental and daughter strands plus the displaced single parental strand.
Figure 1-9: Adenovirus DNA replication.

DNA replication starts at either ends of the DNA molecule, and replacing the non templates strand which is circularized in order to form a complementary strand.

(From Fields and Knipe, 1990)
The displaced single strand then is circularized through annealing of its complementary termini by base pairing to form a panhandle shape, thereby allowing the synthesis of a complementary strand to take place (Lechner and Kelly, 1977). There are six proteins required for Ad DNA replication, three of which are virally coded: E2B-80Kd pTP, E2B-140Kd DNA pol, and E2A-72Kd DNA binding protein (DBP). The other three are hosts proteins: nuclear factors I-III (Enomoto et al., 1981; Field et al., 1984). Both E2B-80Kd pTP and E2B-140Kd DNA pol function by binding to specific sequence within the ITR called domain A (Chellberg and Kelly, 1981; Tamanoi and Stillman,1983; Lally et al., 1984). E2A-72Kd DBP, which binds to another sequence (domain B) within ITR, seems to play an important role in DNA elongation by enhancing DNA pol efficiency (Rosenfeld et al., 1987). NFI, which binds to the Ad origin of replication between bases 18 to 40, stabilizes pTP at its binding site (Bosher et al., 1990; Chen et al., 1990; Mul et al., 1990; Mul and Van der Vliet, 1992). NFII, which has topoisomerase activity, is involved in enhancing DNA elongation. The mechanism by which NFIII is involved in DNA replication is not yet known, however, it might participate in initiation of DNA replication (Challberg and Kelly, 1989).

1.2.5 Late Gene Expression

While the early genes continue to be transcribed and viral DNA starts replication, the transcription of Ad late genes begins. It is not clear if the onset of viral DNA replication is linked to the switch from early to late genes transcription. Some studies suggested that the switch from early to late phase is controlled by two factors: cis-acting elements and trans-acting factors. The cis-acting elements seem to be time independent, and is characterized by modifications on the viral genome that occur when
the early genes are transcribed and viral DNA starts replication. The trans-acting factors cannot function until cis-acting factor modifications occurred (Thomas and Mathews, 1980). The changes on the viral DNA during DNA replication might create a chance for trans-acting factors to bind to their recognition sites and activate late genes expression.

Three factors have been determined to activate late gene expression: major late transcription factor (MLTF) and two virally encoded transcription factors (Miyamoto et al., 1985). One of the two virally encoded factors is a protein encoded by the IVa2 gene. After the early genes are transcribed, IVa2 can be activated and expressed, suggesting that some early gene products might control the activation of IVa2 (Chen et al., 1994). The IVa2 product then binds to its binding site downstream of MLP (the only promoter that controls late transcription) leading to the activation of transcription (Leong et al., 1990; Mondesert et al., 1992; Lutz and Kendinger, 1996).

Activation of late genes by MLTF is quite different from the way in which IVa2 product works. MLTF activates late transcription by binding to a specific sequence upstream of MLP, immediately after the onset of viral DNA replication (Toth et al., 1992).

The transcription of the Ad late unit can be initiated both early and late by means of MLP. During the early phase, transcription does not run further than L1. However, during the late phase the level of expression is increased and extends to transcribe a great part of viral DNA. The late coding unit and under the control of MLP can form at least 18 different late mRNAs. All of these mRNAs are generated from a single primary transcript approximately 29,000 nucleotide in length (Evans et al., 1977; Nevins and Darnell, 1978). The 18 late mRNAs are classified into 5 families (L1 to L5) based on the utilization of a common polyadenylation sites (Chow et al., 1977a; Nevins and
Darnell, 1978; Ziff and Evans, 1978). Individual RNAs are spliced at their 5' ends to the tripartite leader which is located upstream from the translation initiation site for each protein (Figure 1-3) (Berget et al., 1977; Chow et al., 1977b). The tripartite leader comprises three spliced leader elements at 17, 20, and 27 mu.

1.2.6 Assembly and Release of Viral Particle

The assembly of the virion begins with the production of empty capsids in order to package viral DNA. Production of capsids is a complex process and many factors are involved and the details of this process is not well understood. Nonetheless when enough structural proteins are accumulated and viral DNA replication has occurred, the condition is suitable for the virion assembly. The first protein to be assembled is the hexon protein; L4-100Kd protein participates in making up the trimeric hexon by binding to hexon monomers to facilitate their assembly (Liebowitz and Horwitz, 1975; Oosterom-Dragon and Ginsberg, 1981; Cepko and Sharp, 1982). Assembly of penton capsomers (pentameric penton base and trimeric fiber) takes place in the cytoplasm, and then they are transferred to the nucleus in order to form the capsid (Horwitz et al., 1969).

Different mutations have been made in order to determine the role of different viral proteins in the assembly of Ad virions. Mutations in the region coding for a cysteine protease (L3), an enzyme required for cleaving some of viral proteins during assembly, lead to production of noninfectious virion-like particles (Mangel et al., 1993; Tihanyi et al., 1993; Webster et al, 1993; Greber et al., 1996). Another mutation, which has been made within the region that codes for the L1-52/55Kd protein, inhibits the production of infectious Ads (Edvardsson et al., 1978; Chee-Sheung and Ginsberg, 1982).
Controversy has been raised about the time of entry of the viral DNA into the empty capsids. Some studies suggest that the viral DNA enters the capsid while it is replicating; on the other hand, it is believed that both processes occur at different times. A \textit{cis}-acting DNA sequence located about 260 bp from the left end of the viral DNA seems to play a very important role in packaging of the viral DNA into the capsids, and viral DNA missing this sequence cannot be packaged (Hammarskjold and Winberg, 1980; Hearing \textit{et al.}, 1987; Grable and Hearing, 1992). A few unknown proteins bind to this terminal sequence and participate in viral DNA packaging.

1.3 Bovine Adenoviruses

BAVs are involved in subclinical to mild respiratory and enteric infection in calves (Lehmkuhl \textit{et al.}, 1975; Scanziani \textit{et al.}, 1989). The first BAV was isolated in 1959 by Klein and colleagues. In subsequent years, BAVs have been isolated from variety of animal species (Boros \textit{et al.}, 1985; Belak \textit{et al.}, 1986). BAV3 was first isolated in England from the conjunctiva of a healthy cow in 1965 (Darbyshire \textit{et al.}, 1965). Since then, BAV3 has been isolated from cattle showing signs of respiratory-enteric disease as well as from healthy cattle (Mattson \textit{et al.}, 1988).

BAV3 is a nonenveloped icosahedron of approximately 75 nm in diameter. The 35,000 bp viral genome is a linear double-stranded DNA molecule (Elgadi and Haj-Ahmad, 1992) and the 195 bp ITRs can be found on each end of the DNA molecule (Shinagawa \textit{et al.}, 1987). Hybridization studies on BAV3 and Ad2 revealed extensive homology of these two viral genomes, not only in terms of sequences, but also in terms of transcript location and orientation (Hu \textit{et al.}, 1984).
Previous studies on BAV3 have determined the nucleotide sequences of E1 and pIX regions (Elgadi et al., 1993), E3 and fiber protein (Mittal et al., 1992), and MLP (Song et al., 1996). The ORFs found in BAV3 E1 shows significant homology to E1A and E1B proteins of Ad5. One of the ORFs of BAV3 E1A shows 75.5% homology to Ad5 E1A CRIII. Two ORFs have been found in BAV3 E1B; the first ORF (157 aa) shows homology to Ad5 E1B-19Kd protein and the second ORF shows homology to Ad5 E1B-55Kd protein. An ORF in the pIX region of BAV3 (125 aa) exhibits homology to Ad5 pIX (Elgadi et al., 1993; Zheng et al., 1994). The homology between Ad5 E1A CRIII and the corresponding region in BAV3 genome has raised the fact that BAV3 E1A CRIII can activate in trans other Ad5 genes. In fact, it has been shown that the E1A of BAV3 is able to activate some of Ad5 genes when bovine cells in culture were contransfected with BAV3 and Ad5 lacking E1A (Zheng et al., 1994). The aa sequences, which have been determined for the BAV3 E3 ORFs, have been compared to Ad5 E3 proteins. One of BAV3 E3 proteins exhibits homology to Ad5 E3-14.7Kd protein. The fiber protein of BAV3 has homology to Ad2 fiber protein; however, it is quiet longer (Mittal et al., 1992).

Most studies on constructing recombinant adenovirus vectors have been based on human adenoviruses. Generally, many recombinant human adenoviruses have been constructed with insertions of foreign genes in E1 and E3 (Berkner and Sharp, 1984; Bett et al., 1993; Ghosh-Choudhury et al., 1986; Haj-Ahmad and Graham, 1986). Since E1 of BAV3 shows homology to HAd5 E1, it is possible to construct BAV3 recombinant virus lacking E1 and with insertion of foreign gene in this region. However, as in the case of Ad5 E1 mutants that can only be grown in 293 cells, it is necessary to generate and propagate E1 recombinant BAV3 in a cell line that expresses
complementing BAV3 E1 proteins. In addition, as for HAds, BAV3 E3 has been shown not to be essential for virus replication in cultured cells (Mittal et al., 1995). A deletion in BAV3 E3 would generate a helper-independent recombinant BAV3.

1.4 Use of Adenoviruses as Gene Transfer and Expression Vector

1.4.1 Advantages of Recombinant Adenoviruses

Many features have made recombinant Ads a suitable tool for vaccination and gene therapy (reviewed by Graham and Prevec, 1991, 1992). One of the most important features is the tremendous understanding of the biological properties and the structure of Ads.

Ad genome is medium sized, so it can be easily manipulated by the available biotechnological methods: convenience for deletion and insertion using restriction endonucleases, and the viral DNA can be incorporated in bacterial plasmids and cosmids. The incorporated viral DNA in a plasmid is infectious; introducing the viral DNA to cells (transfection) regenerate viruses.

Ads can accept insertion of large foreign genes. The capacity of recombinant Ads for insertion of foreign genes can be increased by deleting many regions of the viral genome to produce helper dependent/independent recombinant Ads. Moreover, the inserted foreign gene sequences do not undergo any changes during the virus replication cycle. Foreign genes inserted in recombinant Ads are expressed with correct postranslation mechanism and biologically active. The expression of foreign genes can be controlled and detected; high level of expression can be obtained either by relying on the viral promoters or by using exogenous promoters.
Ads infect a variety of animal species and can be grown in different cell lines. Upon permissive infection, 1,000 to 10,000 viral particles can be produced per cell, and are concentrated within the cell and easy to be harvested and prepare high titer stocks.

The study of Ad infection has provided a good deal of information about diseases they cause and the response of immune system. Generally, HAds do not cause serious diseases in humans and have not been demonstrated to induce tumors; thus they appear to be more attractive than other viruses to be used as a vaccine. HAds type 4 and 7 have been used as unattenuated vaccines to prevent the spread of acute respiratory disease, and a great deal of information has been obtained about their advantages and disadvantages. It has been shown that recombinant Ads carrying antigens can induce both humoral and cellular response in vaccinated animals, a feature that characterizes good vaccines.

1.4.2 Construction of Recombinant Adenoviruses

In order to insert foreign genes into the Ad genome, certain regions of the viral genome should be deleted to meet the packaging capacity of 105% of the wild type genome size (Haj-Ahmad and Graham, 1986). At least three regions have been commonly used to insert foreign DNA: E1, E3, and a region between E4 and the right ITR (reviewed in Graham and Prevec, 1991, 1992), and a compensating deletion must be made in case of inserting large fragments of foreign DNA. Deleting parts of viral genome should be applied carefully since each region of viral genome codes for more than one protein. A deletion in a certain position of the viral genome could result in the loss of several viral proteins since both strands code for genes. In case of deletions that code for necessary proteins for viral replication cycle, the missing proteins should
and other services. In order to ensure that the descriptions and instructions are as accurate and effective as possible, the developers of the software have conducted extensive testing and validation. This includes rigorous testing of the software's performance, reliability, and compatibility with various hardware and operating systems.

Another important aspect of the software is its user interface. The developers have worked diligently to create an intuitive and user-friendly interface that makes it easy for users to navigate and perform the necessary tasks. This has been achieved through careful consideration of the interface's layout, color schemes, and the placement of buttons and controls.

In conclusion, the software being developed is a powerful and versatile tool that promises to revolutionize the field of [field]. Its combination of advanced features, robust performance, and user-friendly interface makes it an ideal choice for professionals and enthusiasts alike. The developers are confident that this software will meet the needs of its users and exceed their expectations.
be provided *in trans* either by constructing cell line expressing these proteins or by cotransfection along with a plasmid containing the deleted sequences.

Deletion in E1 region should not reach ITR packaging signal and protein IX (Haj-Ahmad and Graham, 1986; Grable and Hearing, 1990, 1992). Because E1 is essential for virus replication, the E1 mutant virus is helper-dependent during its replication cycle. However, E1 proteins can be provided *in trans* by two different ways: generating and propagating the recombinant virus in cells expressing E1 proteins or by coinfection along with wild-type virus. The level of expression of a foreign gene inserted in E1 may vary depending on: (a) the inserted gene itself, (b) promoters (viral or exogenous promoters), and (c) the orientation of the foreign gene; expression off E1 promoters in case of parallel insertion, and off MLP if the insertion is antiparallel to E1 (Berkner *et al*., 1987).

E3 region is not required for virus replication in tissue culture, and E3 mutant virus is helper-independent (Haj-Ahmad and Graham, 1986). The deletion within E3 of an Ad vector should not extend to the structural proteins which is encoded by L4 and L5 (pVIII and fiber). If the foreign gene is inserted in a parallel orientation within E3 region, high level of expression can be achieved due to the expression off E3 promoters (early) and off MLP (late), suggesting that such inserts might not need exogenous promoters. Antiparallel insertion results in low level expression even in case of exogenous promoters.

As it is mentioned previously, E3 proteins play an important role in modulating the host immune response. Deletion in E3 results in less persistence due to effectiveness of the host immune response in the absence of E3 proteins, specifically E3-19Kd glycoprotein. It has been shown that cells infected by an E3 mutant Ad are
susceptible to CTL cytolysis in the host. Since E3 modulates host cellular response, infected cells by E3 mutant could be eliminated (Ginsberg and Prince, 1989) and such response is not favored in a vaccine.

Insertion upstream from E4, with compensatory deletion of the non essential E3, is particularly useful for constructing replication-competent viral vector. Such recombinant Ads have been considered candidates for live vaccines, possibly more effective than replication deficient recombinant Ads (Gao et al., 1996).

Viruses with E2A deletions have been constructed and grown in cells that provide E2A in trans (Rice and Klessig, 1985). Since the E2A protein is required in very large amounts during viral replication cycle, these mutant viruses were difficult to grow in these cells due to the difficulty to complement E2A product. On the other hand, the E2B proteins (pTP and DNA pol) are required in low amounts, suggesting that construction of cell line expressing E2B proteins would be suitable to grow E2B deleted viruses. In fact, cell lines expressing pTP have been constructed (Schaack et al., 1995; Langer and Schaack, 1996). The resultant cell lines should offer more powerful tools for the construction of pTP mutant Ads and analysis of pTP function.

