THE ROLE OF BOVINE ADENOVIRUS-3 PROTEIN V (pV) IN VIRUS REPLICATION

A Thesis
Submitted to the Faculty of Graduate Studies and Research
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy
in the
Department of Veterinary Microbiology
University of Saskatchewan
Saskatoon

By
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ABSTRACT

Bovine adenovirus type 3 (BAdV-3), which is a non-enveloped icosahedral particle with a double-stranded DNA genome of 34,446 base pair, has been developed as a vaccine vector. It belongs to *mastadenovirus* genus in *adenoviridae* family. Like other *mastadenovirus* members, BAdV-3 genome is composed of early, intermediate and late regions. The seven late regions (L1-7) of BAdV-3 genome all belong to one major late transcription unit (MLTU), which is controlled by the major late promoter (MLP). L2 region encodes only one protein V (pV), which is a core protein and functions as a bridge between the viral capsid and DNA genome. It also has been shown to interact with cellular protein p32, which shuttles between nucleus and mitochondria, and redistribute nucleolar proteins B23 and nucleolin. Both of the cellular protein interaction and redistribution suggest that pV may have other functions in viral replication rather than just being a structural protein. Therefore, the objective of this study is to characterize pV, elucidate the functions of pV in viral replication and identify the viral/cellular proteins interacting with pV.

The open reading frame of BAdV-3 pV was confirmed by DNA sequencing analysis to be shown to correspond to 423 amino acids rather than 412 amino acids reported previously. A 55 kDa protein indicating pV was detected by Western blot using anti-pV sera at 24 to 48 hours post-infection in BAdV-3 infected cells, and the same sized protein was detected in pV expressing plasmid transfected cells. pV was distributed in the nucleus, especially in the nucleolus in both infected and transfected cells. Analysis of BAdV-3 pV protein sequence by protein analysis program “PredictProtein” predicted potential motifs act as nuclear localization signals. However, indirect
immunofluorescence result suggested that amino acids 21-50 and 380-389 are essential for the nucleolar localization of pV. Further analysis identified three motifs, which are essential for amino acids 21-50 mediated nucleolar localization of pV. GST pulldown assay identified pV nuclear localization was mediated by the importin α/β pathway since pV was detected to interact with importin α3 rather than other nuclear transport factors. Three nuclear localization signals (NLS) were detected in pV by sequential deletion and confocal microscopy. Deletion of all of these NLSs abrogates not only pV nuclear localization but also its interaction with import α3.

To determine the function of pV nucleolar localization signals (NoLSs) in BAdV-3 replication, we isolated recombinant BAdV-3s, BAV.pVd1 (deletion of amino acids 21-50), BAV.pVm123 (containing substitutions of basic residues of all three motifs of amino acids 21-50), BAV.pVd3 (containing deletion of amino acids 380-389) and BAV.pVd1d3 (deletion of amino acids 21-50 and 380-389). Analysis of mutant BAdV-3s suggested that the deletion of single pV NoLS did not influence the replication of BAdV-3 in vitro. However, deletion of both NoLSs rendered BAV.pVd1d3 replication incompetent in non-pV expressing cells. Moreover, the double, but not single, deletion of pV NoLSs, decreased late viral protein expression, viral particle assembly in MDBK cells and viral thermostability. These results demonstrate that pV contains two NoLSs, which are essential for BAdV-3 replication.

To elucidate the biological significance of pV in BAdV-3 replication, a plasmid pUC304A.dV, which contains BAV304a genome except pV gene was replaced by an SbfI site, was constructed and used to transfecVIDO DT1 (cotton rat lung [CRL] cells expressing I-SceI) cells. However, pV deleted BAdV-3 cannot be rescued in this non-pV
expressing cells, suggesting pV is indispensible for BAdV-3 replication. Therefore, a pV expressing CRL cell line (CRL.pV) was constructed to support the pV deleted recombinant BAdV-3 (BAV.dV) replication in vitro. Protein analysis revealed that deletion of pV from BAdV-3 genome decreased viral late proteins expression such as pX, 100K and pVII. Electron microscopy analysis revealed that pV deletion results in the dramatic reduction in viral assembly. Moreover, thermostablity assay suggested that the viral thermostablity was significantly decreased without pV incorporation.

To determine viral/cellular proteins interacting with pV, an eight amino acids Strep-tag was introduced between pV amino acids $^{18}$E$^{19}$ to construct a recombinant BAdV-3 with strep-tagged pV. Although expression of viral late proteins was decreased to some extent, the introduction of Strep-tag in pV did not influence viral growth characteristics significantly. Mass spectrometry analysis identified many viral/cellular proteins can interact with pV. The interaction of pV with nucleolin was confirmed by Co-immunoprecipitation (Co-IP) and co-localization. Further analysis indicated that deletion of pV NoLSs did not abrogate pV interaction with nucleolin, suggesting that pV NoLSs are not binding sites for nucleolin. Future work remains to elucidate the function of nucleolin in BAdV-3 replication.
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<td>Adenovirus death protein</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndromes</td>
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<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ARD</td>
<td>Acute respiratory disease</td>
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<td>BAdV-3</td>
<td>Bovine adenovirus 3</td>
</tr>
<tr>
<td>BCV</td>
<td>Bovine coronavirus</td>
</tr>
<tr>
<td>BHV</td>
<td>Bovine herpes virus</td>
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<tr>
<td>BiFC</td>
<td>Bimolecular fluorescent complementation</td>
</tr>
<tr>
<td>BoPSAP/BoMtch1</td>
<td>Bovine presenilin-1-associated protein/mitochondrial carrier homolog 1</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
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<tr>
<td>BRSV</td>
<td>Bovine respiratory syncytial virus</td>
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<tr>
<td>BVDV</td>
<td>Bovine viral diarrhea virus</td>
</tr>
<tr>
<td>CAR</td>
<td>Coxsakievirus and adenovirus receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>cNLS</td>
<td>Classical nuclear localization signal</td>
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<tr>
<td>Co-IP</td>
<td>Co-immunoprecipitation</td>
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<td>CPE</td>
<td>Cytopathic effect</td>
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<td>CR</td>
<td>Conserved regions</td>
</tr>
<tr>
<td>CRL</td>
<td>Cotton rat lung</td>
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<tr>
<td>CsCl</td>
<td>Cesium chloride</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
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<tr>
<td>DBP</td>
<td>DNA binding protein</td>
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<tr>
<td>dCMP</td>
<td>Deoxyctydine monophosphate</td>
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<td>DDX</td>
<td>DAED box</td>
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<tr>
<td>DFC</td>
<td>Dense fibrillar component</td>
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<td>Dlg1</td>
<td>Cellular protein Discs Large 1</td>
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<td>DMEM</td>
<td>Dulbecco’s minimal essential medium</td>
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<tr>
<td>DYNLT</td>
<td>Dynein light chain</td>
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<tr>
<td>E</td>
<td>Early</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>E2F</td>
<td>E2 promoter factor</td>
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<td>Eukaryotic initiation factor 4G</td>
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<td>ER</td>
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<tr>
<td>ETSs</td>
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<td>FBS</td>
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<td>FC</td>
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<td>Frog Adenovirus 1</td>
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<td>Human adenoviruses</td>
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<tr>
<td>I</td>
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</tr>
<tr>
<td>ITR</td>
<td>Inverted terminal repeats</td>
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<tr>
<td>ITSs</td>
<td>Internal transcribed spacers</td>
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<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>L</td>
<td>Late</td>
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<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography–mass spectrometry</td>
</tr>
<tr>
<td>MDBK</td>
<td>Madin Darby bovine kidney</td>
</tr>
<tr>
<td>Mdm2</td>
<td>Murine double minute 2</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
</tr>
<tr>
<td>MERS-CoV</td>
<td>Middle east respiratory syndrome coronavirus</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MLP</td>
<td>Major late promoter</td>
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<td>MLTU</td>
<td>Major late transcription unit</td>
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<tr>
<td>MMP</td>
<td>Mitochondria membrane potential</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
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<td>MTOC</td>
<td>Microtubule organization center</td>
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<td>NCL</td>
<td>Nucleolin</td>
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<td>Nuclear export signal</td>
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<td>NFBP</td>
<td>NFB-binding protein</td>
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<td>Nucleolar localization signals</td>
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<td>Nuclear pore complex</td>
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<td>NPM</td>
<td>Nucleophosmin</td>
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<tr>
<td>Nups</td>
<td>Nucleoporins</td>
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<tr>
<td>ORFs</td>
<td>Open reading frames</td>
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<tr>
<td>PAdV-3</td>
<td>Porcine adenovirus-3</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKR</td>
<td>Protein kinase R</td>
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<tr>
<td>PML</td>
<td>Promyelocytic leukemia protein</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
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<tr>
<td>pol</td>
<td>Polymerase</td>
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<tr>
<td>pTP</td>
<td>Precursor terminal protein</td>
</tr>
<tr>
<td>pV</td>
<td>Polypeptide V</td>
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<tr>
<td>QUICK</td>
<td>Quantitative immunoprecipitation combined with knockdown</td>
</tr>
<tr>
<td>RAdV-1</td>
<td>Raptor Adenovirus 1</td>
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<tr>
<td>RE</td>
<td>Restriction endonuclease</td>
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<tr>
<td>RGD</td>
<td>Arginine-glycine-aspartic acid</td>
</tr>
<tr>
<td>RID</td>
<td>Receptor internalization and degradation</td>
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<td>RNP</td>
<td>RNA-protein complexes</td>
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<td>RP</td>
<td>Ribosome protein</td>
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<td>rRNA</td>
<td>Ribosomal RNA</td>
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<tr>
<td>RRP1B</td>
<td>Ribosome RNA processing 1B</td>
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<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gels</td>
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<tr>
<td>shRNA</td>
<td>Small hairpin RNA</td>
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<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>SNF2h</td>
<td>Sucrose non-fermenting-2h</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>snoRNA</td>
<td>Small nucleolar RNAs</td>
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<tr>
<td>snoRNP</td>
<td>Small nucleolar RNA proteins</td>
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<tr>
<td>SPINE</td>
<td>Strep protein interaction experiment</td>
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<tr>
<td>SRP</td>
<td>Signal recognition particle</td>
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<td>ST</td>
<td>Strep tag</td>
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<tr>
<td>SV40</td>
<td>Simian virus 40</td>
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<tr>
<td>TBP</td>
<td>TATA binding protein</td>
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<tr>
<td>TCID</td>
<td>Tissue culture infectious dose</td>
</tr>
<tr>
<td>TIF</td>
<td>Transcription intermediary factor</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>TP</td>
<td>Terminal protein</td>
</tr>
<tr>
<td>TPL</td>
<td>Tripartite leader</td>
</tr>
<tr>
<td>UBF</td>
<td>Upstream binding factors</td>
</tr>
<tr>
<td>VA RNAs</td>
<td>Viral associated RNAs</td>
</tr>
</tbody>
</table>
| WSA
dV-1        | White Sturgeon Adenovirus 1 |
| YBX-1        | Y box binding protein 1 |
| ZO           | Zonulae occludentes |
1.0 LITERATURE REVIEW

This literature review consists of four sections: Adenovirus; protein nucleocytoplasmic transport; nucleolus and protein-protein interaction. In the first section, I talk about the characteristics of adenovirus, especially human adenovirus and bovine adenovirus 3 (BAdV-3), as well as the feasibility of adenovirus as gene delivery vectors. The second section discusses the mechanism of protein transportation between the cytoplasm and the nucleus including the structure and components of the nuclear pore complex (NPC), protein signal peptides that direct the transportation and cellular transport factors. The third section summarizes the structure, components and functions of the nucleolus. Moreover, the most important nucleolar function, ribosome biosynthesis, is described in detail. In the last section, I discuss the characteristics of protein-protein interactions and what roles they play in adenovirus replication.

1.1 ADENOVIRIUS

Adenoviruses are non-enveloped icosahedral particles of 70-100 nm in diameter. The outer capsid is composed of 240 hexons, 12 pentons, and 12 fibers, which projects from each penton base and determines adenovirus tropism (Russell, 2009). The capsid contains a double stranded (ds)-DNA genome of 26-48 kilobases pairs (bp) (Davison et al., 2000; Kovacs et al., 2003) with 29-368 base pair (bp) inverted terminal repeats (ITRs), which are essential for viral DNA replication. The linear viral genomic DNA is tightly associated with histone like protein VII (pVII) to form the core (Russell, 2009). Terminal protein (pTP), which plays a role in initiating viral genome replication, covalently binds to the ends of the viral genome (Russell, 2009). Structural proteins
hexon, penton base, pVIII, pVI, IIIa and non-structural protein 100K and 52K proteins are common to all adenoviruses. However, significant differences were found in proteins encoded by Early (E) region 1, E3 and E4 region among members of different adenovirus genera (Davison et al., 2000; Kovacs and Benko, 2009; Pitcovski et al., 1998).

So far, more than 100 adenovirus members have been identified, which can infect a broad range of mammal, birds, reptile, amphibian and fish hosts (International Committee on Taxonomy of Viruses, 2012). One of the salient contributions of adenovirus research to the modern biology is the discovery of the splicing mechanism and the existence of introns (Berget et al., 1977).

### 1.1.1 Adenovirus classification

*Adenoviridae* family is composed of five genera: *Mastadenovirus, Aviadenovirus, Atadenovirus, Siadenovirus* and *Ichtadenovirus* based on phylogenetic distance, genome organization, host range, and cross-neutralization (Davison et al., 2003) as shown in Fig 1.1.

Members of *Mastadenovirus* genus only infect mammals (Davison et al., 2003). The genomes of *Mastadenoviruses* range from 30,536 (Morrison et al., 1997) to 37,860 bp (Roy et al., 2009) with relatively long and complex ITRs. The longest ITR of 368 bp has been identified in BAdV-10 (Dan et al., 2001). Adenovirus proteins pV and pIX are only encoded by genomes of *Mastadenoviruses*. Protein pV is a core protein which bridges the core and the capsid via the binding of capsid protein (Chatterjee et al., 1985) and the viral genome DNA (Chatterjee et al., 1986). Protein pIX is a minor structural protein which not only stabilizes the capsid by binding to adjacent hexons (Boulanger et
al., 1979) as shown in Fig 1.2, but also acts as a transcription factor and in nuclear reorganization (Rosa-Calatrava et al., 2001).

Members of Aviadenovirus genus infect only birds, including chicken (Chiocca et al., 1996), falcon (Schrenzel et al., 2005), goose (Zsak and Kisary, 1984) and turkey (Kajan et al., 2010). Although their genomes are relatively large and range from 43,804 bp (Chiocca et al., 1996) to 45,667 bp (Griffin and Nagy, 2011), their ITRs are relatively short and present in the same locations as Mastadenoviruses. Each vertex of aviadenoivirus capsid contains two fiber proteins, no matter the virus encodes two fiber genes like Fowl Adenovirus 1 (Griffin and Nagy, 2011) or only one gene like Duck Adenovirus 2 (Marek et al., 2014). The E1, E3, and E4 regions are missing in aviadenoivirus genomes. Instead, at the left and the right ends of the genome, it is rich in open reading frames (Chiocca et al., 1996), which are predicted to encode proteins to make up the functions of E1, E3 and E4 regions (Chiocca et al., 1996).

Members of Atadenovirus can infect a broad range of hosts, including mammals (Benko et al., 2002), birds (Hess et al., 1997) and scaled reptiles (Benko et al., 2002). Atadenovirus genomes range between 29,576 (Vrati et al., 1995) and 33,213 bp (Hess et al., 1997) in length with high AT content and relatively small ITRs between 46 (Vrati et al., 1995) and 118 (Benko et al., 2002). Similar to aviadenoivirus, the members of Atadenovirus genome does not encode proteins pV and pIX (Benko et al., 2002; Hess et al., 1997; Vrati et al., 1995). There are several unique proteins encoded by atadenovirus genomes, such as a structural protein p32K, which is encoded by the left side of the viral genome (Benko et al., 2002; Hess et al., 1997; Vrati et al., 1995).
Figure 1.1 Phylogenetic analysis of members in *Adenoviridae* family base on hexon amino acids sequence distance matrix analysis. Abbreviations of simian adenoviruses: rh, rhesus macaque; cy, cynomolgus macaque; go, gorilla; ch, chimpanzee; bo, bonobo.

Adapted from (Davison et al., 2003), with permission to use.
Figure 1.2 Structure of adenovirus virion. Adapted from (Russell, 2009), with permission to use.
Members of *Siadenovirus* genus infect reptiles (Kovacs and Benko, 2009), frogs (Davison et al., 2000) and turkey (Pitcovski et al., 1998). So far, only three adenoviruses namely Raptor Adenovirus 1 (RAdV-1) (Kovacs and Benko, 2009), Frog Adenovirus 1 (FrAdV-1) (Davison et al., 2000) and Turkey Adenovirus 3 (TAdV-3) (Pitcovski et al., 1998) are included in this genus. These *siadenoviruses* contain smallest genomes ranging from 26,163 bp to 26,282 bp, as well as the shortest ITRs, from 29 to 39 bp (Davison et al., 2000; Kovacs and Benko, 2009; Pitcovski et al., 1998). The unique feature of members of *Siadenovirus* is the encoding of Sialidase enzyme (Davison et al., 2000). Unlike *Mastadenoviruses*, E1, E3 and E4 early regions and genes of pV, pIX are missing from their genomes, and replaced with 5 potential novel protein encoding ORFs (Davison et al., 2000; Kovacs and Benko, 2009; Pitcovski et al., 1998).

After the phylogenetic analysis of the first fish adenovirus isolated from white sturgeon in 2003, a new genus, *Ichtadenovirus*, was added in *adenoviridae* family (Davison et al., 2003). The White Sturgeon Adenovirus 1 (WSAdV-1) is the only number of *Ichtadenovirus* genus. The genome of *Ichtadenovirus* is 48,396 bp, which is the longest in all known adenovirus members. Although positional homologue of fiber gene is not deleted in WSAdV-1, a fiber homologue encoded by an ORF at the left end (Davison et al., 2003) of the genome has been reported.

### 1.1.2 Human adenovirus

Human adenovirus (HAdV), which belongs to *Mastadenovirus* genus was first isolated in early 1950s from human adenoids (Rowe et al., 1953). So far, 67 serotypes have been identified, and they are grouped in 7 subgroups (A-G) (Ghebremedhin, 2014;
Rosen, 1960). The fiber induced red blood cell agglutination can be inhibited by the antisera of the viruses from the same subgroup, but not other subgroups.

1.1.2.1 Human adenovirus pathogenesis

Human adenoviruses most often infect the upper respiratory tract and can be asymptomatic or cause mild fevers, gastroenteritis and conjunctivitis. About 5% of acute upper respiratory infection in children under 4-year-old are because of human adenovirus type 4 and 7. Adenoviral infection also causes lower respiratory infection, but it is not as common as the upper respiratory infection (Murtagh et al., 1993). Eye infection of serotype 8, 19 or 37 may cause epidemic keratoconjunctivitis, while infection of serotype 3, 7 or 11 may cause conjunctivitis (Kitamura, 2001). HAdV-40 and 41 are also responsible for gastroenteritis in children (Wilhelmi et al., 2003). Moreover, HAdV-36 has been identified to cause obesity (Gabbert et al., 2010).

Two fatal cases caused by adenovirus infection were reported by the US military recruitment department (Potter et al., 2012). Acute respiratory disease (ARD) is usually caused by infection of HAdV-4, 7 and 2. Adenoviral infections cause much severe symptoms in immunocompromised hosts, like patients with severe combined immunodeficiency (SCID) (Al-Herz and Moussa, 2012) or acquired immunodeficiency syndromes (AIDS) (Adeyemi et al., 2008) and organ receivers (Klein et al., 2015). Although infection of human adenovirus type 12 causes malignant tumors in newborn hamster at the injection site (Trentin et al., 1962), the oncogenic potential of human adenovirus for humans have not been reported (Mackey et al., 1976).
1.1.2.2 Human adenovirus structure

Human adenoviruses are members of Mastadenovirus (International Committee on Taxonomy of Viruses, 2012). The adenovirus virion is composed of 13 structural proteins and a linear double stranded DNA of 26-48 kb (Russell, 2009) as shown in Fig 1. Of these structural proteins, there are 7 capsid proteins, which are part of the capsid, and 6 core proteins, which connect the capsid and viral DNA or bind to viral DNA to form a nucleosome-like structure (Mackey et al., 1976). Hexon, penton and fiber are the major capsid proteins. Each capsid contains 240 trimeric hexons and 12 pentons (van Oostrum and Burnett, 1985). Each hexon is surrounded by 6 hexon proteins or 5 hexon proteins and 1 penton protein, and each penton is surrounded by 5 hexon proteins. Proteins IIIa, VI, VIII and IX are minor capsid proteins that play roles in stabilizing the virion structure via interaction with the major capsid proteins (Russell, 2009).

The adenovirus core contains the viral genome core proteins IVa2, V, VII, terminal protein (TP), Mu (X) and virus encoded protease (San Martin, 2012). Protein VII and V are the most abundant core proteins of adenovirus. In the mature virions, pVII functions as a histone like structure around which viral DNA is wrapped (Daniell et al., 1981). pV mediates the transportation of the viral genome from the cytoplasm to the nucleus (Wodrich et al., 2006). Because of its interaction with viral DNA, it may play a role in viral DNA replication and transcription, as well as the viral DNA encapsidation (Haruki et al., 2006; Spector, 2007). Although pV seems to be dispensable in human adenovirus replication (Ugai et al., 2007), it still acts to bridge between the viral core and capsid by interaction with viral DNA and capsid proteins. Since pV has been proved to associated with cellular protein p32, it is possible that pV helps adenovirus hijack the cellular routes
to reach the nucleus (Matthews and Russell, 1998a). Because of the capability to redistribute nucleolar proteins B23 and nucleolin from the nucleolus to the cytoplasm, pV might play some unknown function in the nucleolus (Matthews, 2001). For other core proteins, IVa2 is involved in activation of late transcription and viral DNA encapsidation (Ostapchuk et al., 2005; Tyler et al., 2007); protease activates viral protein precursors by cleaving them (Mangel et al., 2003); Mu plays a role in condensing viral prechromatin (Lee et al., 2004) and pTP binds at the end of viral DNA and primes viral DNA replication (Webster et al., 1997).