To increase the packaging capacity of exogenous sequences, an attempt to rescue defective Ad5 which had L1, L2, VA RNA I and II, and pTP deleted was successful. The recombinant virus was rescued in the presence of wild-type Ad5 as a helper (Mitani, 1995). It should be possible to develop a helper-dependent adenoviral vector, which does not encode any viral proteins, and could be rescued and propagated in the presence of wild-type Ads or in a cell line that expresses viral proteins.

Construction and rescue of recombinant Ads can be achieved by different approaches. The most common protocol was developed by Haj-Ahmad and Graham in
1986. In this protocol, a part of the viral genome (left or right end) is first cloned into a plasmid. A deletion can then be made within the viral sequences, followed by insertion of a foreign gene, with or without exogenous promoters. The resulting plasmid containing the viral DNA and foreign gene is then contransfected along with the other end of the viral genome (overlapping is required) in mammalian cells, and the recombinant virus is generated by homologous recombination. This procedure could be time consuming and parental virus might be regenerated, so screening for recombinant virus needs extensive labor.

There are a variety of procedures for rescuing recombinant Ads available (reviewed in Graham and Prevec, 1991). Ligation of the both ends of the viral genome (one or two of them carry foreign gene) in vitro before transfection is another choice. However, the appropriate restriction sites for insertion would be limited due to the size of the genome. The most recent procedure is cotransfection with two plasmids carrying the whole genome apart. These two recombinant plasmids are noninfectious separately. The recombinant virus can be generated by homologous recombination between the overlapped viral sequences in the two plasmids. Other recombinant Ads have been constructed by inserting the whole viral genome into a cosmid or plasmid, then making the necessary deletion and insertion of foreign genes, then finally transfecting the cells with DNA (Miyake et al., 1996). This technique is quite helpful, but, sometimes, the appropriate restriction sites for insertion are not available.
Table 1-3: This table shows the most recent recombinant adenoviruses vaccine vectors (for HAd2 and 5). The promoters which have been used are shown: [MLP, SV40 (SV40 early promoter), tk pro (HSV1 thymidine kinase promoter), and HCMV (human cytomegalovirus immediate early promoter)]. Orientation to E1 and E3 regions is shown (parallel/antiparallel) (reproduced from Todd, 1995).

Notes: Ex (expression studies), co (construction), V/D (vaccination of dogs), V/R (vaccination of rodents), V/P (vaccination of primates), and V/O (vaccination of other species).
<table>
<thead>
<tr>
<th>Foreign insert</th>
<th>Orientation</th>
<th>Promoters</th>
<th>Notes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inserts into HAd2 or HAd5 E1</td>
<td>parallel</td>
<td>MLP</td>
<td>Co, Ex</td>
<td>Levrero et al., 1991</td>
</tr>
<tr>
<td>chloramphenicol acetyltransferase</td>
<td>anti.</td>
<td>HCMV</td>
<td>Co, Ex</td>
<td>Wilkinson and Akrigg, 1992</td>
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<tr>
<td>human cytomegalovirus IE1</td>
<td>parallel</td>
<td>HCMV</td>
<td>Co, Ex</td>
<td>Wilkinson and Akrigg, 1992</td>
</tr>
<tr>
<td>E. coli β-galactosidase</td>
<td>parallel</td>
<td>ICP4 pro</td>
<td>Co, Ex</td>
<td>Spessot et al., 1989</td>
</tr>
<tr>
<td>HSV 2 ribonucleotide reductase</td>
<td>anti.</td>
<td>MLP</td>
<td>Co, Ex, multiple polyA sequences</td>
<td>Lamarche et al., 1990</td>
</tr>
<tr>
<td>hepatitis B pre-S2 epitope</td>
<td>parallel</td>
<td>MLP</td>
<td>Co, Ex, V/O, V/CH</td>
<td>Levrero et al., 1991</td>
</tr>
<tr>
<td>hepatitis B surface antigen</td>
<td>parallel</td>
<td>MLP</td>
<td>Co, Ex, E1A cloned downstream of insert</td>
<td>Levrero et al., 1991</td>
</tr>
<tr>
<td>hepatitis B surface antigen</td>
<td>parallel</td>
<td>E1</td>
<td>Co, Ex</td>
<td>Karlsson et al., 1986</td>
</tr>
<tr>
<td>human globin gene</td>
<td>parallel</td>
<td>none</td>
<td>Co, Ex</td>
<td>Karlsson et al., 1986</td>
</tr>
<tr>
<td>human globin gene</td>
<td>parallel</td>
<td>MLP</td>
<td>Co, Ex</td>
<td>Alkhatib and Briedis, 1988</td>
</tr>
<tr>
<td>measles hemagglutinin</td>
<td>parallel</td>
<td>MLP</td>
<td>Co, Ex</td>
<td>Alkhatib et al., 1988</td>
</tr>
<tr>
<td>measles P/C</td>
<td>parallel</td>
<td>MLP</td>
<td>Co, Ex</td>
<td>Co, Ex</td>
</tr>
<tr>
<td>mouse dihydrofolate reductase</td>
<td>parallel</td>
<td>MLP</td>
<td>Co, Ex, E1B present</td>
<td>Ebata et al., 1992</td>
</tr>
<tr>
<td>human parainfluenza type 3 gp</td>
<td>parallel</td>
<td>MLP</td>
<td>Co, Ex</td>
<td>Berkner et al., 1987</td>
</tr>
<tr>
<td>polyoma middle T antigen</td>
<td>parallel</td>
<td>MLP</td>
<td>Co, Ex</td>
<td>Massie et al., 1986</td>
</tr>
<tr>
<td>polyoma middle T antigen</td>
<td>parallel</td>
<td>MLP</td>
<td>Co, Ex</td>
<td>Eloit et al., 1990</td>
</tr>
<tr>
<td>pseudorabies gp50</td>
<td>parallel</td>
<td>MLP</td>
<td>Co, Ex, V/O, V/R</td>
<td>Jacobs et al., 1992</td>
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<tr>
<td>tick-borne encephalitis virus NS1</td>
<td>anti.</td>
<td>HCMV</td>
<td>Co, Ex, V/R</td>
<td></td>
</tr>
<tr>
<td>Inserts into HAd2 or HAd5 MLP</td>
<td>parallel</td>
<td>MLP</td>
<td>Co, Ex, replication defective vector</td>
<td>Mansour et al., 1985</td>
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<td>polyoma middle T antigen</td>
<td>parallel</td>
<td>MLP</td>
<td>Co, Ex</td>
<td>Yoo et al., 1992</td>
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<tr>
<td>inserts into HAd2 or HAd5 E3</td>
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<td>MLP</td>
<td>Co, Ex</td>
<td>Dewar et al., 1989</td>
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<td>bovine coronavirus HA-esterase</td>
<td>parallel</td>
<td>SV40</td>
<td>Co, Ex, V/R</td>
<td>Prevec et al., 1991</td>
</tr>
<tr>
<td>HIV-1 envelope proteins</td>
<td>parallel</td>
<td>SV40</td>
<td>Co, Ex, V/P</td>
<td>Johnson et al., 1988</td>
</tr>
<tr>
<td>HIV-1 gag antigens</td>
<td>parallel</td>
<td>SV40</td>
<td>Co, Ex</td>
<td>Witmer et al., 1990</td>
</tr>
<tr>
<td>HSV 1 glycoprotein B</td>
<td>parallel</td>
<td>SV40</td>
<td>Co, Ex</td>
<td>Hanke et al., 1990</td>
</tr>
<tr>
<td>HSV 1 glycoprotein C</td>
<td>parallel</td>
<td>SV40</td>
<td>Co, Ex</td>
<td>Hanke et al., 1990</td>
</tr>
<tr>
<td>HSV 1 glycoprotein E</td>
<td>parallel</td>
<td>SV40</td>
<td>Co, Ex</td>
<td>Haj-Ahmad and Graham, 1986</td>
</tr>
<tr>
<td>HSV 1 glycoprotein I</td>
<td>parallel</td>
<td>SV40</td>
<td>Co, Ex</td>
<td>Morin et al., 1987</td>
</tr>
<tr>
<td>HSV 1 thymidine kinase</td>
<td>anti.</td>
<td>tk pro</td>
<td>Co, Ex</td>
<td>Wesseling et al., 1993</td>
</tr>
<tr>
<td>hepatitis B surface antigen</td>
<td>parallel</td>
<td>none</td>
<td>Co, Ex</td>
<td></td>
</tr>
<tr>
<td>mouse hepatitis coronavirus spike, nucleocapsid, membrane proteins</td>
<td>parallel</td>
<td>SV40</td>
<td>Co, Ex, V/R</td>
<td></td>
</tr>
<tr>
<td>rabies glycoprotein</td>
<td>parallel</td>
<td>SV40</td>
<td>Co, Ex, V/R, V/D</td>
<td>Prevec et al., 1990</td>
</tr>
<tr>
<td>rotavirus VP4</td>
<td>parallel</td>
<td>none</td>
<td>Co, Ex</td>
<td>Gorziglia and Kapikian, 1992</td>
</tr>
<tr>
<td>rotavirus VP7sc</td>
<td>parallel</td>
<td>SV40</td>
<td>Co, Ex, V/R</td>
<td>Both et al., 1993</td>
</tr>
<tr>
<td>vesicular stomatitis virus G</td>
<td>parallel</td>
<td>tk pro</td>
<td>Co, Ex, V/R, V/D,</td>
<td>Schneider et al., 1989</td>
</tr>
</tbody>
</table>
Overview of the Project

To develop BAV3 into an expression vector for foreign genes with potential application in gene therapy and vaccine production, the popular approach that has proven effective in generating Ad5-based vectors was adopted for this project. Figures 1-10, 11 outline the basic steps involved in this strategy and how it applies to the BAV3 vector.

The strategy involves the use of cloned viral fragments containing sequence substitutions in either E1 or E3 regions with those of foreign genes. The plasmid DNA bearing the appropriate viral sequences and the foreign insert becomes reconstituted with the complete viral DNA via homologous recombination using overlapping sequences that exist in both plasmid and viral DNA.

The first objective of this project was to construct the appropriate shuttle plasmids suitable for rescuing foreign genes into the BAV3 genome. Two separate systems were envisioned. First, a shuttle vector for inserting foreign genes into the E1 region (Figure 1-10) to generate recombinant BAV3 with substitutions in E1, with or without additional deletion in the E3 region. The second system involves shuttle vectors for inserting in the E3 region (Figure 1-11), also with or without E1 deletions. Both systems take advantage of additional deletions in either E1 or E3 to extend the capacity of the viral vector to accommodate bigger inserts.
Figure 1-10: Strategy for the rescue of E1 recombinant BAV3.

A bovine cell line expressing E1 proteins would be cotransfected with the E1 shuttle plasmid along with BAV3 DNA. The BAV3 DNA is cleaved by one or more restriction enzymes to destroy its infectivity but leaving a contiguous segment on the right side of the genome. The recombinant BAV3 would be generated by homologous recombination between the overlapped DNA sequences in E1 shuttle plasmid and the right end of the viral DNA.
Figure 1-11: Strategy for the rescue of E3 recombinant BAV3.

A bovine cell line would be cotransfected with the E3 shuttle plasmid along with BAV3 DNA. The BAV3 DNA is cleaved by one or more restriction enzymes to destroy its infectivity but leaving a contiguous segment towards the left side of the genome. The recombinant BAV3 would be generated by homologous recombination between the overlapped DNA sequences in E3 shuttle plasmid and the left end of the viral DNA.
To develop a BAV3 as gene transfer vector, first, sequence analysis of BAV3 DNA from 11.7% to 30.8% was carried out to obtain a detailed restriction map of the region. This not only facilitated the construction of the plasmids from this region, but also led to the identification of BAV3 E2B-encoded gene products. Second, shuttle vectors were designed and constructed to facilitate foreign gene insertion into E1 or E3. Such shuttle vectors contained appropriate sequence deletion in either the E1 or the E3, which were expected not to alter recombinant virus growth since functions lost with the deletions could be provided by the complementing packaging cell line (E1) or have been shown to be non-essential for virus growth in tissue culture (E3). Third, to demonstrate the utility of the shuttle vectors, reporter genes were inserted in either E1 or E3. Finally, attempts were made to rescue recombinant virus using several transfection protocols and a number of optimization procedures.
Objectives of the Project

The objectives of this study were:

1) Sequencing analysis of the left end of BAV3 DNA.

2) Construction of a shuttle plasmid that carries at least 50% of BAV3 genome (left end) with a deletion in E1 region from Sacl (1.3%) to Clal (9%) sites.

3) Insertion of a reporter gene in place of E1.

4) Rescue of the E1 shuttle plasmid into infectious virus by using a cell line that expresses E1 of BAV3.

5) Deletion of E3 region of BAV3 between 78.9%-82.5% (Xhol and Hpal sites), and subsequently inserting a reporter gene in place of E3.

6) Rescue of E3 deletion mutant of BAV3 by cotransfecting E3 shuttle plasmid along with digested viral DNA in a bovine cell line.
Chapter 2: Materials and Methods

2.1 Bacterial Culture Techniques

2.1.1 Bacterial Strains

Bacterial strain *Escherichia coli* (E. coli) DH5α (Gibco, BRL) was used as a host for recombinant plasmid DNA.

2.1.2 Propagation and Maintenance of Bacterial Strains

*E. coli* was grown in sterile Luria broth (LB) (10 g bacto-tryptone, 5 g bacto-yeast, 10 g NaCl per liter of dH2O, pH 7.0; sterilized by autoclaving). In case of LB agar, 15 g of bacto-agar per liter of LB was added before autoclaving. Bacterial cultures were stored at 4°C or frozen in 15% glycerol at -70°C. Bacterial cultures were grown for selection in the presence of 50 μg/ml ampicillin, or 100 μg/ml kanamycin or combination of both where appropriate.

2.1.3 Transformation

Competent *E. coli* DH5α cells were prepared as described by Cohen *et al.*, (1972). Cells were grown in 10 ml LB at 37°C. Next day, 100 ml of LB was inoculated with 1 ml of overnight culture and kept in water shaker at 37°C until OD550 was 0.5-0.6.
Bacterial cultures were chilled on ice for 1 h then sedimented by centrifugation at 5,000 rpm (JA-14 rotor) for 15 min at 4°C. the supernatant was discarded and cell pellets were resuspended in 25 ml of ice-cold transformation buffer (50 mM CaCl$_2$, 10 mM Tris, pH 8.0) and kept on ice for 15 min; followed by centrifugation at 4,000 rpm (JA-14 rotor), at 4°C, for 5 min. Cells were resuspended in 3 ml of transformation buffer, and aliquoted and frozen in 10% glycerol at -80°C, frozen stocks were thawed quickly for subsequent use.

Transformation of plasmid DNA was carried out as described by Cohen et al., (1972). To transform bacteria, plasmid DNA (0.1-1 μg) was added to 100 μl chilled competent cells and kept on ice for 30 min with tapping every 10 min. The cells were then heat-shocked for 45 seconds at 42°C, then 900 μl of SOC media (2% Bacto-tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl$_2$, 10 mM MgSO$_4$; 20 mM glucose) was added and cells were recovered by incubating in water shaker at 37°C for 45 min. From 100 to 150 μl of cells were plated on LB agar petri dishes countering the appropriate antibiotic and incubated at 37°C overnight.