1.1.2.3 Genome organization

Adenovirus genome contains two DNA replication origin sites. In several hundreds base pairs in left side of the adenoviral DNA, its also contains a packaging sequence, which directs the viral genome into the empty capsid (Hearing et al., 1987). The viral genome carries four early transcription regions (E1-E4), two intermediate genes (IVa2 and pIX) and one major late unit that is transcribed and alternatively sliced into five groups of late mRNA (L1-5) as shown in Fig 1.3. All of these genes are transcribed by RNA polymerase II (Mishoe et al., 1984). In addition, human adenovirus also carries viral associated RNAs (VA RNAs), which are transcribed by RNA polymerase III (Kidd et al., 1995). E1, E3 and major late unit are transcribed from the forward strand, while E2, E4 and IVa2 are transcribed from the reverse strand (Chroboczek et al., 1992).
**Figure 1.3** Schematic representation of the adenoviral genome. Thick black boxes represent DNA sequences of ITR or MLP. Early, intermediate and late regions are depicted in red, blue and black, respectively. ITR: inverted terminal repeat; $\psi$: packaging sequence; MLP: major late promoter. Adapted from (Wold and Toth, 2013), with permission to use.
1.1.2.4 Replication cycle

The adenovirus replication cycle can be divided into five stages: adsorption and entry, activation of early genes, DNA replication, activation of late genes, assembly and release.

1.1.2.4.1 Adsorption and entry

Adenovirus infection is initiated by the attachment of virus to the cells, which is mediated by fiber protein. The fiber protein of different adenoviruses can bind different cellular proteins exposed on the cells membrane as receptors. The most popular one is coxsakievirus and adenovirus receptor (CAR) (Bergelson et al., 1997), which is used by human adenovirus subgroup A, C, D, E and F (Roelvink et al., 1998). In addition to CAR, other cellular proteins used as receptors by human adenoviruses including: heparin sulphate glycosaminoglycans, cluster of differentiation (CD) 46, CD80, CD86 and sialic acid (Zhang and Bergelson, 2005). Since some adenoviruses cannot enter some cells to which they are capable to bind, it is possible that another protein-protein interaction is involved in adenovirus internalization (Silver and Anderson, 1988). The interaction between penton arginine-glycine-aspartic acid (RGD) sequence and integrin has been proved to be critical for both adenovirus internalization and endosome escape (Shayakhmetov et al., 2005). It is postulated that the fiber-receptor interaction is just facilitating the RGD-integrin interaction by bring the virion to the cell surface.

The RGD-integrin interaction triggers adenovirus internalization via receptor-mediated endocytosis (Varga et al., 1991). The purified penton not fiber has been shown to quickly penetrate the cell membrane in cell culture (Wickham et al., 1993). The
interaction between RGD motif and integrin activates the lipid kinase phosphotidylinosito-3-OH kinase, which is involved in the rearrangement of actin cytoskeleton (Li et al., 1998). Moreover, adenovirus internalization also requires dynamin, which is associated with the regulation of clathrin-mediated endocytosis (Wang et al., 1998).

After internalization of the cell, adenovirus uses two steps to escape the endosome: 1, viral uncoating to become smaller; and 2, disruption of the endosome membrane to pass through. Once in the endosome, the virus starts to stimulate the cell to acidify the cytosol to respond to the infection, which helps adenovirus disassembly. The vertex part, which contains penton base and fiber protein are firstly detached from the capsid. Then, the protein IIIa that bridges hexon and penton is lost, followed by removal of hexon, pVI, pVIII and pIX (Greber et al., 1993). Since both pVI and pVIII connect capsid and core, the removal of these proteins prepares for release of pVII and viral DNA complex. After partial disassembly, pVI is cleaved by the viral protease, and then attaches to endosome membrane causing membrane disruption and adenovirus escape (Wiethoff et al., 2005). Moreover, pVI is also involved in the activation of 23K proteinase (Ding et al., 1996) and cytoplasm to nucleus transportation of hexon (Kelkar et al., 2006).

After escaping from endosomes, partially disassembled adenovirus must inject its viral DNA into the nucleus to start the replication, but firstly it has to get access to the nucleus. To achieve this, the partial disassembled adenovirus is associated with cytoplasmic dynein and transported to microtubule organization center (MTOC), which is adjacent to the nucleus (Kelkar et al., 2006). Once it reaches MTOC, the adenovirus must detach from the MTOC and attach to the nuclear pore complex (NPC). After being
transported to the nucleus by pVI, hexon binds to the NPC, which gives access to nucleus to pVII-DNA complex (Kelkar et al., 2006). Finally, the viral genome is injected into the nucleus via NPC and starts transcription and DNA replication.

1.1.2.4.2 Expression of early genes

Once inside the nucleus, adenovirus DNA starts to express its early regions, which encode mostly non-structural proteins that facilitate viral replication. Adenoviruses express early proteins to fulfill three main goals. The first is to establish the environment optimal for viral replication by inducing the host cell to enter S phase, in which host cell prepares for DNA replication. Proteins involved in this process are E1A, E1B and E4 regions proteins. The second is to activate the transcription of E2 region genes, which are required for viral DNA replication. The third is to antagonize the host immune system so that adenovirus can finish replication before the infected cell is eliminated by the host immune response. Products encoded by E3 and VA RNA genes are associated with this process.

E1A region is first to be transcribed and produce proteins after the viral DNA enters the nucleus. E1A encodes two mRNAs by alternative splicing, 12S and 13S, with co-terminal 5’ and 3’ ends (Frisch and Mymryk, 2002). Three conserved regions (CR1, 2 and 3) were identified in E1A proteins of a variety of human adenovirus serotypes. E1A proteins are capable to immortalize primary cells, as well as induce tumors in newborn rodents with E1B proteins (Ben-Israel and Kleinberger, 2002). Instead of directly binding to DNA, E1A proteins interact with key cellular regulatory proteins and transcription factors to control cell cycle and gene expression (Avvakumov et al., 2002).
To regulate the cell cycle, E1A interacts with pRB to disrupt the pRB-E2F (E2 promoter factor) complex and free E2F. The pRB inhibits E2F transcriptional activation by forming a complex with it, while CR1 and CR2 regions of E1A proteins bind to pRB, dissociating it from E2F (Ikeda and Nevins, 1993). Free E2F activates both viral and cellular genes dramatically regulating the cell cycle. E1A proteins also inhibit other cell cycle control proteins, like the cyclin-dependent kinase inhibitor p21 (Chattopadhyay et al., 2001). E1A proteins have also been reported to regulate chromatin structure by interacting with host factors, such as TRRAP/GCN5 (Lang and Hearing, 2003), pCAF and p300/CBP (Chakravarti et al., 1999; Hamamori et al., 1999). Chromatin remodeling may increase DNA and transcription factor binding, thereby increasing expression of genes regulating cell cycle. Moreover, E1A proteins may regulate target gene expression by directly interaction with cellular transcription factors like CBF1 (Ansieau et al., 2001) and SUR2 (Stevens et al., 2002).

Cells respond to E1A induced cell cycle deregulation by accumulating tumor suppressor p53 protein (Lowe and Ruley, 1993). The cell signaling pathways activated by p53 induce apoptosis of the infected cell to prevent viral replication. In adenovirus-infected cells, E1B transcriptional unit encodes two proteins, E1B-55K and E1B-19K, which are involved in preventing apoptosis. The E1B-55K inhibits p53-dependent apoptosis by directly binding to and degrading p53 (Li et al., 2011b). To degrade p53, E1B-55K forms a complex with another viral protein E4orf6 and other cellular proteins, which act as ubiquitin ligase (Sarnow et al., 1984). In addition, p53 accumulated in the infected cell is ubiquinated and degraded (Querido et al., 2001). Moreover, E1B-55K can bind and repress p53 to inhibit activation of pro-apoptosis genes (Ben-Israel and
Kleinberger, 2002), which results in accumulation of inactive p53. The E1B-19K blocks cell programmed death by mimicking cellular anti-apoptotic protein MCL1 (White, 2001). Pro-apoptotic proteins BAK and BAX form a complex and bind to mitochondria membrane to induce apoptosis (Cuconati et al., 2003). MCL1 directly binds to BAK and BAX complex to inhibit its pro-apoptotic activity. In adenovirus-infected cells, E1B-19K mimics MLC1 to bind BAK and BAX complex and inhibits its pro-apoptotic activity (Cuconati et al., 2003).

The E2 unit encodes three products: DNA binding protein (DBP), terminal protein (pTP) and DNA polymerase (pol), and all of them contribute to viral DNA replication (de Jong et al., 2003). Although, adenovirus replicates in the nucleus, adenovirus encodes its own DNA polymerase. Precursor terminal protein (pTP) binds to the 5’ end of each strand and serves as a primer to initiate the viral DNA replication (Mysiak et al., 2004). DBP forms a complex with pTP and viral DNA polymerase to initiate the DNA replication, and it also plays a role in elongation by unwinding the template (van Breukelen et al., 2003).

The E3 region encodes 4 proteins, include: E3-gp19K, 10.4K, 14.5K and 14.7K. They all help human adenovirus to evade the host immune defense (Horwitz, 2004). E3-gp19K modulates major histocompatibility complex (MHC) class I in two ways. Firstly, E3-gp19K binds and retains MHC class I in the endoplasmic reticulum (ER), therefore, it cannot be transported to the cell membrane and present peptides to cytotoxic T lymphocytes (Burgert et al., 1987). Secondly, E3-gp19K also plays a role in inhibiting the assembly of MHC class I and peptides thus reduces the antigen presentation (Bennett et al., 1999). E3-10.4K and 14.5K bind to each other to form receptor internalization and
degradation (RID) complex, which clears chemokine receptors from cell surface and mediates their lysosome transportation and degradation to inhibit chemokine induced apoptosis (Shisler et al., 1997). E3-14.7K not only inhibits tumor necrosis factor (TNF)-α induced apoptosis, but also decreases the secretion of TNF-α induced arachidonic acid, which is a inflammatory intermediate (Zilli et al., 1992).

E4 transcriptional unit encodes several proteins such as orf1, orf3, orf6, orf4 and orf6/7 with different functions. E4orf1 has been shown to induce mammary tumor in rat by directly binding to tumor suppressor zonulae occludentes (ZO) protein (Javier et al., 1991). Both E4orf3 and E4orf6 facilitate viral mRNA maturation, transport, stability and cytoplasmic accumulation, and thus increase production of the late viral proteins (Imperiale et al., 1995). Additionally, both of them also function to regulate viral DNA replication (Bridge and Ketner, 1989). E4orf6/7 protein plays a role in facilitating E1A proteins to activate other viral or cellular genes by directly binding to E2F (Schaley et al., 2000). E4orf4 has been reported to decrease both E1A and E4 production via protein phosphatase 2A (Muller et al., 1992), which limits the cytotoxic effects and ensures maximum productivity.

1.1.2.4.3 Viral DNA replication

As the accumulation of E2 encoded proteins occurs, adenovirus DNA replication initiates from the ITRs, which act as replication origins (Hay, 1985). Initiation of adenovirus DNA replication requires a protein complex composed of several viral and cellular proteins (Hoeben and Uil, 2013). The three E2 encoded proteins are present in this complex. The pTP is a 80-kD protein covalently binds to 5’ end of the viral genome,
and plays a role in initiation of viral DNA replication (de Jong et al., 2003). Adenovirus encoded DNA polymerase is synthesized as a 140-kD protein and has distinct characteristics from any other eukaryotic DNA polymerase (Field et al., 1984). In addition to the 5’ to 3’ polymerase activity, it also contains 3’ to 5’ exonuclease activity indicating its proofreading activity (Field et al., 1984). Two cellular factors, nuclear factor I (NFI) and NFIII are recruited in the DNA replication complex. The NFI stabilizes the DNA pol-pTP complex at the origin by directly interacting with DNA pol (Mul et al., 1990). NFI also binds to ITRs, which is facilitated by E2 encoded protein, DBP (Stuiver and van der Vliet, 1990).

Adenoviral replication is initiated with the formation of an ester bond between the deoxycytidine monophosphate (dCMP) α-phosphoryl group and the β-OH of a serine in pTP (Lichy et al., 1981). After pTP-dCMP complex formation, 3’-OH group starts to serve to the new strand synthesis by DNA pol. Two E2-encoded proteins, DNA pol and DBP, and a cellular factor NFII are required for DNA elongation. DBP binds to single stranded DNA tightly in a sequence independent pattern and serves as DNA helicase to unwind the double stranded template in DNA replication (Lindenbaum et al., 1986). NFII functions as a topoisomerase, and is postulated to overcome the long DNA problem after extensive replication (Nagata et al., 1983).

1.1.2.4.4 Expression of intermediate genes

Two viral structural proteins, IVa2 and pIX, are known as delayed-early or intermediate proteins since they are expressed at the same time as the viral DNA replication starts. IVa2 is present in both empty capsid and mature virus particles (Zhang
and Imperiale, 2003). IVa2 also functions to regulate major late promoter (MLP) activity (Lutz and Kedinger, 1996; Lutz et al., 1997), regulate empty capsid formation and viral DNA encapsidation by binding to DNA packaging sequence (Zhang and Imperiale, 2003). pIX is involved in holding adjacent hexon proteins and plays a role in adenovirus replication and MLP activation (Lutz et al., 1997). In addition, pIX accumulation induces formation of specific nuclear domains in infected cells, which probably inhibits the antiviral activity of ND10 and contributes to optimal viral replication (Rosa-Calatrava et al., 2003). Since pIX is present on the virion surface, it has been used as a target for addition of ligands for altering adenovirus tropism (Dmitriev et al., 2002).

1.1.2.4.5 Expression of late genes and viral assembly

After the onset of DNA replication, adenovirus starts to express late genes. All late genes are encoded by major late transcription unit (MLTU) and grouped into 5 late (L) regions L1-L5 (Fraser et al., 1979). The MLTU encodes a single pre-mRNA, which is spliced and polyadenylated into approximately 20 mature mRNAs. These mRNAs primarily encode viral structural proteins and scaffolding proteins facilitating virion assembly (Iwamoto et al., 1986).

After DNA replication, the expression of MLTU provides adequate amount of late proteins for virion assembly. Hexon trimerizes in the cytoplasm soon after translation, even though virions assembles in the nucleus (Cepko and Sharp, 1983). After being translocated to the nucleus, the hexon trimmers start to associate with penton base and other minor proteins to form the capsid (Wodrich et al., 2003). After the formation of the
empty capsid, the viral DNA genome is transported to the capsid to complete genome encapsidation (Edvardsson et al., 1976).

A number of viral proteins are required for genome encapsidation. IVa2 is required for capsid formation by directly binding to DNA packaging sequence (Zhang and Imperiale, 2003). L1-52/55K gene mutant virus produces only empty capsid and partially filled virions, suggesting that this protein is required for genome encapsidation (Gustin and Imperiale, 1998). Intriguingly, IVa2 and L1-52/55K protein form a complex, which binds to the packaging sequence and helps in viral DNA encapsidation (Perez-Romero et al., 2005). Moreover, L4 encoded 33K protein is also involved in viral DNA encapsidation since the 33K mutant virus cannot assemble into mature virus particles (Finnen et al., 2001).

The left end of the viral DNA between ITR and ATG of E1A protein contains the DNA packaging sequences; 7 sequence element repeats (A1-A7) with different functional relevance (Hearing et al., 1987). Before virion assembly is complete, several structural proteins are cleaved by virus-coded protease to produce mature virus particles (Mangel et al., 2003). At approximately 30 hours post-infection, virus particle are released from the lysed cell. Although mechanisms involved in the release of adenovirus from cells is not clear, E3 encoded 11.6 K protein or adenovirus death protein (ADP) has been suggested to play a role in this process (Tollefson et al., 1996a). Although ADP is encoded by the E3 region, it is transcribed by the MLP not the E3 promoter (Tollefson et al., 1996b).
1.1.3 Bovine adenovirus-3

1.1.3.1 Classification of bovine adenoviruses

Bovine adenovirus (BAdV) was first isolated from a healthy cow, though they usually cause mild gastrointestinal or respiratory disease (Lehmkuhl et al., 1975). So far eleven BAdV serotypes have been identified (Lehmkuhl and Hobbs, 2008). Based on the difference of phylogenetic proximity, these eleven identified BAdV serotypes are subdivided into four subgroups (group A-D) and classified into two genera, Mastadenovirus and Atiadenovirus. Subgroup A (BAdV-1), B (BAdV-3) and C (BAdV-10) belongs to Mastadenovirus, while subgroup D (BAdV-4, 5, 6, 7, 8 and strain Rus) belongs to Atadenovirus (Harrach, 2000; Lehmkuhl and Hobbs, 2008). Since the Mastadenovirus serotypes are classified as their own species, they are more phylogenetically close to ovine adenovirus rather than to each other, while the Atadenovirus serotypes are more phylogenetically close related to each other.

1.1.3.2 Characteristics of BAdV-3

BAdV-3 virus particle is an icosahedral particle of 75 nm in diameter containing a linear double stranded DNA genome of 34,446 base pairs in its non-enveloped capsid (Reddy et al., 1998). BAdV-3 was first isolated in 1960s from the conjunctiva of an apparently healthy cow in Great Britain (Darbyshire et al., 1965). BAdV-3 infected calves show mild or no clinic symptoms, although this virus has been isolated from cows with respiratory or enteric diseases (Mattson et al., 1988).
1.1.3.3 Genome organization of BAdV-3

The G/C content of BAdV-3 genome is 54%, which is very close to HAdV-12 genome and 20% more than the G/C content of ovine adenovirus genome (Reddy et al., 1998; Vrati et al., 1996). Like other adenoviruses, the BAdV-3 genome is divided into early regions, intermediate regions and late regions (Fig 1.2), which are predicted to encode 33 proteins (Reddy et al., 1999a; Reddy et al., 1998). BAdV-3 genome is flanked by 195 bases ITRs with G/C content of 84%, which is unusually high compared to other adenoviruses (Reddy et al., 1998). Although the BAdV-3 genome packaging sequence is located at the left end of the adenovirus DNA as other adenoviruses, a part of the E1A open reading frame is shown to be essential for BAdV-3 genome packaging (Xing and Tikoo, 2007; Xing et al., 2003). Moreover, there are no TATA or CAAT boxes between the left ITR and E1A open reading frame, suggesting that the left ITR contains the E1A promoter (Xing and Tikoo, 2006). The transcription map of BAdV-3 is shown in Fig 1.4.

1.1.3.3.1 Early genes

The E1A proteins of early regions are first to be transcribed. Because of different splicing, E1A produces several transcripts, which encode three relatively small proteins termed 211R, 115R and 100R with the same N-terminus (Reddy et al., 1999a). As E1A-221R is rich in proline and glutamic acid residues, the observed molecular weight is more than the predicted molecular weight in sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) (Reddy et al., 1999a). Both 211R and 115R are encoded from transcripts
**Figure 1.4** Transcription map of BAdV-3. Transcript locations and directions are indicated by arrows. Adapted from (Reddy et al., 1998), with permission to use.
with the same open reading frames but have different downstream splicing. These two E1A encoded proteins are expressed at 24 hours post-infection, while the 100R was observed from 12 hours to 36 hours post-infection. HAdV-5 genes can be activated by BAdV-3 E1A product, indicating that they are homologous in functions (Zheng et al., 1994). Moreover, BAdV-3 E1A transcripts have unusually long 5'-untranslated region, the function of which is not known (Reddy et al., 1999a). The E1B regions transcribe two different mRNAs, coding 420R and 157R, which are homologous to HAdV-5 E1B-19K and 55K (Zheng et al., 1994), respectively. Phosphorylation has been reported on serine/threonine of E1A and E1B-157R serine/threonine and tyrosine residues of E1B-420R (van Olphen et al., 2002). Unlike HAdV-2 (Berk and Sharp, 1978), BAdV-3 E1A and E1B share the same promoter and polyadenylation sites (Reddy et al., 1998).

Like human adenoviruses, BAdV-3 E2 region encodes proteins that contribute to viral DNA replication. The only protein encoded by E2A is DBP, which is a multifunctional protein and involved in diverse processes (Chase and Williams, 1986). BAdV-3 DBP is 423 amino acids in length, which is shorter than HAdV DBPs (Kitchingman, 1985). Like in other adenoviruses, the DBP of BAdV-3 contains a variable N-terminus and a conserved C-terminus (Tucker et al., 1994). Although the variable N-terminus is not essential for HAdV replication, it determines the host range (Anderson and Klessig, 1984). The conserved C-terminus is involved in DNA binding, DNA replication and activation of MLP (Kitchingman, 1985). HAdV DBPs contain two zinc atom-binding motifs, which maintain the protein three-dimensional structure and function via binding to zinc atoms (Tucker et al., 1994). The BAdV-3 DBP contains a number of threonine and serine residues at the N-terminus that may act as the phosphorylation sites.
Like HAdV DBPs, BAdV-3 DBP contains motifs rich in basic amino acid, including $^{29}$PRKK$^{32}$, $^{35}$RKRR$^{38}$, and $^{53}$KRAK$^{56}$, which are potential nuclear localization signals (NLS) (Reddy et al., 1998). Adenovirus DNA Pol and precursor terminal protein (pTP) are coded from E2B region. As in HAdV-2, BAdV-3 transcripts coding DNA Pol and pTP are 3’ co-terminal (Reddy et al., 1998). BAdV-3 DNA Pol and pTP are 1023 and 649 amino acids long, respectively (Baxi et al., 1998), and share 59-60% and 58-60% amino acid identity, respectively, to their counterparts of other members in Mastadenovirus genus.

The E3 region of BAdV-3 is 1.4 kb, which is relatively small compare to other adenoviruses including HAdV-5 (Davison et al., 2003), and produces transcripts encoding four proteins of 284, 121, 86 and 82 amino acids (Idamakanti et al., 1999). The 284R, which is unique in BAdV-3, is a glycoprotein. The 121R shows limited homology to HAdV-5 E3-14.7 kDa protein (Mittal et al., 1993). It is expressed as 14.5 kDa protein in BAdV-3 infected cells (Idamakanti et al., 1999) and involved in inhibition of TNF-α mediated apoptosis (Gooding et al., 1988; Gooding et al., 1990; Horton et al., 1991). Like HAdV-5 (Morin et al., 1987), the E3 region is dispensable for the replication of BAdV-3 in cultured cells.

Like HAdV-5 (Bridge and Ketner, 1989), the E4 region is located at the right end of the BAdV-3 genome (Reddy et al., 1998). Alternative splicing of E4 transcript generates several transcripts, which encode five open reading frames (ORFs) (1-5) (Reddy et al., 1998). ORF 1, ORF2 and ORF4 are 143, 69 and 143 amino acids long, respectively, and show no homologue to E4 ORFs of HAdV-5 (Bridge and Ketner, 1989). However, ORF3 and ORF5 are proteins of 268 and 219 amino acids, respectively, and
they show some homology with HAdV-5 E4orf6 proteins (Baxi et al., 2001). None of these ORFs appear essential for replication of BAdV-3 in tissue culture cells or cotton rats (Baxi et al., 2001).

HAdV-2 expresses two copies of non-coding RNAs, termed viral associated (VA) RNA, which are transcribed by RNA polymerase III (Ma and Mathews, 1996). They inhibit the phosphorylation of eukaryotic initiation factor 2 (eIF2) by binding to protein kinase R (PKR) (Sano et al., 2006), which facilitate viral protein expression. However, no VA RNAs have been identified in BAdV-3 genome so far (Reddy et al., 1998). VA RNA genes are located between 52/55K and pTP genes in HAdV, while there is no enough space for them in BAdV-3 genome since 52/55K protein is adjacent to pTP unless VA RNA genes overlap both 52/55K and pTP genes. It is possible that BAdV-3 VA RNA genes are probably located at different locations from HAdV-2. Alternatively, it is also possible that BAdV-3 uses other mechanisms to antagonize PKR rather than expressing VA RNAs (Reddy et al., 1998).

1.1.3.3.2 Intermediate genes

Like HAdV-2 (van Oostrum and Burnett, 1985), BAdV-3 expresses two intermediate (delayed-early) proteins IVa2 and IX. Protein IVa2 gene is located downstream of DNA Pol and pTP genes on the complementary strand. Unlike HAdV-2 (Berk and Sharp, 1978), BAdV-3 IVa2 transcripts do not share the 3’-terminus with both DNA Pol and pTP transcripts (Reddy et al., 1998). BAdV-3 protein IVa2 is a structural protein with 376 amino acids, which is shorter than IVa2 proteins of other adenoviruses, sharing 29-69% of homology with its counterparts (Reddy et al., 1998). BAdV-3 protein
IX gene is located downstream of E1A and E1B regions and encodes a 125 amino acids structural protein showing 16-28% sequence identity with protein pIX encoded by other members of Mastadenovirus (Zheng et al., 1999).

1.1.3.3 Late genes

The late (L) proteins of BAdV-3 are encoded by different mRNAs that are generated from a single transcript of MLTU by alternative splicing. Unlike HAdV-5 late region (Fraser et al., 1982), BAdV-3 MLTU can be divided into seven late regions (L1-L7) based on mRNAs sharing the same poly(A) signal (Reddy et al., 1998). BAdV-3 MLP contains a TATA box, inverted CAAT box, USF binding site and initiator element (Reddy et al., 1998). Like HAdV-2 tripartite leader (TPL) (Chow et al., 1977). BAdV-3 MLP also transcribes a TPL of 205nt composed of three leaders with 40nt, 78nt and 87nt in length respectively (Reddy et al., 1998).

Unlike HAdV-2 (Kreivi et al., 1991), the L1 region of BAdV-3 encodes four proteins namely 52K, IIIa, III and pVII since their transcripts share the same poly(A) signal. BAdV-3 52K is a non-structural protein of 331 amino acids showing 61% amino acids sequence identity to HAdV-2 52/55K (Reddy et al., 1998). It is localized in the nucleus in both transfected and infected cells, and three basic residues \textsuperscript{105}RKR\textsuperscript{107} seems essential for 52K nuclear transportation (Paterson et al., 2012). 52K can interact with importin \(\alpha_3\), suggesting that the classical importin \(\alpha/\beta\) dependent nuclear transport pathway mediates 52K nuclear trafficking (Paterson, 2010; Paterson et al., 2012). 52 K was also detected to redistribute NFB-binding protein (NFBP), which is involved in ribosomal RNA (rRNA) processing, from the nucleolus to the nucleoplasm (Paterson,
pIIIa is a structural protein bridging the hexon trimmers to stabilize the capsid in HAdV-2 (Vellinga et al., 2005). BAdV-3 pIIIa is a 568 amino acids protein with a putative viral protease cleavage site at 19 residues from the carboxy-terminus, and showing 25-57% amino acids identities to other adenovirus pIIIa proteins (Reddy et al., 1998). Penton base protein comprises the vertexes of the capsid and plays an important role in virus internalization (Zubieta et al., 2005). BAdV-3 penton base protein has 482 residues and share 44-64% homology to penton base proteins of other adenoviruses (Reddy et al., 1998). BAdV-3 penton base protein contains a MDA motif rather than RGD motif found in HAdV penton, and interacts with αvβ3 and αvβ5 to trigger endocytosis for virus internalization (Wickham et al., 1993). To form the penton, BAdV-3 penton base protein also interacts with fiber protein at the conserved fiber-interaction domain (Caillet-Boudin, 1989). BAdV-3 pVII is a core protein of 171 amino acids in length and shares 53% sequence identity to HAdV-2 pVII protein (Reddy et al., 1998). BAdV-3 pVII contains a mitochondria localization signal (MLS), which directs pVII mitochondria localization (Anand et al., 2014). pVII mitochondria localization inhibits apoptosis by maintaining mitochondria membrane potential (MMP), retention of mitochondria Ca^{2+} and increasing ATP production (Anand et al., 2014).

Unlike HAdV-2 (Davison et al., 2003), the L2 region of BAdV-3 encodes only protein V (pV), a protein unique to Mastadenoviruses (Bahr et al., 2003). BAdV-3 pV is 423 amino acids long and shows amino acids sequence identities of 28-40% to other Mastadenovirus pV proteins (Reddy et al., 1998). Moreover, pV is rich of basic amino acids residues, which indicates pV may localized in nucleus (Reddy et al., 1998).
Unlike HAdV-2 (Anderson et al., 1989), the L3 region of BAdV-3 encodes pX precursor (Anderson et al., 1989). BAdV-3 pX precursor protein is 80 amino acids long and shares 54% sequence identity to HAdV-2 pX (Reddy et al., 1998). Similar to HAdV-2 pX (Anderson et al., 1989), it contains a conserved sequence, which may act as a bipartite NLS for protein, and two predicted viral protease cleavage site (Reddy et al., 1998).

Unlike HAdV-5 (Hayes et al., 1990), the L4 region of BAdV-3 encodes only pVI protein (Reddy et al., 1998). BAdV-3 pVI is a structural protein with 263 amino acids and shares 32% homology to HAdV-2 pVI. In addition to being assembled in the capsid, pVI also mediates the disruption of endosome membrane and pVI precursor transports hexon from cytoplasm to nucleus (Kauffman and Ginsberg, 1976). BAdV-3 pVI protein contains two putative protease cleavage sites at both N- and C-terminus of protein (Reddy et al., 1998).

Unlike HAdV-2 (Chroboczek et al., 1992), the L5 region of BAdV-3 encodes hexon and protease (Reddy et al., 1998). BAdV-3 hexon is a 910 amino acids protein, showing 65% sequence identity to HAdV-2 hexon. Although there are some differences in external loops between BAdV-3 and HAdV-3 hexon proteins, the difference did not influence the structure of hexon proteins (Athappilly et al., 1994; Hu et al., 1984). BAdV-3 protease is 204 amino acids long, and showing 65% sequence identity to HAdV-2 protease. In BAdV-3, protease cleaves structural proteins pIIIa, pVI, pVII, pVIII, pX, and pTP precursors before virus assembly (Reddy et al., 1998).

Unlike HAdV-2 L4 region, which encodes only 100K and 33K (Oosterom-Dragon and Anderson, 1983), the L6 region of BAdV-3 encodes non-structural proteins 100K,
33K and 22K, and a structural protein pVIII (Kulshreshtha et al., 2004). The BAdV-3 100K protein is 850 amino acids long, and showing 52% sequence identity with HAdV-2 100K. It functions to facilitate hexon trimerization (Oosterom-Dragon and Ginsberg, 1981). Moreover, BAdV-3 100K contains two putative protease cleavage sites, and the cleaved C-terminus product is localized in the nucleolus, although the cleavage of 100K is not essential for viral replication (Makadiya et al., 2015). 100K interacts with viral protein 33K and cellular protein dynein light chain (DYNLT) 1, while co-expression of 100K with 33K or DYNLT1 did not alter the cellular localization of 100K (Makadiya, 2013). In BAdV-3, 33K and 22K share the same N-terminus while differs in C-terminus because of splicing (Kulshreshtha et al., 2015). BAdV-3 33K interacts with 100K and pV, and functions to transactivate MLP (Kulshreshtha and Tikoo, 2008). 100K was also found to interact with cellular protein bovine presenilin-1-associated protein/mitochondrial carrier homolog 1 (BoPSAP/BoMtch1), and colocalize in the mitochondria with BoPSAP (Kulshreshtha, 2009). BAdV-3 pVIII is the only structural protein encoded by L6 region. It is 216 amino acids in length, and showing 52% sequence identity with HAdV-2 pVIII (Reddy et al., 1998). Two proteases cleavage sites have been identified in BAdV-3 pVIII, and after cleavage, the 8 kD C-terminus is assembled into the virions (Ayalew et al., 2014). pVIII can abolish translation of capped mRNA via its interaction with cellular protein DEAD box (DDX) 3, and knockdown of DDX3 significantly reduces the BAdV-3 viral yield and expression of viral late proteins (Ayalew, 2014).

The fiber protein is coded by BAdV-3 L7 region rather than L5 region in HAdV-2 (Reddy et al., 1998). BAdV-3 fiber protein is 976 amino acids long and shows 23.7%
amino acids identity to HAdV-3 fiber protein. The speculated motif interacting with penton is highly conserved in both HAdV-2 and BAdV-3 (Caillet-Boudin, 1989). Replacing its knob region of BAdV-3 fiber with HAdV-5 knob region can alter its tropism (Wu and Tikoo, 2004).

1.1.3.4 BAdV-3 as vectors for vaccination

With the development of DNA editing, genomics and immunology, it has become possible to manipulate viruses to use them as vaccine delivery vehicles (Draper and Heeney, 2010). Adenovirus is one of the few viruses being developed and evaluated as vaccine delivery vehicles (Wold and Toth, 2013). Although human adenoviruses are widely evaluated for delivery of vaccine antigens of HIV (Herath et al., 2015), influenza viruses (Coughlan et al., 2015), rabies (Shen et al., 2012), dengue (Raviprakash et al., 2008), middle east respiratory syndrome coronavirus (MERS-CoV) (Kim et al., 2014) and Ebola virus (Ledgerwood et al., 2015) to human and animals, safety concerns and efficiency of vaccination has hindered its widespread use (Fausther-Bovendo and Kobinger, 2014). Currently, non-human adenoviruses such as chimpanzee adenovirus-7/63 (Kobinger et al., 2006; Nebie et al., 2014), bovine adenovirus-3 (Mittal et al., 1995; Reddy et al., 1999b; Singh et al., 2008), porcine adenovirus-3/5 (Hammond et al., 2001; Tuboly and Nagy, 2001) and canine adenovirus-2/3 (Tordo et al., 2008; Wright et al., 2013), ovine adenovirus-7 (Tang et al., 2012), and fowl adenovirus-9 (Deng et al., 2013) are being evaluated as vaccine delivery vehicles both in humans (Kobinger et al., 2006; Nebie et al., 2014) and animals (Tang et al., 2012).
A number of properties of BAdV-3 including high titers, low virulence, easy manipulation, stability and safety make it a vector of choice for developing BAdV-3 based vaccines for humans (Singh et al., 2008; Wu and Tikoo, 2004) and animals (Mittal et al., 1995; Reddy et al., 1999b; Singh et al., 2008). E1A 286R or E1B 55 kDa are essential for BAdV-3 replication since their deletion renders BAdV-3 replication-defective in cultured cells (van Olphen et al., 2002). Thus, the replication-defective BAdV-3 vectors are generated by the deletion of E1A region, therefore E1 deleted vectored vaccines are thought to be safer than replication-competent vectored vaccines (Ayalew et al., 2015). However, deletion of a small region of E1B (Zhou et al., 2001) or the E3 region (Reddy et al., 2000; Zakhartchouk et al., 1998) does not alter the replication of BAdV-3 in cultured cells. Thus, replication-competent vectors are generated by deletion of E1B (Zhou et al., 2001) and \ or E3 (Reddy et al., 2000; Zakhartchouk et al., 1998). Several BAdV-3 vectored vaccines based on insertion of foreign antigenic genes, such as bovine herpes virus (BHV)-1 gD (Zakhartchouk et al., 1998), bovine viral diarrhea virus (BVDV) E2 (Baxi et al., 2000) and bovine coronavirus (BCV) HE (Reddy et al., 2000), into the E3 deleted region have been developed and evaluated in cotton rats (Zakhartchouk et al., 1998). In addition, the E4 region has been identified as an insertion region for foreign ORFs (Baxi et al., 1999; Baxi et al., 2001). The C-terminal 1.3 kb or N-terminal 1.5 kb fragment can be deleted from E4 region without influencing BAdV-3 replication (Baxi et al., 1999; Baxi et al., 2001) and suggested that foreign ORFs can be inserted at these positions to increase BAdV-3 vectors capacity (Baxi et al., 1999; Baxi et al., 2001). Protecting animals from multiple antigens is feasible for BAdV-3 vectors since they can express multiple antigenic foreign
genes. Two antigens BHV-1 gD and bovine respiratory syncytial virus (BRSV) gG are inserted into a BAdV-3 based vector that induces production of protective antibodies against both BHV-1 and BRSV in cotton rats (Brownlie et al., 2015).

BAdV-3 structural proteins can be modified to expand the tropism of the BAdV-3 based vectored vaccines. Two viral proteins are the potential targets for tropism modification. Firstly, fiber protein is a major capsid proteins used by BAdV-3 for cellular receptor binding (Wu and Tikoo, 2004). The replacement of BAdV-3 fiber knob region with that of HAdV-5 increases the BAdV-3 vector transduction efficiency significantly in non-bovine cells, which are transduced by the unmodified BAdV-3 vectors with low efficiency (Wu and Tikoo, 2004). Secondly, pIX is a minor protein that exposed on the BAdV-3 virion surface that provides its potential for tropism modification. Insertion of RGD motif into the C-terminus of pIX enhances fiber-independent tropism of BAdV-3 (Zakhartchouk et al., 2004). Tropism alterations of BAdV-3 vectored vaccines not only improve vaccine delivery efficiency, but also broaden the range of target animals (Ayalew et al., 2015).

1.1.3.5 Protein-protein interaction in adenoviruses

Proteins play indispensible roles in different steps of adenovirus replication, including attachment, internalization, DNA replication, protein expression and vial particle assembly. Many of these viral proteins perform their function by interacting with other viral \ cellular proteins (Arnberg, 2009). A variety of assays have been developed to detect these protein-protein interactions, such as co-immunoprecipitation (Co-IP) (Takahashi, 2015), bimolecular fluorescent complementation (BiFC) (Hu et al., 2002;
Kong et al., 2015), pull down assay (Fonseca and Solano, 2013; Wang et al., 2014b), yeast two hybrid (Maruta et al., 2016), strep protein interaction experiment (SPINE) (Herzberg et al., 2007), quantitative immunoprecipitation combined with knock-down (QUICK) (Selbach and Mann, 2006), label transfer (Liu et al., 2007), phage display (Sidhu et al., 2003), photo-reactive amino acid analogs (Hetu et al., 2008), tandem affinity purification (Rohila et al., 2006), chemical cross-linking (Tang and Bruce, 2009), proximity ligation assay (Soderberg et al., 2006). Moreover, recently methods based on Machine-learning-based computational approaches have been developed and utilized for prediction of protein-protein interaction sites (Liu et al., 2015).

1.1.3.5.1 Protein-protein interactions in adenovirus attachment and internalization

Adenovirus infection starts with an interaction between fiber knob and cellular receptors, which is followed by the interaction of penton base RGD or LDV motifs with integrin on the cell membrane (Arnberg, 2009). Although there is no RGD or LDV motifs present in BAdV-3 penton base, the LDV motif seems to be replaced by MDV motif, which may interact with \( \alpha_4\beta_1 \) integrin (Komoriya et al., 1991; Reddy et al., 1998). After endosomal escape, partially uncoated adenovirus is transported to the nucleus along the MTOC, which is possibly mediated by an interaction between viral pV and cellular p32 protein. Cellular protein p32 can shuttle between mitochondria and the nucleus; therefore adenovirus may hijack this route to reach the nucleus after escape from the endosome (Matthews and Russell, 1998a). Since knockdown of Nup214 reduced adenovirus genome entry into the nucleus, it has been suggested that the entry of the adenovirus
genome into the nucleus is mediated by the interaction between hexon and nucleoporin Nup214 (Cassany et al., 2015).

1.1.3.5.2 Protein-protein interactions of adenovirus early proteins

After entry of adenovirus genome in the nucleus, it starts to express E1A proteins (Curtois and Berk, 1984). E1A reprograms the cell cycle and gene expression by directly binding to key cellular regulatory proteins and transcription factors (Avvakumov et al., 2002). As described previously (1.1.2.4.2), E1A products can regulate the cell cycle by interacting with pRB to disrupt pRB-E2F complex and release E2F, a trans-activator of genes involved in the cell cycle (Ikeda and Nevins, 1993). In addition, chromatin can be remodelled by the by E1A protein by interacting with TRRAP/GCN5 (Lang and Hearing, 2003), pCAF and p300/CBP (Chakravarti et al., 1999; Hamamori et al., 1999). Finally, E1A proteins regulate target protein expression by protein-protein interaction with CCBF1 and SUR2 (Ansieau et al., 2001).

The two E1B proteins are involved in inhibition of apoptosis. E1B-55K directly binds to tumor suppressor p53 to degrade it (Ben-Israel and Kleinberger, 2002) or it also forms a complex with other proteins including an ubiquitin ligase to degrade p53 (Sarnow et al., 1984). The interaction of E1B-19K with BAK and BAX can disrupt BAK and BAX complex binding to the mitochondria membrane therefore inhibits its pro-apoptosis activity (Cuconati et al., 2003).

The E2 region proteins are involved in viral DNA replication. DBP, pTP and DNA pol are complexed by interacting with each other to initiate the DNA replication,
maintain the elongation and mediate the termination of the DNA replication (Mysiak et al., 2004; van Breukelen et al., 2003).

The protein-protein interaction between E3-gp19K and MHC class I helps to retain MHC class I in the ER, therefore it cannot migrate to cell surface and present antigens to cytotoxic T lymphocytes (CTLs) (Burgert et al., 1987). The RID complex formed by the interaction of E3-10.4K and 14.5K removes the chemokine receptors on the cell surface and mediates their degradation and hence inhibits chemokine induced cell apoptosis (Shisler et al., 1997).

E4 region encoded proteins are involved in modulation of cell cycle, transcription, DNA repair and cell signalling via protein-protein interactions (Weitzman, 2005). The interaction of E4-orf1 and cellular protein Discs Large 1 (Dlg1) promotes cell survival and viral replication by activating phosphatidylinositol 3-kinase (PI3K) (Kong et al., 2014). E4-orf3 promotes viral replication by interacting with cellular proteins transcription intermediary factor (TIF) 1α, TIF1β and TIF1γ and relocating them to nuclear track structures (Vink et al., 2012). Unlike other E4 proteins, E4-orf4 induces apoptosis and down regulates adenovirus early protein expression (Muller et al., 1992) by interacting with ATP dependent chromatin-remodeling factor ACF, which is composed of an Acf1 regulatory subunit and a sucrose non-fermenting-2h (SNF2h) ATPase (Brestovitsky et al., 2011). Moreover, the interaction of E4orf4 and PP2A inhibits the activity of ACF and enhances the chromatin activity of WSTF-SNF2h (Brestovitsky et al., 2011). E4-orf6 interaction with E1B-55K not only shuts off cellular protein translation but also degrades tumor suppressor p53 by ubiquitination (Evans and Hearing, 2003).
1.1.3.5.3 Protein-protein interactions of adenovirus intermediate proteins

The intermediate protein IVa2 is involved in viral capsid assembly (Zhang and Imperiale, 2003), viral DNA encapsidation (Zhang et al., 2001) and activation of the major late promoter. In HAdV-5 infected cells, IVa2 interacts with L1 protein 52/55K to facilitate the viral DNA packaging (Zhang and Imperiale, 2003). Recently, IVa2 was found to function as a packaging ATPase and interact with another viral protein 33K, which is important for viral DNA packaging (Ahi et al., 2015). In another study, IVa2 forms a complex with L4-22K and attaches to the DNA packaging sequence to direct viral DNA packaging (Pardo-Mateos and Young, 2004; Yang and Maluf, 2012). Moreover, IVa2 interacts with pVIII in porcine adenovirus-3 (PAdV-3) infected VIDO R1 cells, although the purpose is not clear (Singh et al., 2005).

Adenovirus pIX is a structural protein, which plays an important role in the stabilization of the capsid by interacting with the C-terminus of hexon (Parks, 2005). Moreover, pIX also functions as a transcription activator and causes virus-induced nuclear reorganization by interacting with the major late promoter (Rosa-Calatrava et al., 2001). In another study, pIX has shown to colocalize and associate with promyelocytic leukemia protein (PML), which is a major component of nuclear structure nuclear domain 10 (ND10) and involved with an antiviral response (Chee et al., 2003). The interaction of pIX with PML probably leads to the sequestration of PML and inhibition of its antiviral activity (Parks, 2005).
1.1.3.5.4 Protein-protein interactions of adenovirus late proteins

The HAdV-5 L1-52/55K mediates viral DNA packaging via its interaction with IVa2 (Perez-Romero et al., 2005). L1-52/55K was also detected to serve as the packaging scaffold protein in viral assembly (Mangel and San Martin, 2014). Recently, it has been demonstrated that L1-52/55K interacts with the core and capsid components and is present in empty capsid but not in mature viral particles (Perez-Berna et al., 2014). In the maturation process of adenovirus particles, the L1-52/55K molecules are cleaved by the viral protease and transported out from the capsid (Perez-Berna et al., 2014). The other L1 protein IIIa is a structural protein that interacts with hexon to stabilize the viral capsid (Ma and Hearing, 2011). In addition, IIIa interacts with L1-52/55K to improve viral DNA packaging, and the interaction has been detected both in vivo and in vitro (Ma and Hearing, 2011).