2.2 Recombinant DNA Techniques

2.2.1 Identification of Bacterial Colonies by Colony Hybridization

This method was carried out as described by Grunstein and Hogness (1975). Bacterial colonies were transferred to 82 mm nitrocellulose membrane (NEN™ Life Science products) by laying the membrane directly on the petri dish. The cells on the nitrocellulose membrane were lysed by putting the membrane (colonies upside) on a
filter paper soaked with 10% SDS for 3 min. The membrane was then transferred to a second filter paper soaked with denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 5 min and then placed on a filter paper soaked with neutralizing solution (1.5 M NaCl, 0.5 M Tris.C1 pH 7.4) for 5 min followed by 5 min on filter paper soaked with 2X SSC (3 M NaCl, 0.3 M Na-Citrate, pH 7.0). The membrane was dried at room temperature (RT) for 30 min baked for 1-2 h at 80°C in a vacuum oven to fix the DNA on the membrane.

Prehybridization was carried out for 1-3 h at 68°C in a polyethylene heat-sealable bag with 15 ml of hybridization buffer (6X SSC, 2X Denhardt's solution, 0.5% SDS). An appropriate plasmid or virus DNA as probe was labeled with [α-32p]-dCTP using a nick translation kit following the supplier's recommended procedure (Promega).

The labeled probe was denaturated by heating and then added to the bag and hybridization was carried out for 6 h to overnight at 68°C. After hybridization, the filter was removed from the bag and washed in 2X SSC for 15 min at RT, followed by 2X SSC, 1% SDS at 60°C for 30 min, and 0.1X SSC at RT for 15 min. The membrane was air-dried and exposed to Kodak X-Omat AK film (Bio Max™) for 1 h to overnight at -80°C. The X-ray film was developed and fixed with Kodak GBX solutions.

2.2.2 Plasmid DNA Preparations and Purification

2.2.2.1 Small-Scale Plasmid DNA Preparation (mini-prep)

This method of plasmid DNA preparation is based on the alkaline lysis of bacterial cell (Birnboim and Doly, 1979). Desired colonies were picked and grown in 2-3 ml of LB at 37°C overnight in the presence of an appropriate antibiotic. The following
day, 1.5 ml of overnight cultures were transferred to Eppendorf tubes and cells were centrifuged at 13,000 rpm for 1/2 min. The supernatant was discarded, and the pellet was resuspended in 100 μl of lysis buffer (10 mM EDTA, 50 mM glucose, 25 mM Tris.HCl, 2 mg/ml lysozyme (Sigma), pH 8.0). The tubes were incubated on ice for 30 min then 200 μl of alkaline-SDS (0.2 N NaOH, 10% SDS) was added and mixed by inverting the tubes several times. The tubes were kept at RT for 15 min or until the solution became clear. To neutralize the reaction, 150 μl of 3 M NaAc (pH 4.8) was added to each tube and mixed and kept on ice for 30-45 min; followed by centrifugation at 13,000 rpm for 5 min. The supernatant was transferred to new tubes and 2 volumes of ice-cold 95% ethanol were added to precipitate the DNA. The tubes were kept at -20°C for 30 min and DNA was pelleted by centrifugation at 13,000 rpm. The samples were then washed with ice-cold 75% ethanol. The DNA was pelleted by centrifugation at 13,000 rpm. The DNA sample was dried from ethanol and resuspended in 100-200 μl of dH2O or TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5).

2.2.2.2 Large-Scale Plasmid DNA Extraction

This method is based on the alkaline lysis of bacterial cell with some modifications. A test tube containing 2 ml of LB media with the appropriate antibiotic was inoculated with the desired colony. Then the tube was incubated for 7 h at 37°C in a shaking waterbath. A flask containing 500 ml of LB media (containing selection antibiotic) was inoculated with 0.5 ml of culture from the first tube. After overnight incubation in a shaking waterbath at 37°C, the culture was transferred to a centrifuge tube and sedimented at 8000 rpm (JA-14 rotor) for 8 min at 4°C. The cell pellet was
resuspended in 10 ml lysozyme solution (2 mg lysozyme/ml lysozyme buffer) and kept on ice for 30 min. Then 20 ml of alkaline-SDS was added, and the tube was incubated at RT for 15 min or until the solution became clear. Then, 15 ml of 3M NaAc (pH 4.8) was added followed by incubation on ice for 45 min. The cell debris was pelleted through centrifugation for 15 min at 10,000 rpm (JA-14 rotor), 4°C. The supernatant was collected into fresh centrifuge tubes using a funnel filled with glass wool to remove any insoluble material that did not pellet out. To the supernatant, 2 volumes of cold 95% ethanol were added, then tubes were kept at -70°C for 20 min. The DNA was pelleted by centrifugation at 10,000 rpm (JA-14 rotor) for 15 min. The DNA pellet was washed further with ice-cold 70% ethanol. The DNA then was resuspended in 3-5 ml dH2O or TE buffer.

2.2.2.3 DNA Purification using CsCl Gradient Centrifugation

The plasmid DNA was purified by using CsCl gradient centrifugation (Sambrook et al., 1989). For every ml of DNA solution 1 g of CsCl was added. Then the salt was dissolved by mixing gently. One hundred and fifty µl of Ethidium bromid (EtBr: 10 mg/ml) was added to every 3 ml of DNA/CsCl solution. The density of the solution was adjusted to between 1.55 and 1.59 g/ml (by weight measurement). The solution then was centrifuged using 3 ml Beckman tubes (3 ml of solution in each tube) in Beckman TL-100 ultramicrocentrifuge at 65,000 rpm (TLA-100 rotor), at 22°C, for 20 h. The DNA band was visualized and removed using a sterile syringe. EtBr was then removed by using several extraction with isoamyl alcohol until the pink color disappeared from both aqueous and organic phases. The DNA was precipitated by adding two volumes
of water and 3 volumes of 95% ethanol, followed by centrifugation. The DNA pellet then was resuspended in appropriate volume of dH₂O or TE buffer and kept at -20°C.

2.2.3 DNA Ligation

All DNA ligations were carried out using T4 DNA ligase in the presence of 10X ligase buffer [10 mM MgCl₂, 20 mM dithiothreitol, 50 mM Tris, 1 mM ATP, pH 7.5, supplied by New England Biolab (NEB)]. Generally, in a microfuge tube, 10-100 ng of the digested DNA was mixed with 10-100 ng of plasmid DNA (pUC 19) in the presence of 0.5-1 μl (400-800 U/μl) T4 DNA ligase and 2 μl of 10X ligase buffer. The volume was then raised to 20 μl with ddH₂O and the reaction mixture was incubated overnight at 16°C or for 1-3 h.

2.3 Analysis of DNA

2.3.1 DNA Digestion

All restriction enzymes used in this study were used according to the supplier's recommendation (Promega or NEB). Typically in a microfuge tube, 0.1-2 μg DNA, 2 μl of the appropriate 10X restriction enzyme buffer, and 0.5-1 μl (10-20 U) enzyme were mixed together, then the final volume was raised to 20 μl with sterile ddH₂O. The reaction was incubated at the appropriate temperature (usually 37°C). Where required, the enzyme was heat-inactivated (if it was heat labile) at 70°C for 15 min or phenol/chloroform extracted and ethanol precipitated.
2.3.2 Agarose Gel Analysis

The digested DNA was analyzed by using agarose gel electrophoresis. Appropriate amounts of 6X loading buffer (20% glycerol, 2% SDS, 0.05% bromophenol blue) was added to the digestion reaction. The mixture was then loaded on 0.9% agarose gel which was placed in electrophoresis chamber, and covered with TAE buffer (40 M Tris, 20 mM acetic acid, 1 mM EDTA; pH 8.0), just until wells were submerged. The DNA fragments were separated electrophoretically to an appropriate distance, depending on the size of DNA to be visualized. To visualize the DNA, the gel was stained with EtBr and placed on short wave UV light. The gel was then photographed with a mounted Polaroid Land Camera using Polaroid film. The size of the digested DNA fragments was estimated using lambda-Hind III digests (Promega) as molecular-weight marker.

2.3.3 Gel Purification of DNA Fragments using Gene Clean™

The fragment to be purified was excised from the agarose gel and placed in a microfuge tube to which 2.5 to 3 volumes of 6 M NaI solution was added. The agarose was dissolved by incubation at 55°C for 5 to 10 minutes, cooled on ice, and 5 µl of Glassmilk suspension (specially prepared suspension of silica matrix in H₂O) was added to the solution. The Glassmilk/DNA complex was pelleted by centrifuging for 5 seconds, and the supernatant was removed. The pellet was washed 3 times in NEW wash (50% ethanol (v/v), 50% of a solution containing 20 mM Tris (pH 7.4), 1 mM NaCl) (v/v) stored at -20°C, and the DNA was eluted into 20 µl of H₂O by heating to 45°C.
2.3.4 Southern Blot Analysis

Southern blot analysis was carried out according to Southern (1975).

2.3.4.1 Preparing Nitrocellulose Membrane and Transferring DNA

Digested DNA was run in 0.9% agarose gel in the presence of a marker. The gel was then stained with EtBr and photographed. The gel was then soaked in 500 ml of 0.25 N HCl for 15 min at RT. Then the gel was rinsed with dH2O several times. The gel was placed in 500 ml of 0.4 N NaOH, 0.6 M NaCl (denaturating solution) for 30 min at RT. After the denaturation, the gel was placed in 500 ml of neutralizing solution (1.5 M NaCl; 0.5 M Tris. HCl pH 7.5) for 30 min at RT. While the gel was in the neutralizing solution, a nitrocellulose membrane (NEN™ Life Science products) was cut to exact size of the gel and layered gently on fresh ddH2O until the surface of the membrane was saturated. Then, the membrane was soaked in 10X SSC for 15 min.

Two layers of blotting paper (at least 0.5 cm wider than gel on all 4 sides) were placed on surface of sponge already saturated with 10X SSC, and the blotting papers were rolled flat until they became saturated with 10X SSC. Then, the gel was placed on the top of the wet blotting paper and air bubbles were squeezed out by rolling a glass pipette over the gel. Using forceps, the nitrocellulose membrane was placed gently on top of the gel. Three layers of blotting paper were placed on top of the membrane and air bubbles were smoothed out. A 1-inch stack of paper towels were put on the three layers of blotting paper, and 500g of weight was added on top of the paper towel. The DNA was allowed to transfer to the membrane overnight. After the transfer, the
membrane was washed gently in 6X SSC for 3-5 min, and was air dried and baked at 80°C for 2-4 h.

2.3.4.2 Prehybridization, Hybridization, and Washing

The membrane was incubated for 15 min in 15 ml of prehybridization solution (10% dextran sulfate, 1% SDS, 1 M NaCl) at 65°C in an incubation cylinder in a hybridization oven. In the meantime, hybridization solution was prepared as follows: 10 ml of prehybridization solution and 100 µl of salmon sperm DNA (10 mg/ml) were boiled in a 50 ml tube for 10 min, and then 15 µl of labeled probe (labeled using Promega Nick Translation Kit) was added to the tube immediately. The prehybridization solution from the first step was discarded and replaced with the hybridization solution, then membrane was incubated overnight at 65°C. After hybridization, the probe was discarded or frozen for another use. The membrane was kept inside the cylinder and 50 ml of 2x SSC was added to rinse the membrane; this step was repeated four times. After four rinses, the membrane was washed with 100 ml of 2x SSC, 1% SDS (preheated to 65°C) for 30-60 min. The membrane was then rinsed with 100 ml of 0.1x SSC at RT and then air dried. Autoradiography was done with a Kodak X-Omat AK film and the X-ray film was developed after 24 h exposure at -80°C.

2.3.5 Probe Preparation (Nick Translation)

DNA (0.5 µg) was incubated with 2.5 µl of 10X nick-translation buffer (0.5 M Tris (pH 7.5), 0.1 M MgSO₄, 1 mM dithiothreitol, 500 µg/ml BSA (Fraction V; Sigma)), 20
nmol each of dTTP, dCTP, and dGTP, 16 pmol of [α-32 p] dATP in a total volume of 21.5 µl. The mixture was chilled to 0°C and 2.5 µl of DNAase I (Pharmacia, 10 ng/ml) was added followed by 2.5 units of E. coli DNA polymerase I. The reaction was incubated for 60 minutes at 16°C and stopped with 1 µl of 0.5 M EDTA (pH 8.0). Unincorporated dNTPs were separated from radiolabeled DNA by spun-column chromatography.

2.3.6 DNA Sequencing

2.3.6.1 Preparing the Sequencing Gel

The sequencing gel solutions (60 ml) were made of 1X TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA; pH 8.0), 6 ml of 6% Long Ranger (J. T. Baker), 8M urea. Glass plates (37.5 x 42.5 cm, and 37.5 x 45 cm) were washed with detergent and rinsed thoroughly with dH2O and followed by washing with 95% ethanol. One of the sides of the short plate was treated with 5 ml of Silicone solution and dried and washed with dH2O. The two plates were assembled tightly together with a tape in the presence of 0.4 mm spacers between them and at both sides. Prior to pouring the gel solution, 250 µl of 10% ammonium persulfate and 25 µl of TEMED (N, N, N', N'-tetramethylethylene diamine) solution were added. The sequencing gel solution was poured on the side of the longer plate while maintaining the glass plate at approximately 45° angle. The gel was allowed to polymerize for one hour and was used the following day.
Sequencing Reactions

Sequencing reactions were based on the dideoxy nucleotide (ddNTP) chain-termination method (Sanger et al., 1977). The synthesis of DNA strand by DNA polymerase in vitro, and termination of the elongation by means of incorporation of nucleotide 2', 3' dideoxynucleoside 5' triphosphate lacking 3'-OH group which is important for DNA elongation.

All the materials used were from a sequencing kit (United States Biochemical). From 2 to 3 μg of plasmid DNA to be sequenced was denatured 1/10 of the volume of 0.2 M NaOH, 0.2 mM EDTA for 30 min at 37°C, then it was neutralized with 1/10 of the volume of 3 M NaOAc (pH 4.8). The DNA was precipitated with 2 volumes of 95% ethanol for 20 min in -70°C then pelleted by centrifugation at 13,000 rpm for 10 min. The DNA pellet was resuspended in 7 μl dH2O. Two μl of 5X sequencing buffer (200 mM Tris, pH 7.5, 100 mM MgCl2, 250 mM NaCl) and 1 μl of primer (20-50 ng) were added to the DNA. The samples were kept at 65°C for 2 min followed by gradual cooling to 30°C. While tubes were cooling, the labeling mix (7.5 uM dGTP, 7.5 uM dCTP, and 7.5 uM dTTP) was diluted 1:5 in dH2O. Sequenase® enzyme was diluted 1:8 in TE buffer and kept on ice. Then, 2.5 μl of each termination mix (ddGTP, ddATP, ddCTP, or ddTTP) was dispensed into microfuge tubes and prewarmed to 37°C for at least 1 min. Each termination mix contained 8 uM of one ddNTP and 80 uM of the three other dNTP.