The HAdV-5 L2 pVII is a core protein that binds to the viral DNA non-specifically (Zhang and Arcos, 2005). In viral DNA nuclear import, pVII interacts with nuclear transport factors importin alpha, importin beta, importin 7 and transportin via its three nuclear localization signals (Wodrich et al., 2006). The use of multiple nuclear import pathways by pVII is thought to improve the efficiency of viral genome nuclear import (Wodrich et al., 2006). Moreover, pVII also functions in viral DNA encapsidation by interacting with two viral DNA packaging proteins IVa2 and L1-52/55K (Zhang and Arcos, 2005). Another HAdV-5 L2 protein pV bridges the capsid and core via interaction with capsid component pVI (Matthews and Russell, 1998b) and core components pVII (Harpst et al., 1977). pV also interacts with cellular protein p32, which shuttles between the mitochondria and nucleus, and this interaction may mediate adenovirus transportation
form the cytoplasm to the nucleus (Matthews and Russell, 1998a). In addition, pV has been detected to interact with nucleolar protein B23. This interaction not only redistributes nucleolar protein nucleolin from the nucleus to the cytoplasm but is also involved in core protein dissociation (Samad et al., 2007). The L2 penton base protein also functions as a structural protein and plays an important role in adenovirus internalization (Varga et al., 1991). The interaction between the penton base RGD motif and integrins triggers clathrin-mediated endocytosis, which facilitates adenovirus cellular entry (Varga et al., 1991). In addition, the penton protein also interacts with a highly conserved fiber-interacting motif (HSRLSNLLGIRKR) to form the penton (Caillet-Boudin, 1989).

The HAdV-5 L3 pVI interacts with other viral components to stabilize the viral particle (Chatterjee et al., 1985; Matthews and Russell, 1998b). It also plays a role in viral endosomal release and capsid assembly. pVI can interacts with the newly synthesized hexon (another L3 protein) and facilitates its import to the nucleus where viral assembly occurs (Wodrich et al., 2003).

The HAdV-5 L4 protein 100K interacts with eukaryotic initiation factor 4G (eIF4G) to form a complex involved in ribosome shunting to improves late viral protein translation (Xi et al., 2004). In addition, 100K also functions in viral capsid assembly of hexon. The 100K serves as a chaperone for hexon protein folding and trimerization by interaction with globular domain of hexon protein, and mediates its nuclear import in insect cells (Hong et al., 2005). 100K can interact with granzyme, a serine proteases released by cytotoxic T cells or natural killer cells to induce programmed cell death, to inhibit its activity against viral replication (Andrade et al., 2003). The HAdV-5 L4 33K
protein interacts with viral packaging proteins, such as L1-52/55K, IVa2 (Ahi et al., 2013), IIIa (Wu et al., 2013) and 22K (Wohl and Hearing, 2008). These interactions are thought to be involved in viral assembly (Wu et al., 2013).

The only protein encoded by the HAdV-5 L5 region is fiber, which interacts with penton base to form penton (Caillet-Boudin, 1989). Moreover, adenovirus infections start with the interaction between fiber knob and the cellular receptors, such as coxsackie virus and adenovirus receptor (Tomko et al., 1997), Heparan sulfate glycosaminoglycans (HS-GAGs) (Dechecchi et al., 2001), CD46 (Stevenson et al., 1995), CD80 and CD86 (Short et al., 2004), and sialic acid (Arnberg et al., 2002).

1.2 PROTEIN NUCLEOCYTOPLASMIC TRANSPORT

The nucleus is the repository of genetic material and control center of eukaryotic cells. Important processes like DNA replication, RNA transcription and processing take place in the nucleus (Cooper GC, 2006). The nuclear envelope separates the genetic materials in the nucleus from the cytoplasm, which provides eukaryotic cells opportunities to regulate protein expression at the levels of transcription and post-transcription. By controlling the nuclear transport of transcription factors, eukaryotic cells regulate gene expression at the transcriptional level (Cooper GC, 2006). On the other hand, eukaryotic cells regulate gene expression at post-transcription level by alternative splicing, which does not happen in prokaryotic cells (Cooper GC, 2006).
1.2.1 Nuclear pore complex

The nuclear pore complex is the only channel through which cellular materials can travel between the cytoplasm and the nucleus (Tonini et al., 1999). The RNAs are synthesized in the nucleus, and must be transported to the cytoplasm for translation leading to protein expression (Cooper GC, 2006). Similarly, proteins are synthesized in the cytoplasm, and need to be transported to the nucleus to perform certain functions (Cooper GC, 2006). Thus, the NPC plays a fundamental role in eukaryotic cells by regulating the transport of molecules between the cytoplasm and the nucleus (Tonini et al., 1999).

1.2.1.1 Nucleoporin

Nucleoporins (Nups) are a family of proteins that comprise the NPC and regulate the transportation of macromolecules between the cytoplasm and the nucleus. Nucleoporins contain some conserved FG (phenylalanine-glycine) motifs, which serve as binding sites for the transport factors (Bayliss et al., 2000). FG repeats consist of several tandem repeats, having hydrophobic cores with hydrophilic spacers. The two most common FG motifs are GLFG and FxFG (x represents small amino acid residues, like alanine, glycine and serine) (Bayliss et al., 2000). Multiple copies of these FG motifs are present in nucleoporins, for example Nup116p and Nsp1p contain 33 GLFG and 19 FxFG, respectively (Bayliss et al., 2000), which bind directly to soluble transport factors (Finlay and Forbes, 1990). Protein nuclear import can be blocked by Mab414 (Davis and Blobel, 1986), which directly interacts with FxFG motifs, and RNA nuclear export can be blocked by anti-Nup98 antibody (Powers et al., 1997). Intriguingly, purified Nup153
FxFG motifs can inhibit the nuclear transport mediated by importins due to its recognition and interaction with classic nuclear localization signals (NLSs), while it has no effect to the nuclear import mediated by transportin, which binds to a different NLS (Shah and Forbes, 1998). In addition to FG motifs, some nucleoporins also contain coiled-coils, which are probably involved in interacting with other nucleoporins for NPC assembly (Bayliss et al., 2000).

Nucleoporins are assembled into functional sub-complexes prior to the construction of NPC (Krull et al., 2004). Of the known nucleoporins (Harel et al., 2003), the Nup107-160 sub-complex is the most extensively studied. Nine nucleoporins present in Nup107-Nup160 sub-complex include: Sec13, Seh1, Nup37, Nup43, Nup85, Nup96, Nup107, Nup133 and Nup160, and knocking out of any of these nine leads to the nuclear envelope devoid of NPC (Harel et al., 2003). This sub-complex plays a role in NPC assembly and facilitates the anchoring of Nup153 at the face of the envelope. The Nup93 sub-complex is composed of Nup35/53, Nup188, Nup155, Nup205 and anchors NPC on the nuclear envelope via an interaction with the cellular protein Ndc1. The interaction of Nup93 with Ncl1 seems to be essential for the assembly of NPC (Mansfeld et al., 2006).

1.2.1.2 Structure of nuclear pore complex

The nuclear pore is a large protein complex composed of three components, including the cytoplasmic ring and filaments, the nuclear ring and bucket, and the central cylinder (Stoffler et al., 1999) (Fig 1.5). More than 30 different types of nucleoporins are present in the NPCs in eight or multiples of eight copies due to the eight-fold symmetry of NPC structure (Stoffler et al., 1999). Recent studies have shown that the NPC is
approximately 50 nm thick with an outside diameter of 80-120 nm and an inner diameter of approximately 40 nm (Bui et al., 2013).

As shown in Fig 1.5, the NPC is composed of an assembly of eight spokes that surrounds a central channel, which spans the outer and the inner membranes of the nuclear envelope (Wente and Rout, 2010). Spokes, which are comprised of the Nup93 sub-complex in vertebrates, connects the rings at the cytoplasmic and the nuclear surfaces (Wente and Rout, 2010). The ring-spoke assembly is anchored on the site of fusion between the outer and the inner membrane of the nuclear envelope (Wente and Rout, 2010). While some nucleoporins protrude into the cytoplasm to form cytoplasmic filaments, some nucleoporins extend into the nucleoplasm to form the nuclear bucket (Kabachinski and Schwartz, 2015). Moreover, the nucleoporins with FG motifs are located in the central channel to provide the transport-binding sites (Wente and Rout, 2010).

1.2.2 Protein selective import

Small proteins with molecular weight less than approximately 40 kDa can freely pass through the nuclear envelope in either direction (Patel et al., 2007), although proteins larger than 60 kDa have been proved to diffuse through the NPC (Wang, 2007). Large proteins, however, pass through the NPC by an active process in which proteins are transported via interactions with transport factors in a specific direction. Proteins that can be transported through the NPC contain specific amino acids sequence termed nuclear localization signal (NLS) or nuclear export signal (NES), which can be recognized and
Figure 1.5 Schematic diagram of nuclear pore complex structure. Adapted from (Strambio-De-Castillia et al., 2010; Wente and Rout, 2010), with permission to use.
bound by transport factors to mediate active transportation (Kabachinski and Schwartz, 2015).

The NLS is a motif rich in basic amino acids arginine (R) and lysine (K), and the best described among them is the classical NLS (cNLS), which is monopartite with a consensus sequence of (K/R)_{4-6} (Dingwall and Laskey, 1991). The first cNLS was identified in simian virus 40 (SV40) large-T antigen (Kalderon et al., 1984). The NLS of large-T antigen was defined as a seven amino acids sequence (PKKKRKV), where all lysine residues appeared to be essential for the protein nuclear transport (Kalderon et al., 1984). A different type of NLS (KRPAATKKAGQAKKKK), which is composed of two basic domains and a ten amino acids spacer that tolerates site mutations, was identified in nucleoplasmin (Robbins et al., 1991). Since it contains two separate domains, it was called bipartite NLS. Though most NLSs are rich in basic amino acids residues, some NLSs do not resemble the consensus sequence. While NLS activity in some proteins may be realized by their 3 dimensional structure (Boulikas, 1993), others may use different factors for their transportation (Lee et al., 2006a). Some proteins contain amino acids sequences that mediate their nuclear export, termed nuclear export signals that are rich in leucine residues (Fornerod et al., 1997).

Protein nucleocytoplasmic transport is a complex process that starts with the interaction of NLS/NES containing target protein with nuclear transport factors. As shown in Fig 1.6, in the importin-α/β mediated nuclear import, importin-β can bind to the NLS of the target protein directly or by using importin-α as an adaptor. After the formation of transport factor-cargo complex, it binds to the cytoplasmic filament, and move toward the nucleus by sequential binding to specific nucleoporins inside the NPC.
In the nucleus, the importin-cargo complex is disrupted by the interaction of importin-β with Ran-GTP to release the cargo protein in the nucleus. The importin-Ran-GTP complex is recycled by being transported back to the cytoplasm where importins participate the next round of protein nuclear import (Wente and Rout, 2010). Nuclear export is a similar process where the binding of NES by exportins and the Ran-GTP form the nuclear export complex that is composed of cargo protein, exportins and Ran-GTP. After the translocation of the complex through the NPC to the cytoplasm, the hydrolysis of GTP to GDP disrupts the complex and leads to the release of the cargo protein into the cytoplasm (Wente and Rout, 2010).

1.2.3 Nuclear transport receptor

The NLSs/NESs are recognized and bound by karyopherin proteins that serve as nuclear transport receptors. The karyopherin protein family are subdivided into importins and exportins depending on their function in nuclear import or nuclear export, respectively. Importin-α recognizes and binds to classical NLSs and serves as the bridge between importin-β and cargo protein, while importin-β recognizes non-classical NLSs, such as PY-NLS that is rich of proline and tyrosine (Lee et al., 2006a). Nuclear transport receptors also contain domains that bind to nucleoporins and Ran-GTP (Bednenko et al., 2003; Kutay et al., 1997).

The Ran is a small GTPase that interacts with the transport receptor-cargo complex and regulates nuclear transport (Steggerda and Paschal, 2002). In the nucleus, the importin-cargo complex is disrupted by a conformational change in importin-β induced by the binding of Ran-GTP. The importin-Ran-GTP complex is then transported back to
Figure 1.6 Importin α/β mediated protein nuclear transportation. Adapted from (Flather and Semler, 2015; Kuersten et al., 2001), with permission to use.
the cytoplasm through the NPC. The Ran-GTP is hydrolyzed in the cytoplasm by Ran-GTP-activating protein (Ran GAP) to Ran-GDP (Hutten et al., 2008), which causes the release of importins so that they can participate the next round of nuclear import. The newly generated Ran-GDP is transported back to the nucleus by NTF2, and then Ran-GDP is transformed to Ran-GTP by Ran guanine nucleotide exchange factor Ran GEF (Macara, 2001).

1.3 NUCLEOLUS

The nucleolus is a prominent body in the nucleus. It is present in the nucleus during the interphase but not at the stage of mitosis. The nucleolus serves as a site for ribosome RNA transcription, processing and ribosome subunits biosynthesis (Sirri et al., 2008). Its size increases during ribosome synthesis (Altmann and Leblond, 1982; Kononowicz and Janick, 1988).

1.3.1 Structure and functions of the nucleolus

The nucleolus is a solid structure with deceptive appearance under the light or electron microscope. In eukaryotic cells, the nucleolus consists of three main blocks with different functions, including: fibrillar center (FC), dense fibrillar component (DFC) and granular component (GC), which functions as sites for ribosomal RNA (rRNA) transcription, processing and ribosome assembly, respectively (Caudron-Herger et al., 2015).
1.3.1.1 Structure of the nucleolus

The nucleolus is a structure composed of granules and fibrils. The low contrast areas of the fibrils are called FCs. The FCs contain rDNA as well as factors for rDNA transcription (Thiry, 1992), such as DNA polymerase I subunits, upstream binding factors (UBF) and DNA topoisomerase I (Thiry and Goessens, 1991). However, the rDNA transcription is detected at the interface between FCs and DFCs rather than in the FCs (Hozak et al., 1994). After the transcription, the pre-rRNA transcripts need to be processed to generate functional rRNAs (Casafont et al., 2007). DFCs are featured with densely packed filamentous materials. It contains nascent pre-rRNA transcripts, which are still attached to the rDNA and pre-rRNA transcripts complex with some proteins and small nucleolar RNAs (snoRNA) (Azum-Gelade et al., 1994; Nazar, 2004; Qiu et al., 2008). The third block designated GC is featured with the presence of the granules, which are possibly pre-60S subunits at different stages of the assembly and the maturation process (Falini et al., 2007). The GC is the site for 60S ribosome subunits assembly because of the presence of proteins for the 5.8S and 28S rRNAs processing (Gadal et al., 2002). Many other proteins involved in ribosome biosynthesis have been detected in the GC, such as B23 (nucleophosmin) (Falini et al., 2007), Nop52 (Yoshikawa et al., 2015), Bop1 (Rohrmoser et al., 2007), and ribosome RNA processing 1B (RRP1B) (Chamousset et al., 2010). It also has been reported that RRP1B containing complexes play functions at the middle and the late stages of 60S assembly (Chamousset et al., 2010).
1.3.1.2 Non-traditional Functions of Nucleolus

Only 30% of the nucleolar proteins are involved in ribosome biosynthesis and suggests that the nucleolus may engage in functions other than ribosome biosynthesis (Ahmad et al., 2009). Recently, reports suggested that nucleolus might be involved in cell cycle regulation, p53 activity regulation and signal recognition particle (SRP) assembly.

1.3.1.2.1 Role of nucleolus in regulating tumor suppressor p53

Though p53 is involved in apoptosis induction and cell cycle regulation to respond the cell stress, the main function of the cellular protein p53 is to suppress tumor formation (Zilfou and Lowe, 2009). Some nucleolar proteins are found to regulate p53 activities, which suggests the nucleous is involved in cell cycle regulation via p53 (Rubbi and Milner, 2003). The cellular protein murine double minute 2 (Mdm2), which is considered the major negative regulator of p53, can induce p53 degradation via its E3 ubiquitin ligase activity to keep p53 at a stable level (Bouska and Eischen, 2009).

The expression of another nucleolar protein ARF is inhibited in many different kinds of tumor cells suggesting that it also plays a role in tumor suppression (Maggi et al., 2014). ARF activates Mdm2 E3 ubiquitin ligase to inhibit the ribosome biosynthesis of tumor cells. One possible explanation is that AFR directly binds to Mdm2 to sequester it in the nucleolus; thereby stabilizing p53 because of the absence of Mdm2 (Tao and Levine, 1999). In addition to binding to Mdm2 alone, the ARF also interacts with a complex of p53 and Mdm2 to inhibit p53 ubiquitination and degradation (Kamijo et al., 1998). In addition to the p53-denpendent AFR function, the antitumor activity of ARF also shows in p53-independent manner (Weber et al., 2000a).
The nucleophosmin (NPM) also known as B23, is one of the most abundant nucleolar proteins. It is a multifunctional protein involved in ribosome biosynthesis, pre-rRNA splicing (Savkur and Olson, 1998) and RNase activity regulation (Herrera et al., 1995). It is also involved regulating p53 oncogenesis by inhibiting (Li et al., 2004) and activating p53 activity (Grisendi et al., 2006). Overexpression of B23 inhibits the cell proliferation by directly binding to and activating p53 (Colombo et al., 2002). In addition, B23 stabilizes ARF by sequestering it in the nucleolus and ARF cannot be transported to the nucleolus without B23 (Colombo et al., 2005). On the other hand, tumor cells express higher levels of B23 than normal cells, which suggests B23 may be associated with oncogenesis and cell proliferation (Zhang, 2004). The activity of p53 is also inhibited by the interaction between B23 and ARF, which sequesters ARF in the nucleolus, thus not letting ARF stabilize and activate p53 (Colombo et al., 2005).

Some ribosome proteins (Harpst et al., 1977) are also associated with the regulation of p53 activity (Dai and Lu, 2004; Dai et al., 2004; Lohrum et al., 2003). If ribosome biosynthesis is disrupted, RPs will be released from the nucleolus and directly interact with Mdm2 to prevent the Mdm2 mediated p53 degradation (Zhou et al., 2012). For example, p53 induced cell cycle arrest can be induced by dysfunction of proteins associated with pre-rRNA transcription, such as Wrd36 (Zhang et al., 2008), WDR12 (Holzel et al., 2005) and Bop1 (Pestov et al., 2001). In addition, some drugs, like actinomycin D, can also disrupt the ribosome biosynthesis (Martinez-Ramon, 1979). Pre-rRNA transcription can be inhibited by actinomycin D via the inhibition of RNA polymerase I activity, which facilitates the release of RPs and p53 mediated cell cycle arrest (Ginisty et al., 1999). NPs including RPL5, RPL11 and RPL23 antagonize Mdm2
activity by directly binding to it and inhibiting its E3 ubiquitin ligase activity (Dai and Lu, 2004; Dai et al., 2004; Lohrum et al., 2003). The RPS7, which is substrate of Mdm2 E3 ubiquitin ligase, also binds to and inactivates Mdm2 (Zhu et al., 2009).

1.3.1.2.2 Role of nucleolus in cell cycle regulation

The nucleolar protein nucleostemin is involved in maintaining the continuous proliferation of cancer cells and stem cells, and has been found in the nucleoli of the neural stem cells and the embryonic stem cells (Tsai and McKay, 2002). Nucleostemin is member of the GTP binding protein family, with the GTP free form localized in the nucleoplasm while the GTP bound form is localized in the nucleolus. Deletion of nucleostemin causes cell cycle arrest at either G1/S or S/G2 phases in different tumor cells (Beekman et al., 2006). Overexpression of nucleostemin induces active cell proliferation in low nucleostemin expressing cells; while cell cycle was arrested at G1/S phase in high nucleostemin expressing cells (Dai et al., 2008). Besides regulating the cell cycle, nucleostemin is also involved in pre-rRNA processing since the splicing of 32S pre-rRNA to 28S rRNA is delayed after the knockout of nucleostemin (Romanova et al., 2009).

The function of nucleostemin in cell cycle regulation is achieved by its interaction with other proteins. The interaction of nucleostemin with Mdm2 stabilizes Mdm2 and improves its E3 ubiquitin ligase activity and sequesters it in the nucleoplasm to inhibit p53 activity (Meng et al., 2008). The interactions between Mdm2 and RPL5 or RPL11 can be detected after the deletion of nucleostemin, which induces the stabilization and activation of p53 (Dai et al., 2008). In summary, the function of the nucleolus in cell
cycle regulation depends on the nucleolar protein nucleostemin, which inhibits p53 activity by binding to and stabilizing Mdm2.

1.3.1.2.3 Role of nucleolus in Signal recognition particle assembly

Signal recognition particle (SRP) is a family of cytoplasmic RNA-protein complexes (RNP) that recognize and target membrane and secretory proteins in the endoplasmic reticulum (Janda et al., 2010). The SRP recognizes and binds to the signal peptide of a nascent protein to arrest the synthesis of the polypeptide, and then the protein is transported to the ER by the subsequent co-translational translocation (Shan and Walter, 2005). The SRP is a complex composed of 6 proteins, SRP9, SRP14, DRP16, SRP54, SRP64 and SRP72, and SRP RNA transcribed by RNA polymerase III (Walter and Johnson, 1994). Micrococcal nuclease cleaves SRP into the two domains and S domain. Alu domain is composed of SRP9 and SRP14; while S domain consists of the other 4 SRP proteins (Walter and Johnson, 1994). Different SRP proteins play different roles (Egea et al., 2008). For example, SRP 54 recognizes the peptide signal (Egea et al., 2008), and SRP 68/72 are involved in the nascent protein translocation (Iakhiaeva et al., 2009). The nucleolar localization of SRP components supports that the nucleolus is involved in the SRP assembly. The SRP RNA nucleolar localization has been demonstrated by situ hybridization and microinjection (Jacobson and Pederson, 1998). The S domain proteins SRP9 and SRP14 are localized only in the nucleolus (Andersen et al., 2002). Three Alu domain proteins ARP19, ARP68 and ARP72 are found in the nucleolus, the nucleoplasm and the cytoplasm, while the SRP54 is localized only in the cytoplasm (Politz et al., 2000). The nucleolar localization of SRP components suggests...
that the nucleolus is partially involved in SRP assembly. Moreover, it is also likely that
the nucleolus provides a potential site for SRP RNA processing since the nucleolar
localization of SRP and the presence of RNA processing machinery in the nucleolus.