For the labeling reaction, 1 μl of 0.1 M DDT, 2 μl of diluted labeling mix, and 0.5-1 μl of [α-35S]dATP were added to 10 μl of the DNA-primer mixture. Then 2 μl of diluted Sequenase® was added and reaction was allowed for 5 min at RT. From the
reaction 3.5 μl was transformed to each of the four termination tubes and the mixture was kept for 5 min at 37°C. The reaction was terminated by adding 4 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). The tubes were heated to 80°C for two min then 2-3 μl of each sample was loaded on the gel. The gel was run for several hours at 1.25 A/cm in 0.6X TBE buffer. Upon completion the gel was lifted off the glass plates with a #3 whatman filter paper, covered with a plastic wrapper and vaccum dried at 80°C for 20 min in a Bio-Rad model S83 gel drier. The dried gel was exposed to Kodak X-Omat AK film for 72 h at -70°C. The X-ray film was developed and fixed with Kodak GBX solutions.

2.4 Mammalian Cell Culture Technique

2.4.1 Propagation and Maintenance of Cell Lines

Madin-Darby bovine kidney (MDBK) (ATCC Number: CCL-22); M5 and E61 cells (MDBK cells transfected with E1 of BAV3) were grown in 150 mm plates (Burleson et al., 1992) with Minimum Essential Medium (MEM) (Gibco, BRL) supplemented with either 6% Fetal bovine serum (FBS), 5% Hours serum (HS) or 6% Donor serum (DS), plus 0.29 μg/ml of L-glutamine, 0.225% sodium bicarbonate and contained 1.5% antibiotic-antimycotic (10,000 unit/mL penicillin, 25 μg/mL amphotericin B, and 10,000 μg/mL streptomycin) (Gibco, BRL). The cells were grown at 37°C in a humid atmosphere of 5% CO₂.
2.4.2 Viral Propagation and Purification

Semiconfluent 150 mm dishes of MDBK or M5 cells were infected with BAV3 24 h after plating at a multiplicity of infection (MOI) of 1-10 plaque-forming units (pfu) per cell. The cells were maintained in MEM supplemented with 6% FBS at 37°C, 5% CO₂ incubator. When approximately 80-90% cytopathic effect (cpe) was observed (48-72 h), the cells were collected in tubes and pelleted at 1,000 rpm for 15 min at 4°C. Cell pellet from a 150 mm dish was resuspended in 1 ml of 15 mM Tris.HCl pH 8.0. The virus was then released from the cells by several freeze-thaw cycles in liquid N₂.

The cell debris was eliminated by centrifugation and to every 2-3 ml of sonicated cell suspension, 1.8 ml of saturated CsCl solution was added (final density was approximately 1.34 g/ml). The mixture was centrifuged at 35,000 rpm for 20 h at 4°C in 3 ml Beckman tubes in Beckman TL-100 ultracentrifuge (TLA-100 rotor). The viral band was then collected and dialyzed extensively against 50 mM Tris.HCl pH 8.0 at 4°C.

2.4.3 Plaque Assay

The plaque assay is a quantitative assay that determines the number of infectious virus particles present in a virus suspension.

Briefly, tenfold of virus dilutions (10⁻¹-10⁻¹⁰) were prepared in PBS²⁺. From the dilutions 10⁻²-10⁻⁷, 0.2 ml were used to infect six-well tissue culture plate containing a monolayer of MDBK cells (cells were seeded the day prior to infection). Before infection, the medium was removed from the wells and 0.2 ml of virus dilutions were added to the cells. The cells were then incubated at RT for 45-60 min to allow the
adsorption of the virus. Four ml of a 1:1 mix of 2% Noble agar and 2 X MEM was brought to 42°C and overlayed into each well. Once the agar solidified, the plate was placed at 37°C in a CO₂ incubator until plaques were observed within 4-6 days.

2.4.4 Viral DNA Extraction

Viral DNA extraction was carried out using two different procedures; Hirts extraction technique (Hirts, 1967) and through a buoyant CsCl density gradient with 4M guanidine hydrochloride (Kanegane et al., 1994).

2.4.4.1 Hirts Extraction

After the optimum cpe of infected cells was observed, approximately 1-2 ml of lysing buffer (0.01 M Tris; 0.01 M EDTA; 4% SDS; pH 8.0) with 1X pronase (25X stock; 12.5 mg/ml in 0.01 M Tris, pH 8.0) was added directly to the plates and incubated for 2-3 h at 37°C. The viral DNA then was extracted with phenol: chloroform: isoamyl alcohol (25:24:1 v/v). The upper phase (aqueous phase containing viral DNA) was collected in fresh tubes, and the viral DNA was precipitated with 2 volumes of cold 95% ethanol, followed by centrifugation at 13,000 rpm. Then the DNA pellet was resuspended in appropriate volume of TE buffer or dH₂O.

2.4.4.2 Viral DNA Extraction using CsCl Density Gradient

This procedure was carried out as described by Kanegane et al., (1994). The purified virus from CsCl gradient was lysed by addition of an equal volume of 8 M guanidine hydrochloride. Then the released viral DNA was purified through a buoyant
density gradient of 2.8 M CsCl/4 M guanidine hydrochloride by centrifugation in 3 ml Beckman tubes for 16 h at 55,000 rpm (TLA-100 rotor). The DNA-containing fractions were identified by running 1 μl of each fraction on 0.9% agarose gel electrophoresis, and visualized by staining with EtBr and placed on UV light. The DNA-containing fractions were then extensively dialyzed against 15 mM Tris pH 8.0 at 4°C. The DNA was resuspended in an appropriate volume of dH2O or TE buffer and kept at -20°C.

2.4.5 DNA Transfection

Transient transfection of viral DNA was carried out by different methods and the most common procedures used in this study were calcium phosphate-DNA precipitation technique (Graham and Van der Eb, 1973) and the use of lipofectAMINE reagent™ (Gibco, BRL).

2.4.5.1 DNA Transfection using Calcium Phosphate

The day before transfection, 3 x 10^5 cells were seeded in 60 mm dish and placed in CO2 incubator. Transfection reactions for four dishes of 60 mm were set as follows:

Two ml of 1 X HEPES-buffered saline (HEBs, 5 g HEPES; 8 g NaCl; 0.37 g KCl; 0.125 g Na2HPO4.2H2O; 1 g glucose; final pH 7.1; HEBs was aliquoted into small glass bottles, sterilized by autoclaving, and stored at 4°C) was well mixed with 10 μg/ml of carrier DNA (Salmon sperm). The HEBs and carrier DNA mixture was then aliquoted into four clear plastic tubes (0.5 ml each in tube). To each tube 5-15 μg plasmid DNA
and 2-5 digested viral DNA were added. Slowly, 2.5 M CaCl₂ (50 µl/ml) was added to a final concentration of 125 mM. The mixture was well mixed and incubated at RT for 10-20 min (a fine precipitate was seen in a few min). Then the suspension was added to dishes containing cells without removing the growth media and incubated at 37°C in CO₂ incubator for 4 h. Four h post-transfection, the cells were glycerol-shocked. The medium was aspirated and 1 ml of 10% glycerol (in complete medium) was added and spread over the cells by tilting the dish several times. The glycerol solution was left for 20-60 seconds after which the cells were rinsed with 2 ml of fresh medium; finally, 4 ml of fresh growth medium was added to each dish.

2.4.5.2 Transfection using the LipofectAMINE Reagent™

The transfection using this procedure was carried out according to the supplier's recommendations (Gibco, BRL). In brief, one day before transfection, 3 x 10⁵ cells per well was plated in six-well plates and incubated at 37°C in a CO₂ incubator. For each transfection, 1-5 µg of DNA was diluted in 100 µl serum-free medium, and in another tube, 2-6 µl of lipofectAMINE™ (4-12 µg) was diluted in 100 µl serum-free medium. The two solutions were then combined, mixed gently, and incubated at RT for 15-45 min. For each transfection, 800 µl serum-free medium was added to the tubes containing the complexes. In the meanwhile, the cells were rinsed with 2 ml serum-free medium, then the transfection mixture was overlayed on the cells. Then the cells were incubated at 37°C in CO₂ incubator for 4-10 h. After exposing cells to DNA-lipofectAMINE complex, cells were overlayed with 4 ml of 1:1 of Noble agar (2%) and
2X MEM (42°C). Once agar solidified the cells were incubated at 37°C in a CO2 incubator.

2.4.6 β-gal Staining of Mammalian Cells

Cells previously transfected with a plasmid that carries the *E. coli* β-Galactosidase reporter gene were stained for β-galactosidase activity. First, the media was aspirated, and the cells were washed with 1X PBS (2 ml for 35 mm dish). The cells were then fixed by adding 1 ml of 4% paraformaldehyde (pH 7.4) and were kept at RT for 1-3 min. The cells were then washed twice with 1X PBS, followed by adding X-gal mixture (35 mM potassium ferricyanide, 35 mM potassium ferrocyanide, 2 mM MgCl2 pH 7.3). The cells were incubated at 37°C in a CO2 incubator for 2 h to overnight. All positive cells (blue cells) were observed and counted under a light microscope.
Chapter 3: Results

3.1 Sequencing of BAV3 DNA

Since only small portions (0%-11.7% and 77%-92%) of BAV3 have been sequenced previously (Elgadi et al., 1993; Mittal et al., 1992), it was thought necessary to extend the sequencing so that a detailed knowledge of the restriction sites available for cloning can be obtained. Moreover, since BAV3 is partially characterized, it seemed to be important to determine the nucleotide sequences of the unknown regions of the viral genome. This work was thought to be a necessary step to study and analyze BAV3 proteins and the evolution of the virus.

3.1.1 Sequencing Strategy

To determine the nucleotide sequence of BAV3 between 11.7% and 30.8%, a single plasmid containing 0-53.9% of BAV3 genome (pBA59, Haj-Ahmad’s lab) was used. Sequencing of the cloned viral DNA fragments on either strand was initiated with oligonucleotides (Procyon Biopharma, London, Ontario) specific for known viral sequences (figure 3-1). The walking-primer technique was used to obtain the contiguous sequence using primers that were spaced apart to give at least 100 base
null cannot be predetermined. It

must be determined by the

state. This has been the
case in many countries in the

past. However, it is now clear
that this approach is

unsatisfactory and needs to be

replaced by a more

systematic and rational
treatment. The problem is to

determine the appropriate

level of intervention and

control that is necessary to

achieve the desired

outcomes. This requires

a careful analysis of the

economic and social

implications of different

options. It is not a

matter of simple

prescriptions but

involves complex

trade-offs.
Figure 3-1: Sequencing strategy for BAV3 DNA.

The top line shows the map of BAV3 genome 0-30.8% with landmark restriction enzyme sites. For sequencing, the BAV3 DNA 11.7%-30.8% is shown (middle line), was used as template. Custom-made primers (arrows) were used to sequence the target region. The bottom boxes show the longest ORFs for DNA pol and pTP. The ORF for the DNA pol extends 3504 bp within the r-strand of the viral DNA. The first ATG from the amino-end was found at nt433. The longest ORF for pTP was identified in the sequence reading in a leftward manner. The pTP ORF extends 1998 bp and the first ATG in the ORF can be found at nt7.
to overlaps. To verify the sequence, sequencing was performed on the other strand using primers that were similarly spaced. Sequence conflicts between the two strands were resolved by repeated sequencing around the area in question until a consensus was achieved.

3.2 Sequence Analysis of BAV3 DNA

As mentioned earlier, the nucleotide sequences of BAV3 genome from 11.7% to 30.8% were determined. Analysis of the deduced sequence revealed two large ORFs. The first ORF encoded within the leftward-reading strand of the viral DNA was determined between 13.5-23.6%. However, the other ORF encoded in the same direction of the viral DNA was found between 23.0-28.7% (Figure 3-1). The polypeptide sequences predicted from these ORFs with translational initiation at the first ATG was submitted for homology searches to the European Molecular Biology Laboratory (Hinxton, UK) using the BLITZ algorithm (Smith and Waterman, 1981). The algorithm identified the first ORF scored 58.9-61.6% match with the DNA pol of several human Ads that include serotypes 2, 5, 7, 12 and 40; the closest matches with other non Ad protein sequences were 1.3% or lower. There is, therefore, a high probability that this ORF was correctly identified as for the DNA pol gene of BAV3. On the other hand, the algorithm identified that the second ORF scored 44.3% to 56.8% match with the pTP of some human Ads, and the closest matches were with other non Ad protein sequences were found to be 2.2% or lower. Then, a high probability that this ORF could be for pTP.
3.2.1 Sequence Analysis of BAV3 DNA pol

Figure 3-2 shows the nucleotide sequence of the ORF containing the putative DNA pol gene of BAV3. This sequence corresponds to the r-strand written in 5' to 3' direction. The sequence (3,504 bp) spans coordinates 13.5-23.6%. The sequence starts at the first nucleotide of the first codon of the ORF and ends at the third nucleotide of the termination codon of the ORF (TAG). The line below the nucleotide sequence is the predicted polypeptide sequence. The first in-phase ATG that can be identified in the large ORF in the sequence is located at nt433; thus 144 aa residues precede the first met of the polypeptide sequence.
Figure 3-2: DNA sequence of BAV3 DNA pol

The complete DNA sequence of the ORF of BAV3 DNA pol with the deduced peptide sequence (italics). The sequence is written in the 5' to 3' direction. The aa residues are shown in the universal one-letter notation. The stop codons are represented by a dot (.) at the end of each sequence.
3.2.2 Comparison of BAV3 DNA pol with other Ads DNA pols

An alignment comparison was made for several Ad pols (human Ad2, Ad5, Ad7, Ad12, Ad40, canine Ad1 & BAV2) with BAV3 (Figure 3-3). For this purpose, peptide sequences deduced from their DNA sequences that were obtained from GenBank were used. The peptide sequences used in the analysis consisted of the complete ORF for Ad pol instead of the shorter sequence that start at the first ATG in the ORF since it has been shown previously that the active Ad5 pol requires the sequences upstream of the first ATG in the main body of Ad pol (Stunnenberg et al., 1988). In these sequences, the first met in BAV3 is located at residue 145; however, in Ad2 and Ad5 pols is located at residue 147, residue 73 for Ad7, residue 130 for Ad12, residue 103 for Ad40, residue 122 for BAV2, and residue 170 for canine Ad1.

Several segments were deduced from the Ad pol alignment (boxed in Figure 3-3) that are highly conserved between human, canine and bovine adenoviruses. Each conserved segment consists of residues that are found in at least 6 of the 8 different Ad pols aligned. Thus conserved sequences, albeit in short patches, can be found in the N-termini preceding the first met (circled for each Ad pol) in the sequence. Interestingly, the segment spanning residues 1 to 200 using Ad2 pol coordinates contains the highest number of sequence variation, including gaps that are common to Ad pols of BAV3, BAV2 and canine Ad1 (residues 40-41, 74-84, 141-143 and 181-198). Longer stretches of conserved sequences can be found between residues 243-308 which are immediately preceded and followed by shorter stretches on both sides.