1.3.2 Ribosome biosynthesis

The most important function of the nucleolus is that it acts as a factory for ribosome
assembly (Cmarko et al., 2008). In the nucleolus, rDNA is transcribed to a 47S pre-
rRNA; and then nascent pre-rRNA is processed to form mature rRNAs 5.8S, 18S and
28S. Finally, the mature rRNAs bind to ribosome proteins to form 40S and 60S ribosomal
subunits (Fig1.7) (Woolford and Baserga, 2013).

1.3.2.1 rRNA transcription

In eukaryotic cells, the ribosome contains four different kinds of rRNAs, including
5S, 5.8S, 18S and 28S. Among them, 18S rRNA belongs to 40S small ribosome subunit,
and the other three rRNAs belong to 60S large ribosome subunit. The 5.8S, 18S and 28S
arise from the pre-47S rRNA transcript (Lafontaine, 2015). Transcription of pre-47S
rRNA occurs in the nucleolus and is catalyzed by RNA polymerase I (Engel et al., 2013),
while transcription of 5S rRNA is catalyzed by RNA polymerase III and occurs in the
nucleoplasm (Costanzo et al., 2001). In addition to RNA polymerase I, the complex for
rDNA transcription also contains TATA binding protein (TBP), upstream binding factor
(UPF), several transcription activating factors (Gorski et al., 2007) and the transcription
terminator (Bartsch et al., 1988).
Figure 1.7 The process of eukaryotic ribosome biosynthesis. Adapted from (Lafontaine, 2015), with permission to use.
The higher eukaryotic cells contain rRNA genes in multiple copies to transcribe large amount of rRNA molecules. The human genome contains approximately 200 copies of gene that transcribes 5.8s, 18S and 28S rRNAs, and about 2000 copies of gene that encode 5S rRNA (Conrad-Webb and Butow, 1995). In human cells, five different chromosomes (13, 14, 15, 21 and 22) contain clusters of 5.8s, 18S and 28S rRNAs genes organized in tandem arrays, while there is only one tandem array of 5S rRNA gene on chromosome 1 (Henderson et al., 1972). The 5.8s, 18S, and 28S rRNA genes consists of a non-transcribed spacer and a transcribed sequence. The non-transcribed spacers are sites for the promoters and the terminators that are indispensable for RNA polymerase I transcription (Hillis and Dixon, 1991).

1.3.2.2 rRNA processing

In addition to 5.8S, 18S and 28S rRNAs, the nascent pre-rRNA 47S also contains spacer regions called external transcribed spacers (ETSs) and internal transcribed spacers (ITSs) (Kent et al., 2009). The ETSs are localized at both the 5’ and the 3’ ends of the 47S pre-rRNA and the ITSs are present between 5.8S, 18S and 28S rRNAs (Kent et al., 2009). The transcription of pre-rRNA 47S is followed by different post-transcriptional manipulations, which splice and modify the pre-rRNA 47S into mature rRNAs for ribosome assembly. The pre-rRNA splicing starts with the removal of the 3’-ETS and cleavage within the 5’-ETS, followed by the cleavages to the ITSs to genera mature rRNAs (Fig 1.8). Besides splicing, a substantial amount of modifications at some ribose residues and bases are essential for rRNA maturation (Decatur and Fournier, 2002). Two
main types of modification occur; methylation of specific ribose residues and bases, and conversion of uridines to pseudouridines (Decatur and Fournier, 2002).

Both pre-rRNA splicing and modification require nucleolar proteins and RNAs. A number of small nucleolar RNAs and more than 150 nucleolar proteins are involved in pre-rRNA processing (Nazar, 2004). Single snoRNAs complexes with approximately 10 nucleolar proteins to form small nucleolar RNA proteins (snoRNP). Together multiple snoRNPs for a pre-mRNA spliceosome like complex with the pre-rRNAs to facilitate its processing (Fayet-Lebaron et al., 2009). U3 is one of the snoRNAs associated with pre-rRNA splicing. It is the most abundant snoRNA in the nucleolus and involved in the cleavage within the 5’-ETS (Pleiss et al., 2007). In addition, snoRNA U22 is involved in maturation of 18S rRNA (Tycowski et al., 1994) and U8 is associated with the cleavages that generate the mature 5.8 and 28S rRNAs (Peculis, 1997). SnoRNAs also serve as guide RNA for pre-RNA base modifications. These snoRNAs guides contain an approximately 15-mer sequence that is complementary to pre-RNA and mediate snoRNPs binding to the modification sites (Kishore et al., 2013). Therefore, the snoRNAs functions as guide RNAs to direct enzymes responsible for pseudouridylation and methylation to correct modification sites on pre-RNA molecules by base paring (Kishore et al., 2013).

1.3.2.3 Ribosome assembly

The ribosome that assembles in the nucleolus, consists of 5S, 5.8S, 18S, 28S rRNAs and different types of ribosomal proteins. Its formation involves the assembly of nucleolus-synthesized rRNAs with both ribosomal proteins and another nuclear-
Figure 1.8 Pre-ribosomal RNA processing pathways in eukaryotic cells. Adapted from (Lafontaine, 2015), with permission to use.
synthesized rRNA 5S. These ribosomal protein genes are transcribed by RNA polymerase II. After translation of mRNAs in the cytoplasm, the ribosomal proteins are transported to the nucleolus and assemble with the rRNAs to form pre-ribosomal complexes. Another ribosome component, 5S rRNA, which is transcribed by RNA polymerase III outside of the nucleolus, is transported to the nucleolus and assembled into the ribosome (Ciganda and Williams, 2011).

More than 200 assembly factors are spatially and temporally involved in ribosome assembly (Strunk et al., 2011), which starts while transcription is still in progress. Before pre-RNAs cleavage, more than half of the ribosomal proteins have been associated with them. For 5S rRNAs and the remaining ribosome proteins, they are incorporated into the assembling complexes during pre-RNAs cleavage (Calvino et al., 2015). The two ribosome subunits, 40S and 60S, are assembled separately. In higher eukaryotic cells, small 40S subunits are composed of 18S rRNAs and 45 additional proteins (Rabl et al., 2011; Taylor et al., 2009). The assembly of 40S subunits is a stepwise process, in which several sub-ribosomal complexes can be detected (Krogan et al., 2004). The first purified sub-ribosomal complex is composed of 35S pre-rRNA, some ribosomal proteins, U3 snoRNP and a number of non-ribosomal proteins (Grandi et al., 2002), suggesting that 40S subunits assembly starts before pre-rRNAs processing. The pre-40S subunits change dramatically after the cleavage of pre-35S rRNAs mediated by U3 snoRNP, including the disassociation of non-ribosomal proteins and the recruitment of other ribosomal proteins (Schafer et al., 2003). The pre-40S particles are then transported to the cytoplasm where they become functional subunits after the maturation of 18S rRNAs (Fatica et al., 2003; Vanrobays et al., 2003). The assembly of 60S subunit is also a multistep process like 40S.
The 27SA and 28SB pre-rRNAs, ribosomal and non-ribosomal proteins were detected in the first purified pre-60S particles (Kressler et al., 2008); while no snoRNP was detected indicating that formation of an earlier pre-particle exists. The processing of pre-rRNAs in pre-60S particles is companied by the incorporation of ribosomal proteins and exchanges of non-ribosomal proteins (Kressler et al., 2008). Subsequently, the pre-60S particles are transported to the cytoplasm, followed by the release of non-ribosomal proteins such as Arx1 (Kressler et al., 2010), and the final processing of 5.8S rRNAs (Ansel et al., 2008; Gabel and Ruvkun, 2008).

1.4 ADENOVIRUS pV

*Mastadenovirus* members express two unique proteins, pV and pIX (Davison et al., 2003). In HAdV-2, pV is encoded by the L2 region with other two late proteins, pVII and penton base protein (Davison et al., 2003). The HAdV-2 pV is essential for viral replication in primary cells, but not cancer cells (Ugai et al., 2012). As a structural protein, pV bridges the capsid and DNA core to stabilize the virion. Recently, the C-terminus of pV has been shown to interact with pVI in the interior of adenovirus particle (Reddy and Nemerow, 2014). The pV also interacts with pVIII C-terminus. Therefore, these three proteins can form a ternary complex to glue the peripental hexons and connect them with adjacent group of nine hexons. Moreover, the basic amino acids rich N-terminus of pV may interact with the adenoviral genome to bridge the viral core with the capsid (Reddy and Nemerow, 2014). The interaction of pV and pVI also provide an insight of the pVI release into the endosome leading to the disruption of endosome membrane and release of partially disassembled adenovirus particle into the cytoplasm.
Figure 1.9 Amino acid homology of HAdV-5 and BAdV-3 pV. AAD09725.1 is the gene bank accession of BAdV-3 pV, and AP_000172.1 is the gene bank accession of HAdV-5 pV.
In addition to acting as a structural protein, pV also interacts with cellular protein p32, which shuttles between the mitochondria and the nucleus. This interaction may mediate transport of partially uncoated adenovirus from the cytoplasm to the nucleus (Matthews and Russell, 1998a). In addition, pV has been detected to interact with nucleolar protein B23 and nucleolin. This interaction not only redistributes B23 and nucleolin from the nucleolus to the cytoplasm but also helps in dissociation of core proteins (Samad et al., 2007).

Unlike HAdV-2 (Davison et al., 2003), the L2 region of BAdV-3 encodes only protein pV. BAdV-3 pV is 423 amino acids long and shows amino acids sequence identities of 40% to HAdV-5 pV protein (Reddy et al., 1998) (Fig 1.9). Moreover, pV is rich in basic amino acids residues, which indicates pV may be localized in the nucleus (Reddy et al., 1998). So far, BAdV-3 pV was shown to interact with another viral non structural protein 33K (Kulshreshtha et al., 2004).
2.0 HYPOTHESIS AND OBJECTIVES

2.1 RATIONALE FOR HYPOTHESIS

BAdV-3 has been studied and developed as a vector for vaccination and gene therapy because of the advantages it owns, such as low virulence, easy manipulation, growth to high titers in cultured cells and broad tropism. It is also a promising vector for human vaccination and gene therapy because the pre-existing human adenovirus immunity extensively influences the efficiency of human adenovirus-based vectored vaccines. Though the BAdV-3 genomic organization is similar to other mastadenovirus members, some distinct features have been observed, such as the cell receptor used for attachment, major late transcription unit organization and the absence of the viral-associated RNAs.

Unlike human adenovirus 5, which expresses penton, pVII, pV and pX, the L2 region of BAdV-3 encodes only protein pV. pV is a structural protein that bridges the capsid and viral genome to stabilize the viral particle. However, in addition to its role as a structural protein, pV may also play other functions in viral replication via its interaction with cellular proteins. It is possibly involved in viral transport from the cytoplasm to nucleus because it interacts with the mitochondria-nucleus shuttle protein p32. Moreover, pV redistributes nucleolar proteins B23 and nucleolin from the nucleolus to nucleoplasm and cytoplasm, suggesting pV may regulate nucleolar functions via interactions with nucleolar proteins.

Viral protein interactions with other viral proteins or cellular proteins play indispensable roles in all steps of BAdV-3 replication. Identifying viral/cellular protein-protein interactions and elucidating their mechanisms would be important to understand
BAdV-3 biology as well as the development of more efficient vaccine delivery vectors. BAdV-3 assembly occurs in the nucleus, while structural protein pV is located to the nucleolus in both transfected and infected cells.

2.2 HYPOTHESIS

We hypothesize that pV may interact with other viral or cellular proteins and plays additional biological functions in BAdV-3 replication rather than just being a structural protein.

2.3 OBJECTIVES

The specific goals of this project are:

1) To characterize pV expression, nuclear/nucleolar localization and determine the biological functions of pV nucleolar localization signals in BAdV-3 replication.

2) To elucidate the biological significance of pV in viral replication by isolating and analyzing pV deleted BAdV-3.

3) To identify viral/cellular proteins that interact with pV and study the biological functions of these interactions.
3.0 NUCLEOLAR LOCALIZATION OF BOVINE ADENOVIRUS pV AND ITS CONTRIBUTION TO VIRAL REPLICATION

3.1 INTRODUCTION

Bovine adenovirus BAdV-3, a member of the *Mastadenovirus* genus is a non-enveloped icosahedral particle which contains a double stranded DNA genome of 34,446 bp organized into early, intermediate, and late regions (Reddy et al., 1998). Despite similarity in genome organization with human adenovirus (HAdV)-5, BAdV-3 appears to possess certain distinct features (Bangari and Mittal, 2006; Idamakanti et al., 1999; Reddy et al., 1998; Xing and Tikoo, 2006, 2007; Xing et al., 2003) including organization of late (L) transcriptional unit into seven (L1-L7) regions in contrast to HAdV-5 (Reddy et al., 1998).

The L2 region of HAdV-5 encodes a minor capsid protein named pV, which appears to associate with the viral genome and bridge the core and the capsid proteins (Chatterjee et al., 1985; Lehmberg et al., 1999; Matthews and Russell, 1998a; Vayda et al., 1983). pV appears to be essential for virus replication in primary cells but not in cancerous cells (Ugai et al., 2007). Protein V mainly localizes to the nucleolus utilizing a transportin dependent pathway (Hindley et al., 2007) and over expression of pV redistributes nucleolin and nucleophosmin to the cytoplasm (Matthews, 2001).

The nuclear localization of a protein is a well-characterized process regulated by nuclear pore complexes (NPCs) and requiring active transport mechanisms and specific nuclear localization signal (NLS) sequences on the transported protein. Unlike the nucleus, the nucleolus is a membrane-free subnuclear structure involved in ribosome biogenesis, cell cycle regulation, cellular stress response, apoptosis and viral replication.
Nucleolar localization depends on the interactions of nucleolar constituents with specific protein sequences namely nucleolar localization signals (NoLS) usually rich in arginine and lysine (Reed et al., 2006). NoLS binds to nucleolar proteins or rRNA and acts as nucleolar retention signal (Zakhartchouk et al., 2004) rather than a NoLS (Reed et al., 2006).

Recently, we reported the characterization of late structural/non structural BAdV-3 proteins and demonstrated that BAdV-3 52K (Paterson et al., 2012) and pVIII (Ayalew et al., 2014) utilize the importin α/β pathway to localize to the nucleus in infected cells, BAdV-3 33K utilizes both importin α/β and transportin 3 pathways for localization to the nucleus in infected cells (Kulshreshtha et al., 2014).

The L2 region of the late transcription unit of BAdV-3 encodes pV, which is collinear with pV of HAdV-5 (Reddy et al., 1998). A recent report suggested that pV interacts with 33K in BAdV-3 infected cells (Kulshreshtha and Tikoo, 2008). Although homologs of pV have been identified in other members of Mastadenovirus genus, BAdV-3 pV show 28%-41% amino acid identity with pV proteins of other Mastadenoviruses. Since it does not show significant similarity with pV encoded by other members of Mastadenovirus, we sought to characterize this protein in detail. Here, we report the characterization and identification of signals mediating nucleolar localization of BAdV-3 pV, and demonstrate that nucleolar localization of pV is required for BAdV-3 replication.
3.2 MATERIALS AND METHODS

3.2.1 Cells and viruses

Madin Darby bovine kidney (MDBK), cotton rat lung (CRL) cells, VIDO DT1 cells (CRL cells expressing endonuclease I-SceI) and CRL.pV (CRL cells expressing BAdV-3 pV) (Zhao and Tikoo, manuscript in preparation) cells were cultivated in minimal essential medium (MEM) (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen). Vero cells and HEK293T cells were propagated in Dulbecco’s modified eagle’s medium (DMEM) supplied with 10% FBS. BAV304a (BAdV-3 E3 region was replaced by a EYFP gene) and mutant BAdV-3s were cultivated in MDBK or CRL.pV cells.

3.2.2 Antibodies

The production and characterization of antisera raised against BAdV-3 DBP (Zhou et al., 2001), fiber (Wu and Tikoo, 2004) and 100K (Makadiya et al., 2015) have been described. Anti-hexon serum detects a protein of 98 kDa in BAdV-3 infected cells (Kulshreshtha et al., 2004). Anti-pVII serum detects two proteins of 22 and 20 kDa in BAdV-3 infected cells (Paterson, 2010). Anti-pX recognizes a protein of 25 kDa in BAdV-3 infected cells.

To produce BAdV-3 pV specific sera, two peptides representing amino acid 1-24 (XZ1) and amino acids 180-212 (XZ2) were synthesized by Genscript. Rabbits were immunized with individual (500 μg / rabbit) peptide conjugated to keyhole limpet haemocyanin emulsified with Freund’s complete adjuvant (Sigma) followed by two injections of ovalbumin conjugated individual peptide (300 μg/rabbit) in Freund’s
incomplete adjuvant (Sigma) three weeks apart. Sera were collected ten days after third injection and tested for specificity by Western blotting. Commercial antibodies used in this report are listed as follows:

- RPA194 Antibody (C-1) (Santa Cruz Biotechnology)
- Anti-β-actin monoclonal antibody (Sigma-Aldrich)
- Alexa Fluor 488-conjugated goat anti-rabbit IgG (Jackson Immunoresearch)
- TRITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch)
- TRITC-conjugated goat anti-rabbit IgG (Jackson Immunoresearch)
- Alexa Fluor 647-conjugated goat anti-rabbit IgG (Invitrogen)
- Alexa Fluor 680 conjugated goat anti-rabbit antibody (Invitrogen)
- IRDye800 conjugated goat anti-mouse antibody (Rockland)

3.3.3 Construction of plasmids

Constriction of plasmids used in this section was depicted in the Appendix A.

3.2.4 Isolation of pV nucleolar localization signal deleted BAdV-3 mutants

To isolate mutant BAdV-3s, we constructed full-length BAdV-3 plasmids containing mutant BAdV-3 genomic DNAs as described (Chartier et al., 1996).

- **Plasmid pUC304a.pVd1.** A 972 bp DNA fragment was amplified by PCR using primers M-F and d(21-50)-F1-R (Table 3.1), and plasmid pcV DNA as a template. Similarly, an 1134-bp DNA fragment was amplified by PCR using primers d(21-50) F2-F and pV-XhoI-R (Table 3.1), and plasmid pcV DNA as a template. In the third PCR, these two PCR fragments were annealed and used as DNA template to amplify a 2068-bp DNA
fragment by overlapping PCR using primers M-F and pV-XhoI-R (Table 3.1). A 1171-bp EcoRI-XhoI DNA fragment of the final PCR product (2068 bp) was isolated and ligated to EcoRI-XhoI digested plasmid pcDNA3 to create plasmid pcV.d1. A 528-bp EcoRI-NheI DNA fragment of plasmid pcV.d1 was isolated and ligated to EcoRI-NheI digested pMCS.pV to create plasmid pMCS.pVd1.

At last, a 6.2-kb EcoRV-BstI1107I fragment of plasmid pMCS.pVd1 was isolated and recombined with SbfI digested plasmid pUC304a.dV DNA in Escherichia coli BJ5183 (Chartier et al., 1996) to generate plasmid pUC304a.pVd1.

**b) Plasmid pUC304a.pVm123.** A 986-bp DNA fragment was amplified by PCR using primers M-F and M12-F1-R (Table 3.1) and plasmid pcV DNA as a template. Similarly, a 1025-bp DNA fragment was amplified by PCR using primers M12-F2-F and pV-XhoI-R (Table 3.1), and plasmid pcV DNA as a template. In the third PCR, two fragments were annealed and used to amplify a 2159-bp DNA fragment by overlapping PCR using primers M-F and pV-XhoI-R (Table 3.1). At last, a 1261-bp EcoRI-XhoI DNA fragment of the PCR product (2159 bp) was isolated and ligated to EcoRI-XhoI digested plasmid pcDNA3 to generate plasmid pcDNA3-pV-m12.

To create pcV.m123, a 1059-bp DNA fragment was amplified by PCR using primers M-F and M3-F1-R (Table 3.1), and plasmid pcDNA3-pV-m12 as a template. An 1141-bp DNA fragment was amplified by PCR using primers M3-F2-F and pV-XhoI-R (Table 3.1), and plasmid pcDNA3-pV-M12 DNA as a template. In the third PCR, these two DNA fragments were annealed and used to amplify a 2159-bp DNA fragment by overlapping PCR using primers M-F and pV-XhoI-R (Table 3.1). Finally, a 1261-bp
DNA fragment of the PCR product (2159-bp) was isolated and ligated to EcoRI-XhoI digested plasmid pcDNA3 to generate plasmid pcV.m123.

A 618-bp EcoRI-NheI fragment of plasmid pcV.m123 was isolated and ligated to EcoRI-NheI digested plasmid pMCS.pV to create plasmid pMCS.pVm123. The SbfI digested plasmid pUC304a.dV was recombined with a 6.3-kb EcoRV-Bst1107I DNA fragment of plasmid pMCS.pVm123 in Escherichia coli BJ5183 (Chartier et al., 1996) creating plasmid pUC304a.pVm123.

c) Plasmid pUC304a.pVd3 and pUC304a.pVd1d3. An 1171-bp fragment was amplified by PCR using primers pV-EcoRI-F and F1-R (Table 3.1), and plasmid pMCS.pV DNA as a template. Similarly, a 661-bp fragment was amplified by PCR using primers pV-d(380-389) F2-F and dV-F2-R (Table 3.1), and plasmid pMCS.pV DNA as the template. In the third PCR, two PCR fragments were annealed and used to amplify a 1790-bp DNA fragment by overlapping PCR using primers pV-EcoRI-F and dV-F2-R (Table 3.1). At last, a 650-bp SacI-HpaI fragment of PCR product (1790-bp) was isolated and ligated to SacI-HpaI digested plasmid pMCS.pV and pMCS.pVd1 to create plasmid pMCS.pVd3 and pMCS.pVd1d3, respectively.

The SbfI digested plasmid pUC304a.dV was recombined with a 6.2-kb EcoRV-Bst1107I fragment of plasmid pMCS.pVd3 or plasmid pMCS.pVd1d3 in Escherichia coli BJ5183 (Chartier et al., 1996) to generate plasmid pUC304a.pVd3 and plasmid pUC304a.pVd1d3, respectively.
<table>
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<th>Sequence</th>
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</thead>
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</tr>
<tr>
<td>d(21-50)-F1-R 5'</td>
<td>CGCTTCTTAGAGCCGCGGTAAATCTCAGGCGCCACGA TGTC</td>
</tr>
<tr>
<td>d(21-50)-F2-F 5'</td>
<td>TCGTGCGGCTAGATTACCCGCGGCTCTAGAAAGCGGG CCTTG</td>
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<td>AATACTCGAGAGCGCTTAACGCGGAGCCGGGTTAC-3</td>
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<tr>
<td>dV-F2-R 5'</td>
<td>GTCC-ATGGCGTGTAAACAGCGTGTG</td>
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3.2.5 Western blotting

Protein in purified recombinant BAdV-3s or infected cells were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane and probed by Western blot using protein specific antisera and alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (Sigma) or Alexa Fluor 680 conjugated goat anti-rabbit antibody (Invitrogen).

3.2.6 Immunofluorescence microscopy

Cells infected with BAV304a or mutant BAdV-3, or transfected with plasmid DNAs were fixed with 3.7% paraformaldehyde for 15 min and permeabilised with PBS supplied with 0.5% Triton X-100. Cells were then blocked with 5% goat serum and stained with protein specific sera and fluorophor conjugated goat anti-rabbit antibody (Jackson Immunoresearch). Finally, cells were mounted by mounting buffer (Vector Laboratories Inc.) with DAPI and imagined under confocal microscope TCS SP5 (Leica).

3.2.7 GST-pull down assay

GST fusion proteins (Importin α1, α3, α5, α7, β1 or transportin-1) were purified from plasmids transfected E.coli BL21 by glutathione sephorose beads (GE Healthcare). [35S] Methionine-labeled pV was synthesized and labeled by TNT T7 Quick Coupled Transcription/Translation System (Invitrogen). After incubation of GST fusion proteins and [35S] methionine-labeled pV overnight, the samples were separated by 10% SDS-PAGE. After exposure, the phosphor screen was scanned and analyzed by Molecular Imager FX (Bio-Rad).
3.2.8 Isolation of mutant BAdV-3s

Monolayers of VIDO DT1 (CRL cells expressing I-SceI recombinase) cells or CRL.pV (CRL cells expressing BAdV-3 pV) cells in six-well plate were transfected with 5-7.5 ug of individual plasmid DNA with Lipofectamine 2000 (Invitrogen). At 4 hrs post-transfection, the media were replaced with fresh MEM containing 2% FBS. Transfected cells showing cytopathic effect (CPE) were harvested, freeze-thawed three times and propagated in MDBK cells.

3.2.9 CsCl gradient centrifugation

Monolayers of MDBK or CRL.pV (CRL cells expressing BAdV-3 pV) cells in T-150 flasks were infected with wild type or mutant BAdV-3s at a multiplicity of infection of 5. At 48 h post-infection, the cells were collected and resuspended in 5ml medium. After freeze thawing three times, the cell lysates were subjected to CsCl density gradient centrifugation at 35 000 rpm for 1 hr at 4°C. The bands containing viruses were collected, and subjected to a second centrifugation at 35 000 rpm for 16 hrs at 4°C. At last, the virus band was collected, dialyzed three times to remove the trace amount of cesium chloride and stored in small aliquots at -80°C.

3.2.10 Virus single cycle growth curve

Monolayers of MDBK cells in 24-well plates were infected with wild type or mutant BAdV-3s at a MOI of 1. At indicated time points post infection, the infected cells were harvested, lysed by freeze-thawing three times to release the virus into medium, and then used to determine virus titers by TCID$_{50}$ in CRL.pV cells.
3.2.11 Protein expression analysis

Monolayers of MDBK in six-well plate were infected with BAV304a or mutant BAdV-3s at MOI of 1. At 24h post-infection, infected cells were harvested and probed by Western blot using protein-specific rabbit antisera and mouse anti-β-actin as primary antibodies (Sigma), Alexa Fluor 680 goat anti-rabbit (Invitrogen) and IRDye 800 goat anti-mouse (Rockland), respectively, as secondary antibodies. At last, the membranes were imagined and analyzed by using the Odyssey® CLx Imaging System (LI-COR).

3.2.12 Virus thermostability assay

To determine the thermostability of BAV304a and mutant BAdV-3s, 10^5 purified infectious viral particles were incubated at different temperatures (-80°C, -20°C, 4°C, 25°C and 37°C) for three days in PBS containing 10% glycerol. To assess the different dynamics of viral inactivation, 10^5 infectious purified viral particles were incubated at different temperatures (-80°C, 4°C and 37°C) for 0, 1, 3 and 7 days in PBS containing 10% glycerol. At last, TCID50 was used to titrate the remaining infectivity.

3.2.13 Transmission electron microscopy

Monolayers of MDBK cells were infected with BAV304a or BAV.pVd1d3 at MOI of 5. At 48 h post-infection, the cells were harvested and fixed with 2.5% glutaraldehyde in 0.1 M PBS, followed by post-fixation in 1% OsO4 and dehydration in a graded ethanol series and propylene oxide. Dehydrated cells were infiltrated in mixtures of propylene oxide and EMbed-812 embedding medium, and then polymerized in embedding capsules.
at 60°C for 24-48 h. At last, the pellet was sectioned with a Reichert ultracut microtome, each section was stained with 2% uranyl acetate and viewed on a Philips CM10 TEM.

3.3 RESULTS

3.3.1 Expression of pV during BAdV-3 infection

To characterize BAdV-3 pV, peptides ZX1 (\text{MASSRLIKEEMLDIVAPEIYKRKR}^{24}) and peptide ZX2 (\text{SRKRGVGKVEPTIQVLASKKRRMA}^{212}) were synthesized and used to generate anti-pV sera designated as XZ1 and XZ2 sera, respectively. The specificity of the sera was analyzed by Western blot using BAdV-3 infected MDBK cells. As seen in Fig 3.1A, both XZ1 serum and XZ2 serum detected a protein of 55 kDa in BAdV-3 infected cells. No such protein could be detected in mock-infected cells using XZ1 or XZ2 sera or BAdV-3 infected cells using pre-bleed sera. The protein could be detected at 24-48 hrs post infection (panel B lanes 6-8) but not at 12 hrs post infection (panel B lane 5). Similarly, anti-pV pooled sera detected a 55 kDa protein in HEK293T cells transfected with plasmid pcDNA3-pV (pcV) (Fig 3.1C, lanes 3-4) DNA. No such protein could be detected in plasmid pcDNA3 DNA transfected cells (Fig 3.1C, lane 5).

3.3.2 Subcellular localization of pV

To determine the subcellular localization of pV, CRL cells were transfected with plasmid pDsRed.B23 (Gomez Corredor and Archambault, 2009) and infected with BAdV-3 at 48 hrs post-transfection. At 24 hrs post-infection, the cells were analyzed by indirect immunofluorescence assay using anti-pV serum. As seen in Fig 3.1D, pV co-
localized predominantly with nucleolar protein B23 fused to DsRed (pDsRed.B23) suggesting that pV localizes in the nucleolus of the infected cells. To determine if nucleolar localization is dependent on other viral proteins, we determined the localization of pV in Vero cells co-transfected with plasmid pcV and pDsRed.B23 DNAs by florescence microscopy. As seen in Fig 3.1D, pV co-localizes predominantly with nucleolar marker B23 fused to DsRed in the nucleolus of the co-transfected cells.

3.3.3 Identification of pV nucleolar localization signal

Bioinformatic analysis of pV protein sequence using motif prediction algorithms by such as “PredictProtein”predicted that the amino acids $^{21}$KRKPRRERAAPYAVKQ EEKPLVKAERKIK $^{50}$, $^{190}$RKRGVKVEPTIQVLASKKR $^{210}$ and $^{380}$RRRRRRRTRR $^{389}$ of BAdV-3 pV may act as potential nuclear localization signals (NLSs) (Fig 3.2A). To determine if these domains act as NLS, we constructed plasmids expressing mutant pV containing specific NLS domain deletions (Fig 3.2B). Vero cells co-transfected with plasmid pDsRed.B23 DNA and individual plasmid DNA expressing mutant pV protein were analyzed with immunofluorescence assay at 48 hrs post transfection. As seen in Fig 3.2C, the mutant pV containing deletion of amino acid 21-50 (V.d1) localized both in the nucleus and the nucleolus of the transfected cells. Similarly, mutant pV containing deletion of amino acid 380-389 (V.d3) localized both in the nucleus and the nucleolus of the transfected cells. However, mutant pV containing deletion of amino acid 190-210 (V.d2) localized in the nucleolus of the transfected cells. Interestingly, mutant pV containing a deletion of amino acids 21-50 and 190-210 (v.d1d2) or deletion of amino acids 190-210 and 380-389(V.d2d3) could be detected in the nucleus and
Figure 3.1 Expression of pV. Proteins from BAdV-3 infected MDBK cells A,B) and indicated plasmid DNA transfected cells C) (lane 2 and 3) or mock infected/transfected cells were harvested at different time points, separated by SDS-PAGE and transferred to nitrocellulose membrane. The separated proteins were probed by Western blot using anti-pV serum. The position of the molecular weight marker (lane M) in kD was used for sizing the protein bands. D) CRL cells were transfected with plasmid pDsRed.B23 DNA and infected by BAdV-3 (panels a-d) or co-transfected with plasmid pcV and pDsRed.B23 DNAs (panels e-h) and fixed at 24 hours post-infection\transfection. The DsRed.B23 was visualized by direct fluorescence microscopy (panels b,f). BAdV-3 pV was visualized by indirect immunofluorescence microscopy (panels c,g) using anti-pV antiserum and Alexa Fluor 488-conjugated goat anti-rabbit IgG. The nuclei were stained with DAPI.
**Figure 3.2 Analysis of BAdV-3 pV nucleolar localization signals.** A) Schematic representation of BAdV-3 pV. The thick black line represents BAdV-3 pV. The numbers below represent amino acids of pV. Potential nuclear localization signal (NLS) and nucleolar localization sequences (NoLS1 and NoLS2) are depicted. The name of the plasmid is depicted on the right. B) Schematic diagram represents mutant pV. Thick black lines represent pV gene, thin black lines represent the deleted regions. The name of the plasmid is depicted on the right. C) Sub cellular localization of pV mutants. Vero cells were transfected with those plasmids expressing pV and mutant pV genes individually and fixed with 4% formaldehyde at 48h post-transfection. BAdV-3 pV was visualized by indirect immunofluorescence using anti-pV antiserum and Alexa Fluor 488-conjugated goat anti-rabbit IgG (Jackson Immunoresearch). Nuclei were stained with DAPI and nucleoli were visualized with indirect immunostaining by using RPA194 Antibody (C-1) (Santa Cruz Biotechnology) and TRITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch).
nucleolus of the transfected cells. In contrast, mutant pV containing the deletion of amino acids 21-50 and 380-389 (pV.d1d3), or deletion of amino acids 21-50,190-210 and 380-389 (V.d1d2d3) localized predominantly in the nucleus of the transfected cells.

Earlier, Weber et al (Weber et al., 2000b) suggested that the basic amino acid rich sequence K/R-K/R-X-K/R, wherein X stands for any amino acids, may play a role in protein nucleolar localization. Our analysis of NoLS1 (amino acid 20-50) and NoLS2 (amino acid 380-389) sequence identified three motifs (21KRKR24, 26RRER29 and 47RKIK50) in NoLS1, which have the potential to act as NoLS (Fig 3.3A). To determine the role of each motif (m1, m2, m3) in nucleolar localization, we constructed a panel of plasmids expressing mutant pV proteins (amino acid 380-389 deleted) in which the basic residues of identified potential NoLS motifs were replaced with glycine alanine residues (Fig 3.3A). Vero cells were co-transfected with plasmid pDsRed.B23 and individual plasmid DNA expressing mutant pV protein, and analyzed with immunofluorescence assay using anti-pV sera. As seen in Fig 3.3B, pV is predominantly localized in the nucleolus of cells transfected with plasmids expressing mutant pV containing amino acid substitution in single motif (V.m1d3, V.m2d3, V.m3d3,) or double motif (V.m1m2d3, V.m2m3d3, V.m1m3d3). In contrast, pV is predominantly localized in the nucleus of the cells transfected with plasmid expressing mutant pV containing basic amino acid substitution in all three potential NoLS (V.m1m2m3d3). To confirm the nucleolar retention function of BAdV-3 pV amino acids 21-50 and 380-389, DNA fragments encoding amino acids 21-50 and 380-389 were fused in-frame with enhanced yellow fluorescent protein (EYFP) gene to create plasmids pNoLS1.EY and pNoLS2.EY expressing fusion proteins (Fig 3.3C). Vero cells co-transfected with plasmid
pDsRed.B23 DNA and either plasmid pNoLS1.EY DNA or plasmid pNoLS2.EY DNA were analyzed by confocal microscopy at 48 hrs post transfection. As seen in Fig 3.3D, EYFP was detected both in the nucleus and the cytoplasm of the transfected cells as EYFP could diffuse passively in the nucleus due to its small size (26 kDa). In contrast, NoLS1 (amino acids 21-50) and NoLS2 (amino acids 380-389) were able to direct the heterologous protein (EYFP) predominantly to the nucleus and the nucleolus of the transfected cells.

3.3.4 Identification of pV nuclear localization signal

To determine the nuclear localization signal(s) of BAdV-3 pV, we constructed plasmids expressing mutant BAdV-3 pV containing truncations and/or internal deletions (Fig 3.4A). Vero cells were transfected with individual recombinant plasmid DNA. At 48 hrs post transfection, transfected cells were analyzed by indirect immunofluorescence using anti-pV sera. As seen in Fig 3.4B, mutant pV containing deletions of amino acids 191-423 (V.d4, V.d5, V.d6 and V.d7) appear to be localized in the nucleus of the transfected cells. Moreover, mutant pV containing deletion of amino acid 21-50 and 190-423 (V.d8) also localized to the nucleus of the transfected cells. As expected, V.d4, V.d5 and V.d7 (contain one or both identified NoLS) localized to the nucleolus of the transfected cells, while V.d6 (absence of identified NoLS1 and 2) localized to the nucleus of the transfected cells. In contrast, analysis of mutant V.d9 (containing deletion of amino acids 21-50+101-210+380-423), V.d10 (containing deletion of amino acid 2-100+190-210+380-423) and V.d11 (containing deletion of amino acids 21-50+81-120+190-210+380-423) suggested that amino acids 21-50, 81-120, 190-210 and 380-423 might
**Figure 3.3 Mutation analysis of pV NoLS1.**

**A)** Schematic representation of BAdV-3 pV depicting the amino acid sequence of NoLS1. The thick line represents BAdV-3 pV gene. The thin line represents deleted region. The basic residue rich motifs (m1, m2, m3) are shown in different font size or indicated by small black bar. The numbers above represent amino acid of BAdV-3 pV. The name of the plasmids is depicted on the right of the panel. **B)** Sub cellular localization of pV mutants. Vero cells were co-transfected with plasmid pDsRed.B23 and individual indicated plasmid DNAs. At 48h post-transfection, cells were fixed with 4% formaldehyde. BAdV-3 pV NoLS1 mutant proteins were visualized by indirect immunofluorescence microscopy using anti-pV antiserum and Alexa Fluor 488-conjugated goat anti-rabbit IgG (Jackson Immunoresearch). The DsRed.B23 was visualized by direct fluorescence microscopy. Nuclei were stained with DAPI. **C)** Schematic representation of fusion protein containing BAdV-3 pV NoLSs. The white box represents BAdV-3 pV nucleolar localization signals amino acids 21-50 or 380-389. The black box represents the EYFP gene. The numbers above represent amino acids of BAdV-3 pV. **D)** Sub cellular localization of fusion protein NoLS-EY. Vero cells were co-transfected with individual indicated plasmid expressing fusion proteins and pDsRed.B23 DNAs, and fixed with 4% formaldehyde at 48h post-transfection. The DsRed.B23 (panel c, g, k) and EYFP (panel b, f, j) were visualized by direct fluorescence microscopy. Nuclei were stained with DAPI (panel a, e, i).
contain nuclear localization signal(s) motifs, which may have redundant function. To further determine the importance of these sequences in nuclear localization of pV, we constructed plasmids (Fig 3.4C) containing deletion of three of the four regions of pV containing potential NLS. Vero cells were transfected with individual plasmid DNAs and subcellular localization of mutant pVs were analyzed at 48 hrs post transfection by indirect immunofluorescence using anti-pV sera. As seen in Fig 3.4D, mutant pV containing amino acids 81-120 (V.d12), 190-210 (V.d13) or amino acid 380-423 (V.d14) localized to nucleus\nucleolus. In contrast, mutant pV containing amino acid 21-50 (V.d15) localized predominantly in the cytoplasm of the transfected cell.

To examine if pV NoLSs can serve as NLSs, plasmids expressing pV.d16 (containing deletion of amino acids 81-120+190-210+390-423) (Fig 3.4C) and NoLS1-GFPβGal (Fig 3.4E) were constructed and used to transfet Vero cells. As shown in Fig 3.4D and F, pV.d16 and NoLS1-GFPβGal were localized in nucleus and cytoplasm, respectively.

3.3.5 Interaction of pV with importins

Members of the importin super family play an important role in nuclear transport of proteins. Since transport of some adenovirus proteins requires importins (Kohler et al., 1999; Kulshreshtha et al., 2014; Paterson et al., 2012; Wodrich et al., 2006), we performed a GST pull down assay using purified GST-fusion proteins of importin α1, importin α3, importin α5, importin α7 or importin β1 individually immobilized on glutathione-sepharose beads with radiolabelled in vitro synthesized BAdV-3 pV. As seen in Fig 3.5A, GST-importin α3 was able to bind pV (lane 5) as similar protein was
Figure 3.4 Analysis of BAdV-3 pV nuclear localization signals. A, C) Schematic representation of pV mutants. The thick black line represents BAdV-3 pV gene. Thin black line represents the deleted regions. The numbers above represent amino acids of BAdV-3 pV. The name of the plasmid is depicted on the right. B, D) Sub cellular localization of pV mutants. Vero cells were transfected with individual indicated plasmid DNA and fixed with 4% formaldehyde at 48h post-transfection. BAdV-3 pV mutants were visualized by immunofluorescence using anti-pV antiserum and TRITC-conjugated goat anti-rabbit IgG (Jackson Immunoresearch). Nuclei were stained with DAPI. E) Schematic representation of GFP-βGal fusion protein containing BAdV-3 pV NoLS1. The white box represents BAdV-3 pV NoLS1 amino acids 21-50. The black box represents the fusion protein GFP-βGal. The numbers above represent amino acids of BAdV-3 pV. The name of the plasmid is depicted on the right. F) Sub cellular localization of fusion protein NoLS1-GFP-βGal. Vero cells were transfected with those plasmids individually, and fixed with 4% formaldehyde at 48h post-transfection. The GFP-βGal visualized with direct fluorescence microscopy. Nuclei were stained with DAPI.
observed in input protein control (lane 1). No radiolabelled pV was observed when purified GST alone (lane 7) or GST fusions of importin β1 (lane 2), importin α7 (lane 3), importin α5 (lane 4) or importin α1 (lane 6) bound to glutathione-sepharose beads were used in pull down assays.

Like pV (Fig 3.5C,D), GST-importin α3 bound to glutathione-sepharose was able to bind radiolabeled pV.d18 (deletion of amino acids 190-210 and 380-423), pV.d19 (deletion of amino acids 81-120 and 190-210) and pV.d17 (deletion of amino acids 81-120 and 380-423) albeit with less intensity. However, no such interaction was observed when GST-importin α3 fusion bound to glutathione-sepharose was used to pull down pV.d15 containing deletion of amino acids 81-120, 190-210 and 380-423. Intriguingly, two additional bands were detected in the pV.d15 only lane.

Recently, we demonstrated that BAdV-3 33K interacts with transportin-3 (Kulshreshtha et al., 2014). To determine if pV binds to transportin-3 (Hindley et al., 2007; Kulshreshtha et al., 2014), GST pull down assay was performed using GST alone or GST-Transportin fusion protein and in vitro [35S] methionine labeled pV. As seen in Fig 3.5B, a protein could be observed in input protein control (lane 1). However, no similar protein could be detected bound to GST-TRN-SR2 (transportin-3) fusion protein (Lane 2) or GST alone (lane 3).

### 3.3.6 Construction of BAdV-3s expressing mutant pV proteins

To determine if the potential NoLSs are required for efficient replication of BAdV-3, we constructed full length plasmid genomic clones expressing mutant pV containing
**Figure 3.5 In vitro interaction of pV with transport receptors.**

A) In vitro interaction of pV with importin α3. In vitro synthesized and [³⁵S]-labelled BAdV-3 pV was incubated with purified GST fusion proteins (GST fused with importin α1, α3, α5, α7, β1, or transportin 3) or GST alone and pulled down with glutathione sepharose beads (GE Healthcare).  

B) Schematic representation of pV mutants. The thick black line represents BAdV-3 pV gene. Thin broken line represents the deleted regions. The numbers above represent amino acids of BAdV-3 pV. The name of the plasmid is depicted on the right.  

C) In vitro interaction of pV mutants with importin α3. In vitro synthesized and [³⁵S]-labelled BAdV-3 pV mutants were incubated with purified GST-α3 or GST alone and pulled down with glutathione sepharose beads. Samples from (A) and (C) were separated by 10% SDS-PAGE and exposed to a phosphor screen. The exposed phosphor screen was visualized by Molecular Imager FX (Bio-Rad). 5% of the input radiolabelled pV mutants were used as control.
deletion of potential NoLSs and or substitutions of basic residues with alanine\glycine of potential NoLS1 (Fig 3.6A). Monolayer of VIDO DT1 (cotton rat lung cells expressing I-SceI recombinase) cells were transfected with 5-7.5 μg of individual plasmid DNAs. The cytopathic effects appeared between 9-15 days (Fig 3.6B). However, repeated transfection of VIDO DT1 (cotton rat lung cells expressing I-SceI recombinase) cells with plasmid pUC304a.pVd1d3 did not produce any cytopathic effects. Moreover, reinfection of fresh VIDO DT1 (cotton rat lung cells expressing I-SceI recombinase) with supernatants of infected cell lysates containing mutant viruses (Fig 3.6A) named BAV.pVd1 (deletion of amino acid 21-50), BAV.pVm123 (containing substitutions of basic residues of all three motifs of amino acids 21-50) and BAV.pVd3 (containing deletion of amino acids 380-389) produced infectious virions. In contrast, reinfection of fresh VIDO DT1 (cotton rat lung cells expressing I-SceI recombinase) with supernatant of cell lysates potentially containing mutant BAV.pVd1d3 (containing deletion of amino acid 21-50 and amino acid 380-389) did not produce any infectious virion (Data not shown). To produce mutant BAV.pVd1d3, CRL.PV cells (CRL cells expressing BAdV-3 pV) were transfected with 5-7.5 μg of PacI digested plasmid pUC304a.pVd1d3 DNA. The cytopathic effects were observed in 13 days (Fig 3.6B). To purify the mutant viruses, the MDBK cells infected individually with BAV.pVd1, BAV.pVm123 or BAV.pVd3 (Fig 3.6A) or CRL.PV cells infected with BAV.pVd1d3 were collected, freeze-thawed and purified by CsCl density gradient purification.