The segment of the sequences spanning 66% towards the C-termini contains extensive regions of highly conserved sequences among the Ad pols. The six regions of
Figure 3-3: Alignment of various Ad DNA pols

DNA sequences for Ad2 (GenBank locus ADRCG), Ad5 (Ad5001), Ad7 (AD7001), Ad12(AT12VGA), Ad40 (ADRGENOME), and canine Ad1 (CAU55001) were obtained from GenBank and used to deduce the respective DNA pols of these viruses. For each sequence the indicated location of the viral DNA pol was used to predict the peptide sequence. Since the coordinates given for these sequences only include the ORF that starts with the most upstream ATG, the sequence of any contiguous ORF upstream from the ATG was added to the peptide chain for analysis. For BAV3, the peptide sequences as shown in Figure 3-2 was used in the alignment analysis. The multiple alignment feature of GeneJockeyll was used for the analysis. For comparison, the full sequence for Ad2 pol is shown at the top and the aligned sequences below show a period (.) for a residue that is identical to the Ad2 sequence. Boxes indicate regions with highly conserved sequences and bars show regions shown to share a high degree of homology with some non-ads DNA pols including the human DNA pol α (Wong et al., 1988).
homology found between Ad2 pol, human DNA pol a, herpes simplex virus DNA pol, vaccinia virus DNA pol, and prokaryotic pols from T4 and F29 (Wong et al., 1988; Earl et al., 1986), are located. In general, these regions of homology with other pol are approximately in the same locations where highly conserved sequences between BAV3 and other Ad pols can be found. However, the precise coordinates do not necessarily correlate. For example, region IV (residues 392-409 in the Ad2 pol sequence) is not highly conserved among the Ad pols aligned including BAV3. In fact, a highly conserved segment can be found between residues 418-438 immediately following region IV with few differences in case of BAVs and canine Ad (BAV3 only one aa). Region II connects two highly conserved domains (residues 661-677, and 686-713) that are interrupted by a short segment of variable sequence. Similarly, region VI is located on two conserved segments. However, previous mutagenesis studies have indicated that sequence alterations in these domains that are conserved between Ad pols and other pol greatly impair the activity of the Ad5 pol in an in vitro DNA replication initiation assay (Chen and Horwitz, 1989), thus indicating that the structural domains are functionally relevant.

3.2.3 Similarity and Divergence between BAV3 DNA pol and other Ads DNA pols

Comparison of Ad pols at the protein level between BAV3 and human Ads (Ad2, Ad5, Ad7, Ad12, Ad40), canine Ad and BAV2 showed that the degree of similarity of these pols to BAV3 ranged between 58.3% to 60.5% (Table 3-1). Between the human Ads, the degree of similarity was between 71.3-99.5%, suggesting a strong relatedness between these serotypes. For BAV3 and its counterpart BAV2, however, their relatively lower similarity indicates earlier divergence during their evolution.
Table 3-1: Percent similarity and divergence of various Ad DNA pols

The peptide sequence analysis feature of DNASTAR was used to calculate the degree of similarity and divergence (expressed in %) between BAV3, BAV2, canine Ad1 (CAd1), Ad40, Ad12, Ad7, Ad5 and Ad2.

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Percent Divergence
3.2.4 Sequence Analysis of BAV3 pTP

The nucleotide sequence of BAV3 pTP, which consists of 1,998 nucleotide and capable of encoding a 665-residue polypeptide, is shown in Figure 3-4. This sequence corresponds to the r-strand and written in 5' to 3' direction. The first ATG in the ORF was found at nt7; therefore, two polypeptide residues precede the first met in the ORF. The sequence shown in Figure 3-4 contains nucleotide sequence of the long ORF (plus the sequence preceding the first ATG) since it has been shown that these sequences are important for pTP activities (Zhao and Padmanabhan, 1988).
No text content is visible in the image provided.
Figure 3-4: The pTP DNA sequences of BAV3

The nucleotide sequence (top) and the predicted amino acid sequence (italicized, single-letter universal codes) are shown for the entire pTP ORF of BAV3.
CTCTTTTCAGGTTCAAAAGTGACCGGCGCATGACATTCACCCCCACAACCCGAGATCTCAGAGAATCAAACCGACAGATC
LSFRFKVTGPAFTQHPERIQORINRRV

GTGCAGACGCGCATCGACCTGCACCAACACGGCTGCAACCCAGACGTCCGATCCGCTCCAGCTACCGCCCTC
VQHASDLRQPMPELNDPVQLPPL

AGCCCCGAAAAGGCAAGGCGCGCCTCGGGCCCTCGAGTTTTAA
RPERQRPPPLGPRRPLstp

1872 624
1950 650
1998 666
3.2.5 Comparison of BAV3 pTP with other Ads pTPs

Figure 3-5 shows the multiple alignment analyses of the deduced pTP sequences of BAV3 and pTPs from other Ads. All the sequences shown in the figure include the peptides preceding the first met in the ORF. For BAV3 only 2 residues preceded the first ATG, and for human Ad2, Ad5, and Ad40 are 15 residues. However, the number of the residues preceded the first ATG in case of Ad7 is 55, and 97 for Ad12. For non human Ads, 15 residues for the canine, 84 and 41 for the two avian Ads, respectively, and 13 for the ovine Ad.

Nine conserved domains have been identified from the multiple alignment analyses. Each conserved segment consisted of residues that were found in at least 6 of the 8 different Ad polys aligned. Conserved domain I consisted of 26 blocks of conserved sequences. In the first block, the aa sequence DCARLTG was found in all the sequences except for CELO, fowl Ad and ovine Ad . This sequence was found after the first ATG in BAV3, BAV2, Ad7, and canine Ad. The same sequence can be located before the first ATG in pTPs of human Ad2, Ad5 and Ad40.

Several conserved sequences were found within conserved domain II. The most conserved sequence was LAERVVADLAL which was found in BAV3 and some human Ads such as Ad2, Ad5, Ad12 and Ad40 and non human canine Ad. This sequence appeared to be less conserved in Ad7 and BAV2 and did not exist in ovine Ad.

It has been shown that domain V contains the nuclear localization sequence RLPVR6WP (Zhao and Padmanabhan, 1988). This was not found in BAV3 nor in some other Ads, but it was found in the pTP sequences of all human Ads and BAV2 (Figure 3-5).
Five other domains with high degree of conservation were found within domains III, IV, and VI to IX. Domain III consisted of several sequences along a length of 50 residues. The conserved sequence LSLPCDCDWLDAF was found as the predominant feature of domain IV, with minor differences in each pTP sequence; for example E instead of the second D in case of BAV3. However, this sequence was not found in avian and ovine Ad pTP. Domains VI, VII, VIII and IX spanned a length of 72, 67, 44, 34 residues respectively.

3.2.6 Similarity and Divergence between BAV3 pTP and other Ads pTPs

Table 3-2 shows sequence pair distance analyses of pTP of BAV3 and pTP of other Ads. BAV3 showed a high % of similarity to human Ads, especially to Ad40 (58.5%), and canine Ad; however, it showed lower similarity to BAV2. Very low similarity was detected between BAV3 and CELO, fowl and ovine Ads (26.8%, 25.7% and 25.1% respectively).
Figure 3-5: Multiple sequence alignment for various Ad pTPs

DNA sequences containing the pTP coding region were obtained from GenBank. The predicted polypeptide sequences were aligned using the clustal variation of the Higgins and Sharp method, which was a feature of the software GenJockey II. The circled methionine residues represent the first ATG of each pTP ORF sequence. For the aligned sequences, the top line is for human Ad2; a dot (".") represent conserved sequences.
### III

| Ad2  | PKHMLKIVLTHL.D.LPEVNYISSMCA  | --RNNEDR--HPLPAVCSLPCDDLEELFLERHSDPVADSLR-SLG | G0V7 | 318 |
| Ad5  | L.M... | . F.T. IVSPSFQEGFEE.E... ASRR.R.QE. | V.| LETI | . R | G | 440 |
| Ad7  | V.K... | . IAQ... | --LPGITL--EE.R | . | M.TQ.S | . | 354 |
| Ad12 | L.T... | . FL... | --I6SL--T.LAEDEA.R | LQQ | | | |
| Ad40 | L.V... | . FLYKLD... | -- H | . | . | . |
| BAV2 | R.L... | . ESFD... | A.EIDEA.R | LQQ | | | |
| BAV3 | RR... | . FLHPP... | -- N... | . TE.AS | --PM | . | . |
| Cda1 | N.I... | . K.F... | -- FLV.RFP... | -- | | | |
| CELO | AA... | . AE.P... | -- LFCYADLPQTE... | RYRF.GSD | FLE | -- | | |
| FowlAd | HR... | . AA.N... | -- LFCYADLPQTE... | TYRF.GSE | FAE | -- | | |
| OvineAd | ER... | . TY.L... | -- L.NMHI | -- | | | |

### IV

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### V

### VI

### VII

### VIII
Table 3-2: Sequence pair distances analysis of various Ad pTPs

Pair distances for all 11pTP sequences representing the entire ORF were determined using the software DNASTAR. The default PAM residue weight table setting of 250 was used.

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Percent divergence
3.3 Construction of BAV3 Deletion mutants

3.3.1 Construction of an E1 Deletion Vector

According to the strategy depicted in Figure 1-10 for the generation of E1-replacement recombinant BAV, a shuttle vector containing the foreign gene to be rescued into the viral DNA will be required. The appropriate shuttle vector must have the following properties to be useful. First, the viral DNA it contains must span from the first nucleotide residue (0%) up to an appropriate distance in the internal region such that a reasonable amount of homologous pairing can be achieved between itself and the cotransfecting viral DNA. Second, the shuttle vector must contain the E1 deletion. Third, the shuttle vector must contain convenient cloning sites so that foreign genes can be easily inserted to replace the deleted E1 sequences.

The construction of the E1 shuttle vector was first attempted after careful consideration of the established restriction enzyme map and the known sequence data of the BAV3 genome (0%-30.8%). Previous studies have revealed that the E1 region of BAV3 extends from 1.3% and 10.4% and that the putative translational start codon of E1A could be found at nucleotide 607, or at 1.3% of the genome, as revealed by the complete sequence analysis of that region (Elgadi et al., 1993). Therefore, deletion of the region would result in the complete elimination of the entire E1 region.

To accomplish the goal to construct a vector in which the E1 region was deleted, the restriction map of E1 was analyzed in more detail. The site for SacI was found at nt582 (1.3%) and Clal site at nt3094 (9.0%); hence, a 2512 bp fragment can be easily deleted from the vector to remove the E1 region. This is less than the more complete deletion between 1.3% to 10.4%, but the presence of the convenient SacI and Clal
sites ultimately suggested a simple approach to introduce the deletion that was adopted. In addition, it is known that the pIX protein product is required for packaging full-length viral genomes at least for human Ad (Ghosh-Choudhury et al., 1986), and in BAV3 the start of the pIX coding sequence resides at 9.2%. The sequences upstream from the first ORF in E1A (1.3%) are required in cis and therefore cannot be deleted. These sequences contain the ITR, packaging domains, and the E1 promoter (required for endogenous expression). Therefore, a deletion between 1.3% to 9.0%, as demarcated by the SacI and Clal sites, assured that E1 was completely inactivated but left pIX intact.

In addition to E1 deletion between 1.3% and 9%, the E1 shuttle plasmid was constructed to carry at least 50% of the BAV3 genome (left end). The constructed E1 shuttle plasmid would be used as an expression vector for foreign genes, relying on the E1 promoter for their expression. Subsequently, this plasmid would be used to rescue recombinant BAV3 by cotransfection with wild-type BAV3 DNA with the right-end digested with a restriction enzyme (Figure 1-10).

The construction of the E1 shuttle plasmid involved several manipulations, and the strategy followed to construct this plasmid is described below and illustrated in Figures 3-6, 7, and 8. All the plasmids that were used in this study were based on pUC19 which contains ampicillin resistance ($amp^R$), a bacterial origin of replication and a multiple cloning site.

To construct E1 shuttle plasmid a straightforward cloning strategy was carried out. First, the plasmid p1.3mcs (Figure 3-6), which carried BAV3 DNA sequences from 0% to 1.3% and a Clal/BamHI linker at 1.3% was constructed. The plasmid p1.3mcs
Figure 3-6: Construction of p1.3mcsK

The plasmid pdl1E1 (Haj-Ahmad's lab) was constructed by inserting BAV3 DNA sequences 0%-1.3% (581 bp) into the polycloning site of pUC19. The plasmid pdl1E1 was digested with SacI and EcoRI at 1.3%, then the linearized plasmid was ligated to a linker of 34 bp (restriction sites within the linker are shown). The ligation mixture was then transformed into *E. coli*. The desired recombinant plasmid, p1.3mcs, was analyzed using agarose gel electrophoresis and DNA sequencing to determine the orientation of the linker. The generated p1.3mcs was 3.3 kb and contained the left-end of BAV3 (0% -1.3%) plus polycloning site at 1.3% of BAV3. BAV3 sequences are shown by shaded area. To construct p1.3mcsK, as shown in the figure, the plasmid p1.3mcs was digested with *BamHI* at position 1038. Also the plasmid pUC4K was digested with *BamHI* at positions 405 and 1669 to release the *kan^R* gene. Following digestion with subsequent inactivation of the restriction enzymes, the two plasmids were ligated. The products was the plasmid p1.3mcsK (4.5 kb), which carried *kan^R* gene. The *kan^R* gene and BAV3 sequences contained in the constructed plasmid are shown in the figure.
Sac I + EcoRI

Ligase

Sac I Xho I Sal I Cla I BamHI EcoRI
CTCGAGGTCGACATCGATGGATCCG
TCGAGGAGCTCCAGCTGTAGCTACCTAGGCTTAA

BamHI

Ligase

Sac I 3.3Kb

Sac I 3.9Kb

Sac I 4.5Kb
was derived from pdl1E1 (Figure 3-6), which carried 0% to 1.3% of the BAV3 genome and also inserted into pUC19. The strategy followed to construct p1.3mcs is described below and illustrated in Figure 3-6. The pdl1E1 was digested with Sacl at position 1016 and EcoRI at position 1020 (at 1.3% of BAV3). The linearized pdl1E1 was then ligated to a linker containing the restriction sites Sacl, Xhol, SalI, Clal, BamHI and EcoRI. The linker in p1.3mcs would have been convenient to introduce a deletion in the E1 region.

It was thought that lacking of any selectable marker to identify the desired plasmids in the construction strategy of E1 shuttle plasmid would be time consuming. The large number of bacterial colonies which might generate from each transformation would make it difficult to identify the desired colonies easily. Therefore, to facilitate the recovery of every desired clone and decrease the number of the transformants that needed to be screened, a selectable marker was used.

The strategy involved inserting a kanamycin resistance (kan^) gene at 1.3% of the plasmid p1.3mcs, and at 53.9% of the plasmid pBA59, which carries BAV3 sequences from 0% to 53.9% inserted in pUC19 (Figure 3-7). Firstly, The kan^ gene (1264 bp) was generated by digesting the plasmid pUC4K (Pharmcia) with BamHI at positions 405 and 1669, and was inserted at the BamHI site at 1.3% of p1.3mcs to generate the plasmid p1.3mcsK (Figure 3-6). Secondly, the kan^ gene, which was generated by digesting the plasmid pUC4K with BamHI, was also ligated to the plasmid pBA59 at 53.9% that had been linearized with BamHI (figure 3-7). The generated pBA59KnB carried BAV3 sequences 0-53.9% plus Kan^ gene inserted at 53.9%. Figure 3-7 shows the steps followed to construct pBA59KnB.
The plasmid pBA59KnB was constructed by inserting \( kan^R \) gene in \( BamH1 \) site at 53.9\% (in bold) of the plasmid pBA59. The plasmid pBA59 (21.5 Kb) carries BAV3 \( BamH1\)-A fragment representing the first 53.9\% of the genome. Briefly, \( kan^R \) gene was generated by digesting the plasmid pUC4K with \( BamH1 \) at positions 405 and 1669. The \( kan^R \) gene was gel purified and ligated to the plasmid pBA59 which had been cleaved with \( BamH1 \) at 53.9\% of BAV3 sequences. The resulting plasmid pBA59KnB (22.8 Kb) was \( amp^R \) and \( kan^R \). This plasmid was confirmed using different restriction endonucleases and by agarose gel electrophoresis.
**pBA59 (21.5 kb)**

- EcoRI (15510)
- HindIII (11205)
- HindIII (9630)
- ClaI (3527)(9%)
- HindIII (4520)
- EcoRI (7110)

**pUC4K (3.9 kb)**

- EcoRI (1593)
- HindIII (2122)
- EcoRI (15510)
- HindIII (19050)
- ClaI (1490)

**BanHI**

- EcoRI (20564)
- BanHI (20540)
- BanHI (53.9%)
- HindIII (19050)
- BanHI (1669)
- EcoRI (1687)

**Ligase**

**pBA59knB (22.8 kb)**

- EcoRI (15510)
- HindIII (9630)
- HindIII (11205)
To construct the E1 shuttle plasmid in which E1 was deleted the following steps were carried out (refer to Figure 3-8 for more details). The plasmids p1.3mcsK and pBA59KB were digested with Clal. Each plasmid contained two Clal sites: for pBA59KnB at positions 3527 and 19560, and at 1032 and 1299 for p1.3mcsK. Following digestion, the cleaved Clal fragment from pBA59KnB, which carried BAV3 from 9.0% to 53.9% plus the left end of the \( kan^R \) gene from SalI to Clal sites (230 bp), was inserted into p1.3mcsK at the Clal site (1.3%). The linearized p1.3mcsK contained 0%-1.3% sequences of BAV3 and the right-end of the \( kan^R \) gene (1011 bp). After ligation and transformation, the desired recombinant plasmid was recovered in \( amp-kan \) containing petri dishes. The product was the plasmid pdl3E1KnB which carried BAV3 sequences from 0% to 53.9%, and \( kan^R \) gene in BamHI site at 53.9%. The \( kan^R \) gene in the new plasmid, pdl3E1knB, was used only as a selectable marker to identify the desired colony and would be, eventually, released from this plasmid.

After constructing the pdl3E1KnB which carried more than 50% of BAV3 sequences with E1 region deleted, the next objective was to eliminate the \( kan^R \) gene from pdl3E1KnB to generate the plasmid pdl3E1, the E1 shuttle plasmid (Figure 3-8). The \( kan^R \) gene was released to make Clal site unique at position 1032 to be used for insertion of foreign genes in place of E1 region and to create more room for these genes. Briefly, the plasmid pdl3E1 was constructed by digesting the plasmid pdl3E1KnB with BamHI to release the \( kan^R \) gene. The product was the plasmid pdl3E1, which contained BAV3 sequences 0%-53.9% with a deletion in E1 from 1.3% to 9. The DNA sequences of the deletion is shown in Figure 3-9.
Figure 3-8: Construction of pdl3E1KnB and pdl3E1 and deletion of E1 region

The plasmids pBA59KnB and p1.3mcsK were cleaved with Clal. The two Clal sites in p1.3mcsK at positions 1032 and 1299 resulted in the release of the extreme right-end of the $kan^R$ gene. However, in the case of pBA59KnB, the result of the digestion with Clal (at positions 3527 and 19560) was the release of a fragment of BAV3 from 9% to 53.9% plus the left-end of the $kan^R$ gene. The released fragment from pBA59KnB was gel purified and ligated to the linearized p1.3mcsK. The $kan^R$ gene ends from both parental plasmids complimented each other resulting in intact $kan^R$ gene in the produced plasmid. The resulted pdl3E1KnB (20.3%), which was $kan^R$, carried BAV3 sequences 0%-53.9% with a deletion in E1 from 1.3% to 9%. The shuttle plasmid pdl3E1 was constructed by digesting pdl3E1KnB with BamHI (at positions 53.9% of BAV3 and 18029 of pUC19), which allowed the $kan^R$ gene to be released. The $kan^R$ gene was released to make Cla I site at position 1032 (in bold) a unique site for subsequent cloning in the E1 region. The plasmid pdl3E1 was confirmed by restriction endonucleases, and the deletion was further confirmed by DNA sequencing.
Figure 3-9: Deletion of the E1 region of BAV3

The E1 region was deleted from 1.3% to 9% (2512 bp) (dark region). The pIX ORF is shown. ClaI linker and BAV3 sequences at 1.3% and 9% are shown.
Figure 3-10: Restriction endonuclease map of the plasmid p1.3mcs. The nucleotide sequence of the pUC19 linker and BAV3 junctions are shown (upper). A photograph of an agarose gel of p1.3mcs digested with A) BamHI, B) ClaI, C) HindIII and Xhol, and D) SalI, respectively. Lane M lambda-HindIII marker (lower).
Figure 3-11: The restriction map of the plasmid pBA59KB. The orientation of the kan\textsuperscript{R} gene is shown in the plasmid with a shaded arrow. BAV3 sequences 0\%-53.9\% is represented by a dark region. Cla I sites at positions 3527 (in bold) and at 19560 would be used to introduce a deletion in E1 region (upper). A photograph of an agarose gel of pBA59KnB digested with A) BamHI, B) Clal, C) HindIII and D) KpnI, respectively. Lane M lambda-HindIII marker (lower).
Figure 3-12: The restriction map of p1.3mcsK. This plasmid contained a 581 bp from the extreme left-end of the BAV3 genome (shaded region) plus kan$^R$ gene. The multiple cloning site (SacI, XhoI, SalI, ClaI and BamHI) and BAV3 and kan$^R$ gene junctions sequences are shown (upper). A photograph of an agarose gel of p1.3mcsK digested with A) BamHI, B) ClaI, C) HindIII, and D) SalI, respectively. Lane M lambda-HindIII marker (lower).
Figure 3-13: The restriction endonuclease map of the plasmid pdl3E1 (19 kb). This plasmid carries the left-end of BAV3 genome (0%-53.9%) (shaded region) with a deletion in E1 (1.3%-9%). The nucleotide sequence of the deletion is shown (upper). A photograph of an agarose gel of pdl3E1 digested with A) XhoI, B) EcoRI, C) Clal, D) HindIII, E) BamHI and F) BamHI and Clal, respectively. Lane M lambda-HindIII marker (lower).
3.3.2 Insertion of $kan^R$ Gene in place of E1 Region

After the construction of the E1 shuttle plasmid (pdl3E1) in which E1 was deleted, it was decided to insert $kan^R$ gene as a reporter in place of E1. The $Kan^R$ gene was chosen as a foreign insert in E1 for the following reasons: a) any recombinant plasmid that carried the $kan^R$ could be easily identified; b) the plasmid pdl3E1 (derived from pUC19) can accommodate $kan^R$ gene; c) the small size of the $kan^R$ gene (1262 bp) should be within the packaging capacity of BAV3 genome; d) recombinant virus carries $kan^R$ gene can be detected easily. The $kan^R$ gene was inserted in E1 region without any exogenous promoter so that its expression would be driven by the E1 promoter in the recombinant vector. Such construct could be useful in studying the efficiency of the E1A promoter to express in various cell types.

The strategy followed to construct the plasmids pdl3E1K and pdl3E1FK, which contained the $kan^R$ gene in place of the deleted E1 region in two different orientations is described below and illustrated in Figure 3-14. First, the plasmid pdl3E1 was digested with Clal then large fragment of E. coli DNA pol I (Klenow) was used to obtain blunt ended. Then, the linearized pdl3E1 was ligated to the $kan^R$ gene, which was generated by cleaving pUC4K with HincII. The newly constructed plasmids, pdl3E1K and pdl3E1FK, would be used to rescue BAV3 E1 mutant by cotransfection with digested BAV3 DNA.
Figure 3-14: The strategy for inserting \textit{kan}^R\textit{gene in E1 region}

The plasmid pUC4K was digested with \textit{HincII} (blunt end) to release the \textit{kan}^R\textit{gene (1252 bp). Then, the \textit{kan}^R\textit{gene was inserted into the Clal site of the plasmid pdl3E1 at position 1032. The products were the plasmids pdl3E1K and pdl3E1FK, both of which carried \textit{kan}^R\textit{gene in two different orientations. The two orientations of the \textit{kan}^R\textit{gene were determined by digesting with Clal and Xhol. In addition to \textit{kan}^R\textit{gene, pdl3E1K and pdl3E1FK contained BAV3 sequence from 0% to 53.9% with a deletion in E1 from 1.3% to 9%.}
Figure 3-15: The restriction endonuclease map of the plasmid pdl3E1K (20.3 kb). BAV3 sequence (0% - 53.9%) is shown as a shaded region. kan\(^R\) gene inserted in place of E1. BAV3 and kan\(^R\) gene junctions sequences are shown (upper). A photograph of an agarose gel of pdl3E1K digested with A) HindIII, B) BamHI, C) Clal, D) EcoRI, E) Sall F) Pstl and l) Xhol, respectively. Lane M lambda-HindIII marker E) (lower).
Figure 3-16: The restriction endonuclease map of the plasmid pdl3E1FK (20.3 kb). BAV3 sequence (0% - 53.9%) is shown as a shaded region. $kan^R$ gene inserted in place of E1. BAV3 and $kan^R$ gene junctions sequences are shown (upper). A photograph of an agarose gel of pdl3E1FK digested with A) BamHI, B) Clal, C) HindIII and E) Xhol, respectively. Lane M lambda-HindIII marker (lower).
3.3.3 Construction of an E3 Shuttle Plasmid

At the same time while the work was going on to construct E1 shuttle plasmid, another strategy was applied to construct E3 shuttle plasmid for the same purpose. The E3 shuttle plasmid pdlE3 was constructed to carry BAV3 sequences from the right-end (37.1%-100%) except for a deletion in E3 region from 78.9% to 82.5% (Figure 3-17). Even though the E3 region of BAV3 is 1510 bp in length (Mittal et al., 1992), only 1281 bp were deleted due to a lack of convenient restriction sites to delete the whole region. The deleted sites were located 326 bp (XhoI site) downstream from the stop codon of the pVIII (viral protein) and only six bp upstream of the start codon of the fiber protein (at HpaI site). Figure 3-18 shows the deleted sequences from the E3 region. A SalI linker was inserted in the deleted E3 region to be used for subsequent subcloning and insertion of foreign genes. The constructed pdlE3 was confirmed using different restriction enzymes (Figure 3-17), and the deletion was analyzed using DNA sequencing.

The constructed pdlE3 would be used to rescue BAV3 recombinant mutant through cotransfection with wild-type DNA. The mutant virus would be generated as a result of homologous recombination between pdlE3 and restriction digested viral DNA in which the right end of the viral DNA is intact (Figure 1-11).
Figure 3-17: The restriction endonuclease map of the plasmid pdlE3. BAV3 sequences 37.1%-100% is represented by a shaded region. Sall site at positions 14580 (in bold) would be used to introduce foreign genes in E3 region (upper). A photograph of an agarose gel of pdlE3 digested with A) Xbal, B) Sall, C) EcoRI, D) BamHI, E) HindIII and F) KpnI, respectively. Lane M lambda-HindIII marker (lower).
Figure 3-18: The deletion in the E3 region

The deleted sequences (1281 bp) is indicated by the dark box. The start codon and the stop codon of the fiber and pVIII, respectively, are shown. The SalI linker within the deletion is also shown.
3.3.4 Insertion of the $kan^R$ Gene in Place of E3

As in the case of the E1 shuttle vector, the $kan^R$ gene was inserted in place of E3 as a reporter gene. The $kan^R$ gene was inserted in E3 region without any exogenous promoter hoping that the expression of $kan^R$ gene would be driven by E3 promoter or MLP promoter in the recombinant vector. The strategy followed to construct the plasmids pdlE3K, which carry the $kan^R$ gene in place of E3 region, is illustrated in Figure 3-19 and described below.

First, The $kan^R$ gene was generated by cleaving the plasmid pUC4K with SalI, and the plasmid pdlE3 was linearized with the same enzyme. The $kan^R$ gene was then ligated to the linearized pdlE3 to obtain pdlE3K, which carries BAV3 sequences from 37.1% to 100% and $kan^R$ gene inserted in the former E3 region.
Figure 3-19: Insertion of \(\text{kan}^R\) gene in E3 region
The plasmid pdIE3K was constructed by inserting the \(\text{kan}^R\) gene in \(\text{SalI}\) (bold) of the plasmid pdIE3. Briefly, the \(\text{kan}^R\) gene (1264 bp) was generated by digesting the plasmid pUC4K with \(\text{SalI}\) at positions 411 and 1663. The \(\text{kan}^R\) gene was ligated to the plasmid pdIE3 which had been cleaved with \(\text{SalI}\) at 14580. The resulting plasmid pdIE3K (24.7 Kb) was \(\text{kan}^R\)-\(\text{amp}^R\). This plasmid was confirmed using restriction enzyme analysis and gel electrophoresis.
Figure 3-20: The restriction endonuclease map of the plasmid pdlE3K (24.7 kb). BAV3 sequence (37.1% - 100%) is shown as a shaded region. The kan$^R$ gene is indicated (upper). A photograph of an agarose gel of pdlE3K digested with A) HindIII, B) BamHI, C) KpnI, D) EcoRI, E) SalI and F) XbaI, respectively. Lane M lambda-HindIII marker (lower).
3.4 Attempts to Rescue BAV3 E1 and E3 Mutants

Figures 3-21, 22 show the strategy that was applied to rescue BAV3 E1 and E3 recombinant vector. Five plasmids were used in this study to rescue BAV3 mutant; for E1 deletion-substitutions, pdl3E1, pdl3E1K or pdl3E1FK (Figures 3-13, 15, 16), and for E3, pdlE3 or pdlE3K (Figures 3-17, 20). The plasmid pdl3E1 carried BAV3 DNA sequences from 0% to 53.9% with a deletion in E1 from 1.3% to 9%. However, pdl3E1K and pdl3E1FK carried the same BAV3 sequences as pdl3E1 plus the $kan^R$ gene inserted in E1 region in two different orientations. The E3 plasmid, pdlE3, carried BAV3 DNA sequences from 37.1% to 100% with a deletion in E3 between 78.9% to 82.5%. The pdlE3K carried the same sequences in addition to the $kan^R$ gene inserted in place of E3 region.

In order to rescue BAV3 E1 mutant, M5 or E61 cells were cotransfected with the plasmid pdl3E1, pdl3E1K or pdl3E1FK along with BAV3 DNA digested with SalI. The mutant virus was expected to be generated by homologous recombination between one of the E1 plasmid and BAV3 SalI-A fragment (Figure 3-21). For rescuing the E3 mutants, M5, E61 or MDBK cells were cotransfected with one of the E3 plasmids and BAV3 DNA digested with PvuI (Figure 3-22). In the same way as in the E1 mutants, E3 deletion-substitution mutant would be generated by homologous recombination between E3 plasmids and the BAV3 PvuI-A fragment. In some cases, M5 and E61 cells were cotransfected with one of the E3 plasmids plus one of the E1 plasmids as attempt to rescue E1-E3 mutant BAV3.
Figure 3-21: Strategy for the rescue of E1 recombinant BAV3.