The presence of the desired mutations was confirmed by DNA sequencing and restriction enzyme digestion of virion DNAs. Since an additional XbaI recognition site was introduced into mutant BAV.pVd1 or BAV.pVm123 genomes, the viral genomes
were digested with *Xba*I. As seen in Fig 3.6C, BAV.pVd1 (Lane 1) and BAV.pVm123 (lane 3) genomes had a band of 2.4 kb, which was missing in BAV304a (lane 2). Similar analysis of *Xba*I digested BAVd1d3 (lane 9) genome detected an expected band of 2.4 kb, but not in BAV304a (lane 8). Since an additional *Pst*I recognition site was introduced to the viral genome BAV.pVd3, the viral genome was digested with *Pst*I. As seen in Fig 3.6C, a 3.2 kb band was detected in BAV304a (lane 5) but not in BAV.pVd3 (lane 4). Similar analysis of *Pst*I digested BAV.pVd1d3 genome detected an expected band of 3.2 kb in BAV304a (lane 6) but not in BAV.pVd1d3 (lane 7).

The ability of the mutant BAdV-3s to express pV protein was analyzed by Western blot analysis of proteins from the lysates of virus infected cells. As seen in Fig 3.6E, protein bands of expected molecular weight could be detected in lysates of CRL cells infected with mutant BAV.pVd1, BAV.pVm123, BAV.pVd3 or BAV.pVd1d3. The ability of mutant BAV.pVd1d3 to express pV protein was analyzed in both CRL and CRL.pV (CRL expressing BAdV-3 pV) cells. As expected, two proteins of 55 kDa (representing wild-type pV expressed in CRL.pV cells) and 53 kDa (representing mutant pV expressed in BAV.pVd1d3) were detected in lysates of CRL.pV cells infected with BAV.pVd1d3, while only the 53 kDa band was detected in CRL cells infected with BAV.pVd1d3.

### 3.3.7 Sub cellular localization of mutant pV protein in recombinant BAdV-3 infected cells

To determine the effect of deletions or amino acid substitutions on nucleolar localization of pV, CRL cells were transfected with plasmid pDsRed.B23 DNA. At 48
hrs post transfection, the cells were infected with BAV304a or individual mutant BAdV-3s. At 24 hrs post infection, the cells were analyzed by immunofluorescence using anti-pV sera. As seen in Fig 3.6F, pV appears localized mainly in the nucleoli of BAV304a or BAV.pVd1 infected cells. Similarly, pV appears localized predominantly in the nucleoli of BAV.pVm123 infected cells. In contrast, pV appears localized in the nucleus of BAV.pVd3 or BAV.pVd1d3 infected cells.

3.3.8 Growth kinetics of viruses

To examine if the deletion\mutation of pV nucleolar localization signals affects BAdV-3 replication, we compared the ability of the mutant viruses and BAV304a to grow on MDBK cells. The virus infected cells were harvested at indicated time points post infection, freeze-thawed 3-5 times and cell lysates were used to determine the virus titers by TCID$_{50}$ assay. As seen in Fig 3.6D, at 48 hrs, virus titer of mutant BAVpVd1, BAV.pVm123, BAV.pVd3 appeared 0.6 to 1.0 log less compared to BAV304a. In contrast, mutant BAV.pVd1d3 did not replicate in MDBK cells.

3.3.9 Analysis of gene expression in mutant virus-infected cells

Since the deletion\mutation of pV NoLS influences the viral growth kinetics, we investigated the effects of pV NoLS deletion /mutations on the expression of early and late proteins in mutant BAdV-3 infected cells by Western blot using protein specific antisera. As seen in Fig 3.7A, anti-DBP serum, anti-pVII serum, anti-pV serum, anti-pX serum, anti-hexon serum and anti-100K serum detected proteins of expected
### E) Western Blot

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<th>BAV.pVm123</th>
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### F) Immunofluorescence

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**Figure 3.6 L2 pV.** A) Schematic representation of BAdV-3 Genomes. Dotted box represents BAdV-3 genome, and thick black line represents pV sequence. The thin line depicts deleted regions. The arrows represent the direction of transcription. The amino acid numbers of pV are shown. The substituted amino acids (alanines\glycines) of NoLS1 are underlines and shown in italics. E3 (early region 3); nucleolar localization signal (NoLS1, NoLs2); CMV (human cytomegalovirus immediate early promoter); EYFP (enhanced yellow fluorescent protein). B) Fluorescent microscopy. The VIDO DT1 or CRL.PV cells transfected with indicated plasmid DNAs were observed for appearance of green fluorescent cells and cytopathic effects. The numbers represent the day the observation was made after transfection. C) Restriction enzyme analysis of recombinant BAdV-3 genome. The DNAs were extracted from MDBK or CRL.PV cells infected with BAV304a (lanes 2, 5, 6, and 8), BAV.pVd1 (lane 1), BAV.pVm123 (lane 3), BAV.pVd3 (lane 4), and BAV.pVd1d3 (lanes 7 and 9) as described previously (Farina et al., 2001), digested with XbaI (lanes 1, 2, 3, 8, and 9) or Pst1 (lanes 4, 5, 6, and 7) and analyzed by agarose gel electrophoresis. D) Virus titer. Monolayers of MDBK cells were infected with BAV304a or recombinant BAdV-3s. At different time points post infection, the cells were freeze-thawed and titrated on CRL.PV cells as described. Values represent averages of two independent repeats and error bars indicate the standard deviations. E) Western Blot. Proteins from lysates of CRL or CRL.pV cells infected with BAV304a, BAV.pVd1, BAV.pVm123, BAV.pVd3 and BAV.pVd1d3 were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane and probed by Western blot using anti-pV serum. The membrane was visualized by Odyssey® CLx Imaging System (LI-COR). F) Confocal microscopy. CRL cells were transfected with plasmid pDsRed.B23 DNA and infected
with indicated mutant BAdV-3s. Infected cells were fixed at 48 hrs post-infection. The DsRed.B23 was visualized by direct fluorescence microscopy. BAdV-3 pV was visualized by indirect immunofluorescence microscopy using anti-pV serum and Alexa Fluor 647-conjugated goat anti-rabbit IgG. The nuclei were stained with DAPI.
molecular weights both in BAV304a and mutant virus infected cells. Densitometer analysis of protein production (Fig 3.7B) showed no significant differences in the expression of DBP, pX in any mutant BAdV-3 infected cells compared to BAV304a infected cells. Similarly, there was no dramatic change in the expression of pVII in mutant infected cells compared to BAV304a infected cells. However, expression of hexon and 100K was significantly reduced in BAV.pVd1d3 infected cells compared to BAV304a infected cells. Moreover, the expression of pV was severely reduced in mutant BAVd1d3 infected cells compared to BAV304a, BAV.pVd1, BAV.pVm123, and BAV.pVd3 infected cells.

3.3.10 Structural protein incorporation assay

To determine if the decreased late protein express influences the structural protein incorporation, structural proteins in purified virus were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane and probed with Western blot by anti-hexon (Kulshreshtha et al., 2004), anti-fiber (Wu and Tikoo, 2004), anti-pVII (Paterson, 2010), anti-pV and anti-pVIII (Ayalew, 2014) antisera. As shown in Fig 3.8, there was no detectable difference between hexon, fiber, pVII and pVIII expression among these recombinant BAdV-3s. However, the expression of pV mutants was different. Firstly, different sized pV mutants were detected from different recombinant BAdV-3s, indicating the deletion or mutation of pV NoLS(s). Secondly, different pV expression patterns shown in BAV.pVd1d3 purified from CRL and CRL.pV cells. Two pV bands were detected in BAV.pVd1d3 purified from CRL.pV cells, while only the lower one was detected in the BAV.pVd1d3 purified from CRL cells.
Figure 3.7 Analysis of gene expression in mutant BAdV-3 infected cells. A) Proteins from lysates of MDBK cells infected with indicated mutant BAdV-3s were separated by 10% SDS-PAGE, transferred to nitrocellulose and probed with protein specific antisera and Alexa Fluor 680 conjugated goat anti-rabbit antibody (Invitrogen). β-actin was used as a loading control and was detected using anti-β-actin monoclonal antibody (Sigma-Aldrich) and IRDye800 Conjugated goat anti-mouse antibody (Rockland). Protein names are depicted on the right of the panel. E (Early), L (Late), DBP (DNA binding protein). B) The values were analyzed by using Odyssey® CLx Imaging System (LI-COR). Values represent averages of two independent repeats and error bars indicate the standard deviations.
**Figure 3.8 Structural protein incorporation assay.** Structural proteins from the purified BAV304a (lane 1), BAV.pVd1 (lane 2), BAV.pVd1 (lane 3), BAV.pVd3 (lane 4) or BAV.pVd1d3 (lane 5) grown in CRL cells and BAV.pV BAV.pVd1d3 (lane 6) grown in CRL.pV cells were separated by 10% SDS-PAGE, transferred to nitrocellulose and probed by Western blot using protein specific antisera. Protein bands were visualized by Odyssey® CLx Imaging System (LI-COR). Protein names are depicted on the right of the panel.
3.3.11 Analysis of BAdV-3 capsid assembly.

Since expression of BAdV-3 proteins (hexon, 100, and pV) was significantly reduced in NoLSs deleted BAdV-3 (BAV.pVd1d3), viral capsid assembly was analyzed initially in BAV.pVd1d3 and BAV304a infected MDBK cells. Capsid formation was analyzed in BAV.pVd1d3 infected cells by TEM. As seen in Fig 3.9A, although capsid formation was observed in BAV.pVd1d3 infected cells, many of the capsids were lightly stained. Moreover, there were fewer amounts of capsids observed in BAV.pVd1d3 infected cells than BAV304a infected cells.

Viral capsid assembly was analyzed by using BAV.pVd1d3 and BAV304a viral particles purified from MDBK cells. The infected cells were harvested, freeze-thawed, and the virions were purified using CsCl gradients. As seen in Fig 3.9B, few mature virions were present in purified BAV.pVd1d3. Moreover, the amount of mature virions present in purified BAV.pVd1d3 was significantly lower than the amount detected in BAV304a infected cells.

3.3.12 Thermostability of recombinant BAdV-3

Deletions and mutations in viral genome are always associated with thermo vulnerability (Ugai et al., 2007). To examine if the deletion or mutation of pV NoLSs leads to the decrease of BAdV-3 thermostability, wild-type and recombinant BAdV-3s were treated as described above (Ugai et al., 2007). As seen in Fig 3.10A, there is no titer difference when the temperature was under 25°C. However, when viruses were incubated at 37°C, the titers dropped significantly, especially for BAV.pVd1d3 and BAV.pVm123. To assess the different dynamics of viral inactivation, wild-type and recombinant BAdV-
3s were treated at -80°C, 4°C or 37°C for 0, 1, 3 or 7 days. As seen in Fig 3.10B-F, after seven days incubation at -80°C or 4°C, there was no detectable change in all these five BAdV-3s. However, after seven days incubation at 37°C, BAV304a, BAV.pVm123 and BAV.pVd1d3 lost all their infectivity, while for the single NoLS deleted recombinant viruses BAV.pVd3 and BAV.pVd1, ~10^3 infectious VP were still remaining.
Figure 3.9 Transmission electron microscopic analysis. A) Viral assembly in infected cells. Uninfected MDBK cells (Panel 1, 2), MDBK cells infected with BAV.pVd1d2 (Panel 3, 4) or BAV304a (Panel 5, 6). The arrows depict higher magnification (60,000x) of the areas in the red boxes. B) Negative staining of purified BAV304a (Panel 1, 2) and BAV.pVd1d2 (Panel 3, 4). The arrows depict higher magnification (120,000x) of the areas in the red boxes.
Figure 3.10 Thermostability of the recombinant BAdV-3s. A) Thermostability assay of the recombinant BAdV-3s. $10^5$ TCID$_{50}$ of BAV304a, BAV.pVm123, BAV.pVd1 or BAV.pVd3 virions purified from CRL cells or $10^5$ TCID$_{50}$ of BAV.pVd1d3 virions purified from CRL.pV cells were incubated at -80°C, 20°C, 4°C, 25°C or 37°C for 3 days, and the residual viral infectivity was determined with TCID$_{50}$ on CRL.pV cells. Values represent averages of two independent repeats and error bars indicate the standard deviations. B, C, D, E, F) $10^5$ TCID$_{50}$ of BAV304a, BAV.pVm123, BAV.pVd1 or BAV.pVd3 virions purified from CRL cell or $10^5$ TCID$_{50}$ of BAV.pVd1d3 virions purified from CRL.pV cells were incubated at -80°C, 4°C or 37°C for 0, 1, 3 or 7 days. The residual viral infectivity was determined with TCID$_{50}$ using CRL.pV cells. Values represent averages of two independent repeats and error bars indicate the standard deviations.
3.4 DISCUSSION

Although adenovirus protein homologs are encoded by members of *Mastadenovirus* genus, recent reports have demonstrated the differences in the subcellular localization and function of homologous adenovirus proteins (Blanchette et al., 2013; Cheng et al., 2013; Stracker et al., 2005). Recently, we reported that 100K protein encoded by HAdV-5 and BAdV-3 differ in subcellular localization and protein function (Makadiya et al., 2015). Adenovirus pV is a *Mastadenovirus* genus specific minor core protein, which localizes to both the nucleus and the nucleolus in infected cells (Matthews, 2001). Although transportin appears necessary for the nucleolar localization of pV (Hindley et al., 2007), the molecular mechanism involved in the nucleolar localization is not known. The present study was designed to characterize BAdV-3 pV protein, investigate the mechanism of nucleolar localization and determine its role in virus replication.

The BAdV-3 pV is predicted to encode a protein of 423 amino acids, which is expressed as 55 kDa protein, appears between 12-24 hrs post infection and could be detected till 48 hrs post BAdV-3 infection. pV is almost exclusively detected in the nucleolus of the BAdV-3 infected or transfected cells in the absence of any other viral proteins.

Proteins localizing to nucleolus also localize to the nucleus and thus may contain either overlapping NLS\NoLS (Cheng et al., 2002; Sheng et al., 2004) or separate non-overlapping signals for localizing to both the nucleus and the nucleolus (Cros et al., 2005; Ladd and Cooper, 2004). Amino acid sequence analysis of BAdV-3 pV predicted three clusters of arginine-lysine rich sequences in both N-terminus (amino acid 21-50), central
domain (amino acid 190-210) and C-terminus (amino acid 380-389) of pV with potential to act as NLS.

However, deletion analysis identified N-terminal amino acids 21-50 (NoLS1) and C-terminal amino acid 380-389 (NoLS2) as NoLS, both containing basic residues that can function as NoLS. Both NoLS1 and NoLS2 amino acids were sufficient to direct nucleolar import of EYFP, a non-nucleolar protein. This is consistent with the earlier report, suggesting that NoLS are rich in basic amino acids and are predominantly localized near N or C-terminus of the protein (Scott et al., 2010). Deletion of a potential NoLS did not reduce the nucleolar localization of pV suggesting that NoLS1 and NoLS2 function is redundant. Interestingly, three arginine and lysine rich motifs of NoLS1 also appear to have redundant function as mutation of any arginine lysine rich motif of NoLS1 did not abrogate the nucleolar localization of BAdV-3 pV. Interestingly, deletion of both abrogated the nucleolar localization of pV. However, deletion of potential NoLS did not alter the nuclear localization of pV. Moreover, V.d15 (Fig 3.4C) containing amino acid 21-50 (NoLS1) localized predominantly in the cytoplasm of the transfected cells (Fig 3.4D) and the fusion protein GFPβGal (Fig 3.4E) containing amino acid 21-50 showed no nuclear and nucleolar localization (Fig 3.4F). Unlike EYFP, a 26 kDa protein can diffuse between the cytoplasm and the nucleus, GFPβGal is too big to diffuse through the NPC (Paterson, 2010). These results suggest that the NoLS1 does not contain nuclear localization signal(s) required for pV to localize to the nucleus. In contrast, V.d16 (Fig 3.4C) containing amino acids 21-50 (NoLS1) and 380-389 (NoLS2) located in the nucleus and nucleolus (Fig 3.4D), suggesting that amino acids 380-389 can mediate V.d16 both nuclear and nucleolar localization. Although human adenovirus 2 pV can
redistribute B23 from the nucleolus to cytoplasm (Matthews, 2001), in this study, B23 was used as a nucleolar marker and no B23 redistribution by pV was observed both in the infected and transfected cells. This is probably because of the low identity between BAdV-3 and HAdV-2 pV (40.9%) (Reddy et al., 1998).

The nucleolar transport usually require binding of nucleolar constituents to specific protein sequences namely nucleolar localization signal (NoLS), which helps to retain the protein in the nucleolus. Though, there is no consensus in known NoLS sequences, NoLS are usually rich in lysine and arginine residues, which may interact with nucleolar RNAs or other nucleolar proteins (Olson and Dundr, 2005) for their retention in the nucleolus by a charge dependent mechanism (Musinova et al., 2015). While many nucleolar proteins contain RNA binding motifs (Hiscox, 2007) and are retained by binding to nucleolar RNAs, nucleophosmin protein contains acidic regions which bind to positively charged amino acids in putative nuclear proteins and retain them in the nucleolus (Adachi et al., 1993; Valdez et al., 1994). Though NoLS1 and NoLS2 do not contain a specific amino acid sequence, both are rich in positively charged residues. Since no specific NoLS sequence pattern could be defined in pV, the abundance of positively charged residues appears to mediate translocation of pV from nucleus to nucleolus suggesting that nucleolar retention is due to electrostatic interactions.

Unlike nucleolar transport, nuclear import requires active transport mechanisms, which are dependent on energy, soluble factors and functional nuclear pore complex (Nigg, 1997). Most of the proteins imported into the nucleus contain nuclear localization signals (Boulikas, 1993; Kosugi et al., 2009), which interact with importin α/β and/or transportins in the cytoplasm and are transported through nuclear pore complex into the
nucleus. Though bioinformatic analysis predicted 190-210 to act as potential NLS, deletion analysis identified three regions including amino acid 80-120, 190-210 and 380-423, which can act as NLS. Deletion of all three motifs is required to abolish the nuclear localization and binding of pV to importin α3 suggesting that each motif is functionally redundant. Separate or overlapping redundant NLSs have been identified in viral proteins including polyomavirus large T antigen (Howes et al., 1996; Richardson et al., 1986), influenza virus NS1 protein (Melen et al., 2007), adeno-associated virus 2 assembly activating protein (Earley et al., 2015) and in BAdV-3 33K (Kulshreshtha et al., 2014). It is possible that BAdV-3 pV NLS redundancy may help promote efficient interaction with nuclear transport system leading to an effective nuclear transport. Support for this comes from the fact that increased binding of pV to importin α3 could be observed in the presence of all three NLS regions (Fig 3.5C,D). Two additional bands were detected in the in vitro synthesized pV.d15. It is possible that the deletion of amino acids 81-120, 190-210 and 380-389 induces the leaky scan, which renders 40S ribosomal subunit bypass the first AUG sometimes and starts translation at the second AUG.

A number of viral proteins, including HAdV-5 pVII, use multiple nuclear import pathways (Wodrich et al., 2006). Recently, we also have demonstrated that nuclear import of BAdV-3 33K involves recognition of overlapping NLS motifs located in 40 amino acid long conserved region of BAdV-3 33K by importin α5 and transportin-3 (Kulshreshtha et al., 2014). Though transportin-3 has been shown to be required for HAdV-5 pV nucleolar transport, our data suggest that the nuclear import of pV appears to be mediated only by importin α3 of importin α/β pathway and requires amino acids 81-120, 190-210, and 380-423.
Although deletion of NoLS2 affects the efficient production of progeny virus, both NoLS1 and NoLS2 do not appear essential for the production of viable virus suggesting that each NoLS motif may be functionally redundant. In contrast, deletion of both NoLS1 and NoLS2 prevented the production of viable virus suggesting that nucleolar localization of pV is essential for the production of viable virus. Since nucleolar delocalization of pV appeared lethal for production of progeny virus in MDBK cells, this phenotype could be due to defect in any step of the viral replication including viral protein expression, DNA replication and/or virus assembly. The early protein expression in BAV.pVd1d3 appeared comparable to BAV304a suggesting that the loss of growth is potentially due to an event occurring late in infection. Analysis of late protein expression revealed that the nucleolar delocalization of pV altered the expression of some late viral proteins namely hexon, 100K and pV in BAV.pVd1d3 infected cells compared to BAV304a infected cells. Moreover, progeny virus could be detected in BAV.pVd1d3 infected MDBK cells suggesting that pV NoLSs are not required for assembly of empty capsids and immature virions. Western blot analysis of CsCl purified BAV.pVd1d3 virus grown in CRL cells could not detect difference in pV incorporation, indicating that pV NoLSs are not essential for pV incorporating into the virus particles.