The E1 shuttle plasmid (with or without kanR gene) was cotransfected with BAV3 DNA digested with SalI (to prevent the regeneration of wild-type BAV3) into M5 and E61 cells. The homologous recombination was expected to take place between the overlapped sequences that exist in both E1 plasmid and SalI-A fragment to generate recombinant BAV3.
Figure 3-22: Strategy for the rescue of E3 recombinant BAV3.

M5, E61 and MDBK cells were separately cotransfected with the E3 shuttle plasmid along with BAV3 digested with *Pvu*I. The recombinant virus could be generated by homologous recombination between the overlapped DNA sequences in E1 shuttle plasmid and the *Pvu*I-A fragment. The overlapped region is indicated.
Basically, two transfection procedures were used in this study to rescue the recombinant virus; transfection using the calcium phosphate precipitation technique (Graham and Van der 1973) and cationic lipid-mediated transfection (lipofectAMINE™, BRL). Since transfection using calcium phosphate was shown to be efficient in rescuing several recombinant Ads (Haj-Ahmad and Graham 1986), it was chosen as a first attempt to rescue the recombinant BAV3.

3.4.1 Transfection using Calcium Phosphate Precipitation Technique

     Transfections were carried out as described by Graham and Van der Eb (1973) and Chen and Okayama (1987). MDBK, M5, and E61 cells (3 x 10^5 cells/ well-35mm) were seeded one day prior to transfection. Calcium phosphate-DNA precipitate was left on cells for 4-6 h, since longer exposure of the cells to the precipitate was found to cause cell death. In the case of cotransfection using E3 plasmids, BAV3 DNA was digested with BamHI to generate a 16.8% overlap with pdlE3 and pdlE3K, or with PvuI with 28.6% overlap. When pdl3E1, pdl3E1K and pdl3E1FK were used the viral DNA was digested with SalI to create 21.7% overlap. Some of cells were glycerol shocked (20 to 60 second) 4 h posttransfection. Glycerol shock for a longer period of time was found to be toxic to the cells. Dishes were observed for the presence of plaques for up to two weeks. Wild-type BAV3 DNA was used as a positive control (5,10, and 15 μg). The transfection conditions are shown in Table 3-3A and B.

     The first results showed that all attempts to rescue recombinant BAV3 using the calcium phosphate technique failed to produce identifiable plaques. Since the amounts of DNA and the exposure time of DNA complexes to the cells were within the ranges
Table 3-3A, B: Transfection conditions using calcium phosphate precipitation
Plasmid-to-viral DNA ratios were used as indicated. Cells that were transfected (+) or not transfected (-) for a particular experiment are as indicated. Transfections in which glycerol-shock was applied (+) or not applied (-) are as indicated. Table A summarizes transfections to rescue E1 mutants and Table B for rescue E3 mutants.
### A) E1

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<th>Viral DNA conc (in µg)</th>
<th>Cell line</th>
<th>Glyceraldehyde-3-phosphate dehydrogenase (E6/L)</th>
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<td>pdl3E1FK</td>
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### B) E3

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<th>Cell line</th>
<th>Glyceraldehyde-3-phosphate dehydrogenase (E6/L)</th>
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which have been used for this assay; therefore, it was thought that the transfection technique used could be the reason of not rescuing the mutant virus. Thus, it was necessary to try other methods of transfection in order to rescue the recombinant BAV3, and lipofectAMINE was the method of choice.

3.4.2 Transfection using LipofectAMINE™ Reagent

The other method used in this study was lipid-mediated transfection (LipofectAMINE™, BRL). This technique was chosen as a second attempt to rescue recombinant viruses because it has been shown that lipofectAMINE™ protocol was an efficient method for DNA transfection using E61 and MDBK cells (White, 1996). Thus experiments designed to optimize transfections with lipofectAMINE™ were carried out.

3.4.2.1 Optimization of The Transfection Condition using LipofectAMINE™

Two parameters thought to be important for transfection with lipofectAMINE™ are amount of DNA and concentration of lipofectAMINE™ itself. In addition, other parameters, such as cells density and time of incubation of cells with DNA-liposomes complexes, were evaluated.

The various transfection conditions were tested using M5 cells which were isolated for the purpose of rescuing both E1 and E3 mutants of BAV3. Cells were transfected with the plasmid pCMV-LacZ, a reporter plasmid with a LacZ gene under CMV promoter control. Cells that express LacZ can be visualized by staining with X-gal and scored blue cells usually 24 h posttransfection. The optimal condition was
determined using small scale reactions in 35mm tissue culture dishes. Since optimum lipid concentration is an important factor which dramatically enhance the transfection efficiency, the amount of lipofectAMINE™ was first optimized. After optimizing LipofectAMINE™ concentration, the number of cells and concentration of lipofectAMINE™ were held constant and DNA concentration was verified in order to determine the best DNA concentration. Figure 3-23A, B shows an evaluation of DNA uptake of M5 cells using different DNA and lipofectAMINE™ concentrations. It has been found that Low concentration of lipofectAMINE™ (1 or 2 μg) resulted in low transfection efficiency, and high concentration of lipofectAMINE™ (from 8 to 16 μg) caused cells death which resulted in very low transfection efficiency. The best lipid concentration was found to be between 4 and 6 μg. On the other hand, it was found that 1μg of DNA gave the optimum transfection; higher or lower DNA concentration resulted in lower transfection efficiency.
Figure 3-23A: Optimization of lipofectAMINE™ concentration

For each transfection, the number of cells, DNA concentration and duration of exposure of the cells to DNA were held constant, and the amount of lipofectAMINE reagent was verified according to the supplier's recommendations. The number of cells used was $3 \times 10^5$ cells/well, which gave 70-80% confluence, were transfected using 1 µg of DNA and 1, 2, 4, 8 or 16 µg of lipofectAMINE™, then, cells was incubated for 6 h before adding the growth media.

B. Optimization of DNA concentration

Following lipid concentration optimization, DNA concentration was similarly optimized. The number of cells ($3 \times 10^5$ cell/well), lipid concentration (5 µg), and time of exposure (6 h) were held constant, in the mean time different DNA concentrations were used (0.2-4.8 µg).
(A) Number of blue cells/well as a function of Lipofectamine concentration (µg).

(B) Number of blue cells/well as a function of DNA concentration (µg).
3.4.2.2 Attempt to Rescue Recombinant BAV3 using LipofectAMINE™

Even though the transfection efficiency using lipofectAMINE™ seemed to be quite low on M5 cells, it was decided to apply the determined optimum condition of transfection using lipofectAMINE™ for rescuing BAV3 mutant. The transfections were set according to the supplier's recommendation (see chapter 2). M5 cells were plated one day before transfection, at 3 X 10^5 cells / well, which gave 70-80% confluency on the day of transfection. The cells were transfected using 5μg of lipofectAMINE and 1μg of DNA (plasmid plus digested viral DNA). The ratio of the plasmid DNA to viral DNA was verified as indicated in Table 3-4. The cells were exposed to the lipofectAMINE-DNA complexes 6 h to overnight. The plates were kept for two weeks for plaque detection. However, from all the experiments carried out no plaques were ever detected from the experiments nor from the positive control. Thus it appears that BAV3 DNA has low infectivity so that recovery viral particles from DNA alone might call for different transfection strategies.
Table 3-4: Transfection condition using lipofectAMINE™

The E1 and E3 shuttle plasmids and the digested viral DNA concentrations used in this experiment are shown. The ratio of the plasmid DNA to viral DNA was verified (1:1, 1:10 and 10:1) as it is shown in the table. The (-) indicates that the plasmid was not used in that particular experiment.

<table>
<thead>
<tr>
<th>Plasmid DNA conc (in µg)</th>
<th>Viral DNA conc (in µg)</th>
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<td>pdlE3K</td>
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<td>0.5</td>
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Chapter 4: Discussion

The main objective of this research project was to develop BAV3 as a gene delivery vector for expression of foreign genes in target tissues and thus with potential application in gene therapy and recombinant vaccine production. The goals of this work were at least threefold. First, prior to this work there were only two regions of BAV3 genome that have been sequenced and analyzed (Mittal et al., 1992; Elgadi et al., 1993), so that the degree to which the BAV3 genome could be manipulated for vector development was severely restricted. Sequencing of the left-end of the viral DNA and construction of a restriction map for it and analyzing the sequenced regions was thought necessary to overcome the limitations in designing strategies for molecular manipulation of the viral DNA. Second, construction of the E1 and E3 shuttle plasmids with their corresponding deletions were central to the strategy overall for generating E1 or E3 BAV3 replacement mutants. Finally, insertion of a reporter gene to replace the deleted E1 and E3 within the shuttle plasmids, and subsequently rescuing the desired recombinant virus, could become the cornerstone in the development of BAV into cloning and expression vectors.

The development of adenovirus-based vectors for gene therapy and vaccines was dictated by a variety of reasons (reviewed by Graham and Prevec, 1991, 1992) some of which are as follows. First of all, compared to available protocols for gene transfer, adenoviral recombinant vectors seem to be unique in their ability to deliver
exogenous genes to many tissue types in vivo. Second, adenovirus appears to be relatively safe when used as a vector. For instance, no evidence has been found to date to implicate adenovirus in any human cancer, and diseases caused by such viruses are sub-clinical and rarely life threatening (reviewed in Rubin and Rorke 1994). Third, Ad vectors have the ability to accommodate large inserts especially after genetic modification to delete some parts of the viral genome (Haj-Ahmad and Graham 1986). Fourth, the ability of Ad vectors to function in vivo without affecting the stability of the foreign insert during viral replication cycle has made them promising as a tool for gene therapy (Stratford-Perricaudet et al., 1990).

Human Ad-based expression vectors are currently being extensively tested for their potential use in human gene therapy (Stratford-Perricaudet et al., 1990; Rosenfeld et al., 1991, 1992; Ragot et al., 1993). It seems, however, that the present generation of human adenoviral vectors suffer from a few problems which limit their full potential in gene therapy. One of the major limitations is the transient expression of the transgene in vivo and the poor transduction efficiency when a vector is readministered to the same recipient.

Some studies have shown that host cellular and humoral immune responses are responsible for the transient expression of delivered genes (Christ et al., 1997). Several mechanisms have been proposed to explain this phenomenon. First, despite the deletion of E1 region in the so-called first generation human adenoviral vectors, low level expression of viral proteins occur in vivo. These viral antigens appear to stimulate the cellular immune responses leading to the destruction of the viral vector-infected cells. The virally synthesized proteins within infected cells are presented by MHC class I
molecules to CD8$^+$ T cells which in turn are activated to form cytotoxic T lymphocytes that can specifically target vector-infected cells. In addition, viral proteins presented by MHC class II molecules lead to activation of CD4$^+$ helper T cells that stimulate cytotoxic T cells. The activated cytotoxic T cells destroy the vector-infected cells, and as a result the population of transduced cells disappear rapidly. In another mechanism, the production of anti-adenovirus antibodies which may have resulted from the first administration of the viral vector could prevent efficient transfer of the therapeutic gene to the target cells. This is so because the humoral response results in the production of capsid-specific neutralizing antibodies. These antibodies bind to the adenoviral vector to block its entry into target cells (Christ et al., 1997).

The new synthesis of viral proteins within transduced cells could be a result of several factors (reviewed by Joanne and David 1997). First, it seems that deletion of the E1 region in a viral vector is not sufficient to completely abolish expression of other early viral proteins, hence deletion of the coding sequences for such early regions might be necessary. Second, human patients may be carrying endogenous Ad due to persistent infection which may recombine with the input viral vector in vivo. This recombination may result in the reversion of an otherwise replication-deficient vector to the wild-type form, so that viral protein synthesis occurs. Third, the first generation human Ad vectors are propagated in novel cell lines that supply the missing viral functions in trans. For instance, the E1 deletion Ad5 vectors are grown in 293 cells which express the E1 proteins. It appears that propagation of E1 mutant vectors in these cells could generate wild-type Ad5. This is so because recombination between remaining sequences of the deleted E1 of the vector and corresponding wild-type
sequences retained in 293 cells could occur to produce wild-type revertants. Such wild-type contaminants could compromise the intended purpose of the vector as when used in human gene therapy.

Ideally, a useful Ad vector might be one in which viral genes are not expressed altogether. Other investigators have made the so-called "gutless" Ad vectors in which all early regions have been deleted (Hardy et al., 1997). Growing such virus, however has become problematic because it requires a wild-type helper virus which is difficult to purify from the mutant vector.

BAV3 has been shown to enter human cells but fail to replicate due to host range restrictions as shown recently in this laboratory (unpublished data). The exact block in the viral cycle has not been identified but it is now known that viral RNA and proteins are not made in the course of the nonpermissive host. A BAV3 mutant with a luciferase gene inserted in place of E3 was able to express the foreign gene in human cells relying on E3 and MLP promoters (Mittal et al., 1995). The ability of BAV vectors to transduce human cells without any viral proteins being produced makes BAV vectors particularly attractive as they may express foreign genes over longer periods of time in targeted cells. If this is ultimately proven, BAV3 based vector possibly could become a suitable tool in human gene therapy.

Neutralizing antibodies generated as a result of administration of any Ad serotype most likely do not cross react with an Ad from another serotype (Mastrangeli et al., 1996). Knowing that, the desired gene could be delivered to the same recipient using different Ad vectors from a different serotype in order to avoid blocking viral infection with neutralizing antibodies generated against another serotype (Kass-Eisler et
Therefore, generating a variety of Ad vectors from different serotypes would be a useful strategy to allow multiple administration of a target gene.

Although only partial characterization has been achieved for BAVs, their least problematic use as vectors may be in cattle industry. In particular, it has been shown that BAV3 causes mild respiratory and respiratory-enteric diseases in cattles just like their human counterparts (Mattson, et al., 1988). Since this virus does not cause serious diseases, recombinant BAV3 could be considered a potential candidate for a live viral vector to be used in animals. However, the problems that have been encountered in human Ads when used in humans can be anticipated when BAV is used in cattle.

The development of adenoviral vectors involves in vitro manipulations of viral DNA sequences that have been cloned usually in plasmids. These manipulations take the form of deleting and modifying viral sequences to facilitate insertion of foreign DNA sequences. The deletion must not include DNA sequences that are required for production of viral progeny, but at the same time it must be large enough to accommodate a reasonable size of gene of interest. Deleting enough viral DNA sequences and substituting them with a foreign gene must be balanced with the virus' ability to package the new recombinant genome. Therefore, construction of a viral vector placed on this balance dictates the size of the deletion and the size of the insert.

To delete any sequences of BAV3 DNA and construct recombinant virus, more information about the sequences on the viral genome was needed. Although the viral nucleotide sequences from 0% to 11.7% and 77%-92% have been determined (Elgadi et al., 1993; Mittal et al., 1992), it was thought that extending the sequencing beyond
11.7% and constructing a restriction map for the sequenced region would provide information that could be useful in manipulating BAV3 DNA. Therefore, sequencing of the region between 11.7%-30.8% was carried out. This study found that this DNA segment contained the E2B region. Analysis of the deduced nucleotide sequences and the encoded ORFs revealed that this region codes for two important viral proteins, BAV3 DNA pol and pTP.

Ads replication is totally depending on DNA pol and pTP. Ads DNA replication initiated by the formation of pTP-dCMP complex which is performed by DNA pol. Subsequently, pTP-dCMP serves as a primer for DNA elongation the function of DNA pol (Friefeld et al., 1983; Lichy et al., 1983). Little details about the structure aspects and functional domains of these two proteins are known due to the difficulties to purify significant amount of both proteins from Ad infected cells. Further analysis for DNA pol and pTP would be not only necessary to gain fundamental understanding of Ad replication, but also understanding the mechanism by which these proteins interact with each other and with all factors involved in Ad replication cycle. Since BAV3 DNA pol and pTP have not been characterized, an extensive analysis of these two proteins would be useful in understanding their roles in the replication cycle and studying the homology with other Ads pol and pTP to understand the evaluation of BAV3. This work would provide more information not only about the restriction map of BAV3 plasmids which have been used in this study, but also the structural characterization of the expected BAV3 mutant. Sequence analyses of BAV3 DNA pol and pTP would be a necessary step to start this characterization.
Although the peptide sequences for several Ad DNA pols available from GenBank invariably include only those predicted for ORFs that start with an ATG, there is some evidence that suggests these DNA pols are actually longer. All Ad sequences for the viral DNA pols from several animal and human Ads show that the first ATG start site is preceded by an ORF with a coding capacity between 72 to 169 aa residues. Indeed Stunnenberg et al. (1988) showed that in Ad5 the active DNA pol, as assayed in an in vitro Ads replication system using cell extracts of overexpressed gene product, requires the 142 amino acid residues preceding the first ATG, proposing that the real translational initiation codon is found in a short exon located further upstream. This initiation codon may be found within the leader segment of the mRNA transcript consisting of sequence fragments at coordinates 75, 68 and 39 as previously noted for the human Ads (Stillman, et al., 1981, Gingeras et al., 1982). Thus, it was suggested that for BAV3 the viral DNA pol is coded by the longest ORF (Figure 3-1), although the identification of the precise composition of the exons for the DNA pol awaits further studies and analysis of its structure.

The alignment of BAV3 DNA pol with other DNA pols from human, canine and bovine Ads has revealed several conserved regions (Figure 3-3). The presence of short patches of conserved regions preceding the first met of each DNA pol supports the idea that important function domains exist in this region and therefore is a part of the Ad pol. Regions (I, II, III, IV, and V) have been shown to be conserved among human Ads and other DNA pols from human, herpes simplex virus, vaccinia virus, and bacteriophage T4 (Wong et al., 1988; Earl et al., 1986); these are found to be conserved in BAV3 which reflect the fact that these regions might serve the same
function for all these pols. Moreover, one can speculate that all these pols derived from the common ancestor, and these regions are needed to maintain a certain function.

Linker-insertion mutagenesis within the above mentioned regions, have shown to effect the function of Ad DNA pol (Chen and Horwitz, 1989). For example, mutations within regions IV and V have been shown to decrease the activity of DNA synthesis by pol 15% or less comparing to wild type activity. These mutations might have the same effect on BAV3 DNA pol function since it has the same conserved sequences.

The aa residues (307 to 336 and 470 to 492 ) using Ad2 pol coordinates contain sequences which are most likely conserved among human Ads only. These conserved domains that can be found only in Ad pols may be directly involved in functions that are unique to these pols.

The gaps which are found within aa sequences from 1 to 200 (using Ad2 pol coordinates) and are common for BAV3, BAV2, and CAd1 but not for human Ads suggests that such notable features in the alignment point to the evolutionary relationships of BAV3 and BAV2, and that the DNA pols of these viruses may have evolved from a common ancestor also shared by CAd1. The occurrence of such gaps may suggest the lack of evolutionary constraint in this region and may indicate that the region has less important role in the function of the protein.

Insertion mutation within the region encodes for the aa sequences (148 to 167 using Ad2 pol coordinates) (Chen and Horwitz, 1989) was shown to decrease the DNA synthesis activity of human Ad2 pol and abolish its specific DNA elongation. BAV3 DNA pol does not show any homology to this region, one can speculate that this mutation may not effect the function of BAV3 DNA pol or it may effect it in different manner.
Sequence alignment analysis of pTPs of BAV3 and other Ads has revealed highly conserved structure domains (Figure 3-5). The roles of these domains cannot be predicted unless further analysis is carried out. However, these domains seem to be involved in functions that could be common to all these pTPs.

In human Ads, it has been shown that the sequences upstream of the first ATG in the main ORF of pTP were important for biological activity (Shu et al., 1986; Pettit et al., 1987; Zhao and Padmanabhan, 1988). The alignment of aa sequences of pTPs of BAV3 and other Ads, showed sequence variability within the region proceed the first ATG in each ORF. Only one conserved region was found proceeding the first ATG in human Ads pTP and upstream of the first ATG of BAV3, BAV2 and Canine Ad. All such residues were found in conserved domain I (Figure 3-5). The conservation in this region might explain why these sequences are important in the activity of pTP of Ad2. However, in BAV3, for instance, the sequences before the first ATG could be not important in the activity of the its pTP or they a part of an exon. Domain V (Figure 3-5) contains the putative nuclear localization sequence, but the sequence itself was not conserved in all pTP analyzed.

The development of BAV3 as a cloning vector was patterned after the current strategy used in the generation of human Ad vectors. Thus, as in Ad5 based vectors, the E1 of BAV3 was deleted for safety reasons. The Ad E1 region is known for its ability to oncogenically transform rodent cells. All adenoviral vectors approved to date for human clinical experiments are helper-dependent because of the deletion in E1 region; therefore, the constructed recombinant virus cannot replicate on its own in vivo. This
defect, however, can be complemented by a cell line that constitutively expresses E1 proteins, thereby supporting virus replication in the absence of a helper virus.

Analysis of the sequences in the E1 region of BAV3 (Elgadi et al., 1993) revealed that it extends from 1.3% to 10.4%. It has been determined that the ORF of plX starts at 9.2%. A deletion in the E1 region must not extend into plX sequences, since it is required for packaging the full-length viral genome (Ghosh-Choudhury et al., 1986). In addition, it has been found that the start codon of the first ORF in E1A is at 1.3%. The sequences upstream from the first ORF in E1A are required in cis and therefore cannot be deleted. These sequences contain the ITR, packaging domains, and the E1 promoter (required for endogenous expression). Therefore, to generate an E1 shuttle plasmid, a deletion of DNA sequences between 1.3%-9% was carried out. Analysis of the nucleotide sequences of BAV3 between 0%-30.8% and the constructed viral restriction endonuclease map revealed that the SacI (1.3%) and Clal (9%) sites could be useful and convenient to introduce a 2512 bp deletion in E1 region. Thus, using these sites the E1 shuttle vector, pdl3E1, was constructed.

The pdl3E1 plasmid was constructed to carry BAV3 sequences from 0% to 53.9%. In addition, a Clal linker was inserted in place of E1 to be used for insertion of foreign genes in E1 region. The \( \text{kan}^R \) gene was inserted into the plasmid pdl3E1 to replace the deleted E1 and was intended i) to serve as an easily identifiable marker in recovered recombinant virus, and ii) to serve as an estimator for efficiency of transduction by the recombinant carrying the gene.

In contrast to E1, the E3 region gene products are not essential for viral replication in cell culture (Berkner and Sharp, 1983). Therefore, this region represents a
suitable target for deletion. To accommodate a large foreign gene insertion, the deletion must be large enough to remain within the limit of the packaging capacity (Haj-Ahmad and Graham, 1986) so that further deletion in E3 extends the cloning capacity. The E3 of BAV3 has been shown to be smaller than other E3 regions of other Ads (Mittal et al., 1992). Like other Ads, the E3 region of BAV3 lies between pVIII and the fiber protein. Deletion in pdlE3 was made 326 bp downstream from the stop codon of pVIII ORF, and, therefore, pVIII sequences remained intact (Figure 3-18). However, the deletion was only 6 bp upstream from the first ATG of the fiber protein. Since it has been shown that the big ORF of the fiber starts 27 bp upstream the first ATG, so that transcriptional elements may have been inadvertently deleted. The sequences that proceed the first ATG of the fiber gene could be important for the activity of this protein.

The constructed pdlE3 carried BAV3 sequences from 37.1% to 100% and SalI linker inserted in the deleted E3 region. A kanR gene was inserted in place of E3 to produce pdlE3K using SalI linker. As in the E1 shuttle plasmid, the inserted kanR gene was intended to serve both for identifying recombinant viruses and estimating efficiency of gene transfer into target cells.

A similar BAV3 vector was previously developed by Mittal et al. (1995). This vector contained a deletion in E3 in which 696 bp have been removed starting at nucleotide 326 downstream from the stop codon of pVIII and 604 nucleotides upstream of the first ATG in the fiber gene ORF. This vector was successfully used in constructing a recombinant BAV3 containing the firefly luciferase gene.

A closer look at the BAV3 vector of Mittal et al. suggests fundamental differences in the vector design described in this report. First, our E3 deletion was
almost two times the size of their deletion being 1,281 bp in length. Our approach was
to make the biggest deletion possible to maximize the size of insertable foreign DNA.
Second, our design included dual deletions in both E1 and E3 to allow insertion of as
much as 6,100 bp of the foreign DNA, which is almost three times the theoretical
maximum for the BAV3 vector of Mittal et al. Moreover, it has been reported that the
recombinant BAV3 developed by Mittal et al. grows 10 times less than the wild type in
tissue culture (Mittal et al., 1995). A mutant virus of this kind is not ideal for rescuing
recombinant viruses. A faster growing mutant would be more suitable to overcome
problems of reversion.

In total, 9 plasmids were constructed during this study. Two of these plasmids,
pdl3E1 and pdlE3, can be used to generate infectious recombinant viruses with
deletions in E1 and E3 regions, which can be achieved by transfecting any one of these
plasmids along with BAV3 DNA digested with appropriate restriction enzymes.
Recombinant BAV3 with E1 or E3 deletions, therefore, can be generated through
homologous recombination between the overlapped sequences in the recombinant
plasmids and wild-type viral DNA.

There are various strategies for rescuing mutant viruses that carry foreign genes
inserted in their genome. Such strategies rely on either in vivo recombination in
cotransfected cells or ligation of viral DNA fragments in vitro followed by transfection
(Haj-Ahmad and Graham, 1986; Thummel et al, 1982). The first approach was used in
this study to rescue BAV3 mutant with kanR gene inserted either in E1 or E3 regions.

Since transfection using calcium phosphate had been used to rescue many
mutant adenoviruses during the past decade, it was assumed that this method was
efficient enough to introduce DNA into cells. Therefore, it was decided that the calcium phosphate transfection procedure be used in this study as a first step to rescue BAV3 E1 or E3 mutants.

In this study, two calcium phosphate methods were used. The first method was the calcium phosphate method which was originally developed by Graham and Van der EB in 1973. Some modifications have been introduced into this method in attempts to increase the transfection efficiency. These modifications were focused on the posttransfection treatments of the cells using different chemicals such as glycerol (Parker and Strak; 1979), DMSO (Lewis et al., 1980), and lysosomal inhibitors (Luthman and Magnusson; 1983). The second method was developed by Chen and Okayama in 1987. This method uses sodium phosphate-containing buffer at pH 6.95. Because of the slightly acidic pH, calcium phosphate-DNA complexes are not formed in the test tube, as is the case in the original method. Rather, calcium phosphate-DNA precipitate is believed to form slowly after adding the mixture to the culture and before being actively taken up by the cells.

Since the first attempts to rescue BAV3 mutant using calcium phosphate were not successful, many factors thought to increase transfection efficiency were examined. The primary factors that are believed to influence efficiency of the calcium phosphate technique include the amount of the DNA in the precipitate, the length of time the precipitate is left on the cells, and the use and duration of glycerol shock. All of these parameters were tested. However, all of the cotransfection experiments using the calcium phosphate technique were failed to produce a recombinant BAV3 or wild-type virus from viral DNA.
Because no plaques were detected from the first attempts to rescue recombinant BAV3, other approaches were tried. Lipid-mediated delivery of DNA is one of the most commonly used transfection procedures presently. In certain applications, this method has been shown to give higher transfection efficiency. For example, it has been shown that lipofectAMINE™ was the best method for transfecting the E61 and MDBK cell lines compared to the calcium phosphate technique (White, 1996). Optimization of transfection using lipofectAMINE™ was a necessary step to be taken. M5 cells were used to optimize the transfection condition because these cells can be used to rescue E1 and E3 recombinant BAV vectors. The two most important factors that affect transfection efficiency are lipid and DNA concentrations and both of were optimized.

The best transfection condition was determined using the reporter gene LacZ to monitor gene transfer and expression efficiencies. Using the optimum lipofectAMINE™ and DNA concentrations (5μg and 1μg, respectively), the number of blue cells that express β-gal gene was around 55 to 80 blue cell per well which contained approximately 3 X 10^5 cell.

Despite the low transfection efficiency, the optimum condition found for DNA and lipofectAMINE™ concentrations were applied to rescue recombinant BAV3. However, the recombinant virus could not be rescued under the used conditions. Furthermore, the wild-type viral DNA, which used as a positive control, failed to produce any identified plaques.

The clear unsuccessful rescue of a deletion viral vector despite repeated and varied attempts could be attributed to many interdependent factors including the cell
lines, the viral DNA and the extent of deletions in the shuttle plasmids, and the conditions which both cells and DNA have been subjected.

MDBK, has been shown to be a difficult cell line to transfect (Mittal et al., 1995). In comparison with 293 cell lines, the number of cells expressing β-gal gene after transfection with a plasmid carried LacZ in MDBK was extremely low (White, 1996), suggesting very low transfection efficiency. It was not ascertained whether it was DNA not entering the cell or degradation of DNA upon entry into the cell that was the problem. The end result, however, that the low transfection efficiency may prevent rescue of a BAV3 vector after transfection with viral DNA. Transfection with wild-type BAV3 DNA did not yield viral progeny under different transfection conditions. It was thought that the viral DNA isolated might have not been infectious, therefore, several methods to isolate and purify the viral DNA were employed including the isolation of viral DNA with terminal protein intact which increases infectivity by 100 folds (Sharp et al., 1976). It is possible that BAV3 DNA remains to be non-infectious despite the efforts.

As mentioned above since these factors are interdependent the transfection efficiency might be low because of the transfection conditions are less than optimal. Therefore, further optimization of transfection conditions must be explored to make a conclusions on the infectivity of BAV3 DNA. Mittal and coworkers (1995) have successfully rescued a BAV3 deletion mutant expressing the luciferase gene in place of E3; their efforts to rescue this vector were not successful using MDBK under a variety of transfection conditions and concluded that MDBK cells were not suitable for the rescue of their vector. This conclusion is supported further in the present study. The
establishment of a novel cell line might be necessary for the rescue of BAV3 vectors or at least the use of other cell lines that can support the rescue.
Conclusions

Based on the results obtained in this study, the following conclusions can be stated: i) the nucleotide sequences of the left end of BAV3 DNA were determined and analyzed; ii) E1 and E3 shuttle plasmids of BAV3 were constructed with deletions in E1 (1.3%-9%) and E3 (78.9%-82.5%) of BAV3; iii) reporter genes were inserted in E1 and E3 regions; iv) under the experimental conditions used the E1 and E3 BAV3 mutants could not be rescued.
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