Earlier reports have suggested that trimerization and nuclear transport of hexon by 100K is required for formation of capsid (Hong et al., 2005; Xi et al., 2005). In the protein expression assay (Fig 3.7), the expression of both 100K and hexon was decreased in NoLSs deleted BAdV-3 infected cells. Thus, one explanation of the impaired viral assembly is the reduced expression of hexon, as well as its decreased trimeration and nuclear transport because of the decreased expression of 100K. Moreover, in another
study, the interactions of 33K with pV or 100K were detected (Kulshreshtha and Tikoo, 2008). We also found that pV can interact with 100K and 33K (Zhao and Tikoo, unpublished data). Therefore, one may speculate that pV may form a complex with 100K and 33K to manipulate not only 100K functions but also 33K functions. 33K has been proved to regulate the major late promoter (Ali et al., 2007), capsid assembly and capsid DNA interaction (Finnen et al., 2001; Kulshreshtha and Tikoo, 2008). Therefore, it is possible that pV may improve the viral capsid assembly via its interaction with 33K, while future work need to be done to prove this speculation.
4.0 TRANSITION FROM SECTION 3.0 TO SECTION 5.0

Nucleolar localization signals of pV have been proved to be essential for BAdV-3 replication. Deletion of pV NoLSs blocks pV nucleolar retention, thereby abrogating pV function in nucleolus in viral replication. In addition to this, pV may also play other functions in viral replication. To elucidate the other functions of pV may play, the following chapter was performed. In this chapter, pV was deleted from BAdV-3 genome to construct pV deleted BAdV-3 (BAV.dV) by using homologous recombination. Then, pV functions were examined by comparing the differences between BAdV-3 and BAV.dV in different stages of viral replication.
5.0 DELETION OF pV AFFECTS INTEGRITY OF CAPSID CAUSING DEFECT IN THE INFECTIVITY OF BOVINE ADENOVIRUS-3

5.1 INTRODUCTION

Adenoviruses are non-enveloped icosahedral particles of 70 to 100 nm in diameter (Horne et al., 1959), which infect mammals, birds (Chiocca et al., 1996), reptiles (Benko et al., 2002), frogs (Davison et al., 2000) and fish (Kovacs et al., 2003). Members of Mastadenovirus genus including human adenovirus (HAdV) infect mammals and encode unique proteins including pIX and pV (Davison et al., 2003). Earlier reports suggested that HAdV-5 pV acts as a bridge between the core and the capsid proteins in mature virions (Chatterjee et al., 1985; Lehmberg et al., 1999; Matthews and Russell, 1998a). Additional investigations have revealed that pV promotes viral assembly through nucleophosmin 1 (Ugai et al., 2012) and is essential for virus replication in primary but not in cancer cells (Ugai et al., 2007).

Bovine adenovirus 3 (BAdV-3) is non-enveloped icosahedral virus of 75 nm in diameter (Thompson et al., 1981), which contains a genome of 34,446 bp long and organized into early (E), intermediate (I) and late (L) regions (Reddy et al., 1998). Previously, we reported that the core protein pVII encoded by L1 region of BAdV-3 localizes to the mitochondria using a mitochondrial localization signal, and interferes with apoptosis by altering some mitochondrial functions in infected cells (Anand et al., 2014). Recently, we reported that conserved regions of pVIII encoded by L6 region contain motifs involved in nuclear localization or packaging in mature virions (Ayalew et al., 2014). Similarly, conserved leucines (Kulshreshtha et al., 2015) and conserved arginines (Kulshreshtha et al., 2014) of 33K protein encoded by L6 region appeared
important in binding and the activation of major late promoter, and in nuclear transport of 33K and BAdV-3 replication, respectively.

Though positional homologs are encoded by HAdV-5 and BAdV-3, the structure and function of the homologous proteins may always not be similar (Anand et al., 2014; Kulshreshtha et al., 2004; Li et al., 2009; Reddy et al., 1998). Recently, we demonstrated that unlike HAdV-5, bovine adenovirus-3 protease cleaves 100K protein, which is required for the nuclear transport in the infected cells but not for the virus replication (Makadiya et al., 2015).

The L2 region of BAdV-3, a member of Mastadenovirus genus, encodes pV protein of 423 amino acids, which shows 40.9% homology to pV encoded by HAdV-2 (Reddy et al., 1998). Recently, we have demonstrated that pV is detected as a) 55 kDa protein in CsCl gradient purified BAdV-3 virions or in BAdV-3 infected cells, and b) is localized predominantly in the nucleolus of the virus infected cells, which is required for the replication of BAdV-3 (Zhao and Tikoo, 2016, manuscript in preparation). Here, we report the role of pV in the replication of BAdV-3 in the infected cells.

5.2 MATERIALS AND METHODS

5.2.1 Cells and viruses

Madin Darby bovine kidney (MDBK), CRL (Cotton rat lung) cells (Papp et al., 1997), VIDO-DT1 (CRLcells expressing I-SceI) and CRL.pV cells (described below) were cultivated in minimal essential medium (MEM) (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen). The HEK293T cells (ATCC® CRL-3216™) were cultivated in Dulbecco’s minimal essential medium (DMEM)
(Sigma) with 10% FBS. BAV304a was propagated in MDBK cells, and BAV.dV was propagated in CRL.pV cells.

5.2.2 Antibodies

Production and characterization of anti-DBP (Kulshreshtha et al., 2004), which detect a protein of 48 and 102 kDa in BAdV-3 infected cells, respectively, has been described. The anti-pX serum detects a protein of 25 kDa, anti-hexon serum detects a protein of 103 kDa and anti-pVII serum detects proteins of 22 and 20 kDa (Paterson, 2010) in BAdV-3 infected cells. Production and characterization of anti-pV sera, which recognize a protein of 55 kDa in BAdV-3 infected cells will be described elsewhere (Zhao and Tikoo, 2016, manuscript in preparation). TRITC conjugated goat anti-rabbit IgG (Jackson Immunoresearch), Alexa Fluor 680 conjugated goat anti-rabbit antibody (Invitrogen), anti-β-actin monoclonal antibody (Sigma-Aldrich) and IRDye800 conjugated goat anti-mouse antibody (Rockland) were purchased.

5.2.3 Construction of plasmid pUC304A.dV

A 6.4-kb EcoRV-Bst1107I DNA fragment of plasmid pUC304A+ (E3 deleted BAdV-3 containing CMV.EYFP inserted in E3 region), was isolated and ligated to a 2.1-kb EcoRV-Bst1107I fragment of plasmid pMCS1 (Thanbichler et al., 2007) creating plasmid pMCS.pV. To delete pV from pMSC.pV, a 465-bp fragment was amplified by using primers dV-F1-F: 5’-TGATCCGGTGCGGCGACACAATCGAG-3’; dV-F1-R: 5’-TGTGGCGCTTTGCGGGATGCCTGCAAGCAGCGACTGGGGTTATCGGCCGCG-3’ and plasmid pMCS.pV DNA as a template. Similarly, a 602-bp fragment was amplified by
PCR using primers dV-F2-F: 5’-GCCGATAACCCACTGTGCCTGCAG
GCATCCGCAAGCGCCACAGTAAC-3’; dV-F2-R: 5’-GTCCATGGCGGTGTTAA
CAAGCTGTG-3’ and plasmid pMCS.pV DNA as a template. In the third PCR, these
two fragments were annealed and used as DNA template to amplify the 1040-bp DNA
fragment without pV by overlapping PCR using primers dV-F1-F and dV-F2-R. Finally,
a 622-bp EcoRI-HpaI DNA fragment of the third PCR product was isolated and ligated to
EcoRI-HpaI digested plasmid pMCS.pV creating plasmid pMSC.dV.

A 1.6-kb SbfI fragment (containing kanamycin resistant gene) of plasmid pUC4K
(Taylor and Rose, 1988) was isolated and ligated to SbfI digested plasmid pMCS.dV to
create plasmid pMSC.dV.Kan. The recombinant plasmid pUC304.dV.Kan was generated
by homologous recombination in E. coli BJ5183 between the plasmid pUC304A+ DNA
and a 6.4-kb EcoRV-Bst1107I DNA fragment of plasmid pMCS.dV.Kan. Finally,
plasmid pUC304.dV.Kan was digested with SbfI and large fragment was religated to
create plasmid pUC304A.dV.

5.2.4 Construction of pV expressing cell line CRL.pV

Earlier, we successfully used lentivirus system to isolate VIDO DT1 cells
expressing I-SceI endonuclease) (Du and Tikoo, 2010). To isolate the cell line stably
expressing BAdV-3 pV, we used the second generation replication defective lentivirus
system containing cloning plasmid pTrip-puro, plasmid pSPAX expressing HIV Gag/Pol
proteins and plasmid pMD2.G, expressing vesicular stomatitis virus G protein . Briefly, a
1.2 kb DNA fragment containing BAdV-3 pV gene was ligated to EcoRV-XhoI digested
plasmid pTrip-puro (containing a puromycin resistant marker), creating plasmid pTrip-
pV-Puro. The HEK293T cells were co-transfected with plasmid (pTrip-pV-Puro, pSPAX2 and pSPAX) DNAs. At 48 h post-transfection, the lentivirus in media was collected and used to transduce CRL cells with 8ug/ul polybrene. At 24 h post-transduction, the transduced cells were transferred to 10 cm² dishes. After 24 h, media were replaced by fresh selection media containing 5 μg/ml of puromycin. The puromycin resistant cell clones were picked, propagated in puromycin containing media and tested for the expression of BAdV-3 pV.

5.2.5 Western blot analysis

Proteins from purified virus, virus infected cell lysates or pV expressing cell lysates were separated by Sodium dodecyl-sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membrane (Bio-Rad) and probed by Western blot using protein specific anti-sera and Alexa Fluor 680 or IRDye800 conjugated antibodies. The membranes probed with fluorophore-conjugated secondary antibody were scanned and analyzed by Odyssey® CLx Imaging System (LI-COR).

5.2.6 Confocal microscopy

Monolayer of CRL.pV cells in 2-well chamber slides were fixed with 3.7% paraformaldehyde and permeabilized with 0.1 M PBS containing 0.5% Triton X-100. After blocking with 5% goat serum, the cells were stained with rabbit anti-pV sera and TRITC conjugated goat anti-rabbit IgG (Jackson Immunoresearch). Finally, the cells were mounted by mounting buffer (Vector Laboratories Inc.) containing DAPI and imagined under confocal microscope TCS SP5 (Leica).
5.2.7 Isolation of recombinant BAV.dV

VIDO DT1 (Du and Tikoo, 2010) or CRL.pV (this report) cells in 6-well plates were transfected with *Pac*I digested plasmid pUC304.dV using Lipofectamine 2000 (Invitrogen). At 4 hrs post-transfection, the medium was replaced with fresh MEM containing 2% FBS. The cells showing cytopathic effect (CPE) were harvested and freeze thawed three times. Finally, the recombinant virus was propagated in CRL.pV cells and purified by CsCl gradient centrifugation (Tollefson et al., 1999).

5.2.8 Virus DNA replication

The CRL cells were infected with BAV304a or BAV.dV at a MOI of 2. At 12, 24 and 36 hrs post infection, the infected cells were washed in phosphate buffered saline and used to purify low molecular weight DNA as described (Farina et al., 2001). Equal amount of DNA was digested with *Bmt*I restriction enzyme and separated by agarose gel electrophoresis and analyzed by Gel Doc™ XR+ System (Bio-Rad).

5.2.9 Virus single cycle growth curve

CRL cells in 24-well plates were infected with BAV304a or BAV.dV at MOI of 2. At different times post-infection, the infected cells were harvested, freeze-thawed three times and titrated by TCID$_{50}$ in CRL.pV cells as described elsewhere (Kulshreshtha et al., 2004).
5.2.10 Viral thermostability assay

To determine if deletion of pV alters BAV.dV thermolability, $1\times10^5$ (from CRL.pV) or $1\times10^4$ (from CRL) infectious viral particles purified from CRL.pV or CRL in 10% glycerol containing PBS were incubated at different temperatures (-80°C, -20°C, 4°C, 25°C and 37°C) for three days. To analyze the dynamics of viral inactivation, purified $1\times10^5$ (from CRL.pV) or $1\times10^4$ (from CRL) infectious virus particles in 10% glycerol containing PBS were incubated at different temperatures (-80°C, 4°C and 37°C) for 0, 1, 3 and 7 days. Finally, the infectivity of the treated virus particles was measured by TCID$_{50}$ in CRL.pV cells.

5.2.11 Transmission electron microscopy

CRL cells were infected with BAV304a or BAV.dV at MOI of 2. At 24 hrs post-infection, the cells were collected and fixed in 2.5% glutaraldehyde, and with 1% OsO$_4$ in 0.1M PBS. After dehydration with a graded ethanol series and propylene oxide, the samples were infiltrated with a mixture of propylene oxide and EMbed-812 embedding medium and polymerized in embedding capsules at 60°C for 24-48 hrs. The pellets were sectioned by using a Reichert ultracut microtome, the sections were stained with 2% uranyl acetate and lead citrate. Finally, the stained sections were viewed using a Philips CM10 TEM.
5.3 RESULTS

5.3.1 Isolation of BAV.dV in CRL cells

To determine if pV is essential for BAV304a (Du and Tikoo, 2010) replication, we constructed a plasmid pUC304A.dV containing BAdV-3 genome with deletion of pV and insertion of CMV-EYFP gene cassette in E3 deleted region (Fig 5.1B). Individual plasmid pUC304A.dV or pUC304A+ (containing BAdV-3 genome with insertion of CMV-EYFP gene cassette in E3 deleted region) (Fig 5.1A) DNA were used to transfect VIDO DT1 cells. At 6 days post-transfection, the EYFP expression and cytopathic effects were visible in the cells transfected with plasmid pUC304A+ DNA (Fig 5.1A). However, repeated transfection of VIDO DT1 cells with plasmid pUC304A.dV DNA did show EYFP expression in few cells but no any cytopathic effects even after 20 days post transfection (Fig 5.1B). Moreover, while the lysates from the cells transfected with plasmid pUC304A+ DNA produced cytopathic effects in freshly infected VIDO DT1 cells, the lysates from the cells transfected with plasmid pUC304A.dV DNA did not produce any cytopathic effect or expression of EYFP in freshly infected VIDO DT1 cells (data not shown). These results suggest that pV is essential for the replication of BAV304a.

5.3.2 Construction of CRL.pV cells expressing BAdV-3 pV

To isolate a cell line expressing BAdV-3 pV, CRL cells were transduced with lentivirus expressing BAdV-3 pV and grown in the presence of puromycin as described earlier (Du and Tikoo, 2010). The puromycin resistant clones were analyzed initially for
Figure 5.1 Isolation of pV deleted BAdV-3. A) Schematic diagram of indicated plasmid DNA. Thick black box represents BAdV-3 genomic DNA. Dotted line represents deleted region. Thin lines represent plasmid DNA. The human cytomegalovirus immediate early promoter (CMV), enhanced yellow fluorescent protein gene (EYFP), ampicillin resistance gene (Amp), early region 3 (E3) and pV location is depicted. B) Direct fluorescence. Monolayer of VIDO DT1 cells (Du and Tikoo, 2010) were transfected with 7.5ug indicated plasmid DNA and visualized for the expression of EYFP and development of cytopathic effects using fluorescent microscope TCS SP5 (Leica).
the expression of pV by Western blot and immunofluorescence assay using pV specific antisera. Earlier analysis using anti-pV sera suggested that pV is expressed as 55kDa in BAdV-3 infected cells and localizes predominantly in the nucleolus of BAdV-3 infected cells (Zhao and Tikoo, 2016, manuscript in preparation). As shown in Fig 5.2A, anti-pV serum detected a protein of 55 kDa in BAdV-3 infected cells. Similar protein could be detected in two puromycin resistant clones using anti-pV serum (Fig 5.2A, lanes 1,2). No such protein could be detected in CRL cells (Fig 5.2A, lane 4). Secondly, the sub cellular location of pV in puromycin resistant clones was analyzed by confocal microscopy. As seen in Fig 5.2B anti-pV serum detected protein predominantly localized in the nucleolus of pV expressing cells (CRL.pV1, CRL.pV2). No such protein could be detected in the nucleolus of CRL cells.

5.3.3 Isolation of BAV.dV in CRL.pV cells

To isolate pV deleted BAV304a, CRL.pV cells were transfected with PacI digested plasmid pUC304A.dV DNA and observed for the development of cytopathic effects (Fig 5.3A). As shown in Fig 5.3B, the cytopathic effect and EYFP expression was firstly observed at 7 days post-transfection, which increased by day 12. To confirm the identity of recombinant virus, named BAV.dV, first viral DNA was purified from infected CRL.pV cells, digested with KpnI and analyzed by agarose gel electrophoresis. As shown in Fig 5.3C, BAV304a (Lane 1) contains a fragment of 4.7 kb, which was missing in BAV.dV. Instead, BAV.dV (lane 2) contains a fragment of 3.5 kb because of the deletion of pV gene. Secondly, the expression of pV in virus infected CRL cells was analyzed by
Figure 5.2 Analysis of pV expression in CRL.pV cells. A) Proteins from the cell lysates of CRL.pV cells clone 1 (lane 1) and clone 2 (lane 2), BAdV-3 infected CRL cells (lane 3) and mock infected CRL cells (lane 4) were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane and probed in Western blot by using rabbit anti-pV serum and Alexa Fluor 680 conjugated antibodies goat anti-rabbit antibody (Invitrogen). The position of the molecular weight in kDa is shown on the left of the panel. The molecular weight in kDa of observed protein is shown on the right of the panel. B) Monolayers of CRL.pV (clone 1 or 2) or CRL cells were fixed with 4% paraformaldehyde and visualized by indirect immunostaining with rabbit anti-pV serum followed by TRITC-conjugated goat anti-rabbit IgG using confocal microscope TCS SP5 (Leica). The nuclei were stained by DAPI.
Western blot using anti-pV sera. As seen in Fig 5.3D, a 55 kDa protein could be detected in BAV304a infected CRL cells (lane 1). Similar mol wt protein could be detected in uninfected CRL.pV cells (lane 3). No such band could be detected in BAV.dV infected CRL cells (lane 2).

To determine the influence of pV on the formation of BAdV-3 particle, CRL cells or CRL.pV cells were infected with purified BAV.dV (grown in CRL.pV cells) at a MOI of 2. At 48 hrs post infection, the lysates of infected cells were used to purify virions by CsCl gradient centrifugation. As seen in Fig 5.3E, deletion of pV predominantly produced population of virus representing mature virions. Moreover, no visible decrease in the production of the mature virus particles could be observed in BAV.dV grown in CRL.pV cells or CRL cells.

5.3.4 Growth of BAV.dV in CRL cells

To determine if BAV.dV can produce infectious viral particles in pV negative CRL cells, viral growth characteristics of CsCl purified BAV304a (grown in CRL cells) and BAV.dV (grown in CRL.pV cells) was analyzed. Monolayers of CRL cells in 24 well plates were infected with BAV304a or BAV.dV at MOI of 2. The infected cells were harvested at different times (0, 6, 12, 24, 36, 48 hrs) post-infection. After freeze thawing three times, the samples were titrated by TCID₅₀ in CRL.pV cells. As shown in Fig 5.3F, BAV304a grew to a titer of ~10⁸ TCID₅₀/ml at 48 h post-infection. In contrast, there was no detectable increase in the titer of BAV.dV. Similarly, no detectable increase in the titer of BAV.dV could be observed in MDBK cells (data not shown).
Figure 5.3 Construction and identification of BAV.dV. A) Schematic diagram of plasmid pUC304A.dV DNA. B) Direct fluorescence. Monolayer of CRL.pV cells were transfected with 7.5ug pUC304A.dV plasmid DNA and visualized for the expression of EYFP and development of cytopathic effects using fluorescent microscope TCS SP5 (Leica). C) Restriction enzyme analysis of BAdV-3 genomes. The viral DNA was extracted from CRL cells infected with BAV304a (lane 1) or BAV.dV (lane 2), digested with KpnI and analyzed by agarose gel electrophoresis. Lane M, GeneRuler 1kb DNA ladder (Thermo Fisher Scientific) was used for sizing the viral DNA fragments. Diagnostic bands are indicated with white arrows. Sizes of markers are shown on the left.
of the panel. D) Western blot. Proteins from the lysates of BAV304a infected CRL cells (lane 1), BAV.dV infected CRL cells (lane 2) or uninfected CRL.pV cells (lane 3) were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed in Western blot by using rabbit anti-pV anti-serum and Alexa Fluor 680 conjugated goat anti-rabbit antibody (Invitrogen). The position of the molecular weight in kDa is shown on the left of the panel. The molecular weight in kDa of the observed protein is shown on the right of the panel. E) CsCl gradient purification. The lysates of CRL.pV or CRL infected with BAV.dV were separated by centrifugation through continuous CsCl gradient and centrifuge tubes were photographed. f) Virus growth. Confluent monolayers of CRL cells were infected with BAV304a or BAV.dV at a MOI of 2. At different times post infection, the cell pellets were collected, freeze-thawed, and virus was titrated on CRL.pV cells as described previously (Ugai et al., 2007). Each values represents average of two independent repeats and error bars indicate the standard deviations.
5.3.5 Analysis of protein expression in BAV.dV infected cells

To analyze if deletion of pV influences the expression of viral proteins, monolayers of CRL cells were infected with BAV304a or BAV.dV at MOI of 2. At 24 hrs post-infection, the cells were harvested and lysed. The proteins from the cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed by protein specific anti-serum and secondary antibodies conjugated with fluorophores. Finally, the membranes were scanned and analyzed by Odyssey CLx Imaging System. As expected (Fig 5.4A, B), the expression of pV could be detected in BAV304a infected CRL cells but not in BAV.dV infected CRL cells. No appreciable difference could be detected in the expression of early DBP protein in CRL cells infected with BAV304a or BAV.dV. However, compared to BAV304a, reduced expression of some late proteins, particularly 100K, pX and pVII were observed in BAV.dV infected cells. Moreover, both precursor and cleaved form of pVII could be detected in BAV304a or BAV.dV infected cells.

5.3.6 Analysis of BAV.dV DNA replication

The CRL cells were infected with purified BAV304a or BAV.dV (grown in CRL.pV cells) at a MOI of 2. At 12, 24 or 36 hrs post infection, the cells were collected, washed with PBS and used to extract DNA as described (Farina et al., 2001). The DNA isolated from equal number of cells was digested with restriction enzyme BmtI. Analysis of restriction enzyme digested DNA (Fig 5.4C) suggested that both BAV304a and BAV.dV replicated to similar levels in CRL cells.
Figure 5.4 Analysis of viral protein expression in BAV.dV infected cells. A) Proteins from the lysates of CRL cells were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed by Western blot using anti-DBP (Zhou et al., 2001), anti-pVII (Paterson et al., 2012), anti-pV (Kulshreshtha et al., 2004), anti-pX (Paterson, 2010), anti-Hexon (Kulshreshtha et al., 2004), anti-Fiber (Wu and Tikoo, 2004) and anti-100K (Makadiya et al., 2015) sera followed by Alexa Fluor 680 conjugated goat anti-rabbit antibody (Invitrogen). β-actin was detected by Western blot using mouse anti-β-actin monoclonal antibody (Sigma-Aldrich) followed by IRDye800 Conjugated goat anti-mouse antibody (Rockland). The name of the proteins is depicted on the right of the panel. DBP (DNA binding protein). Early (E), Late (L). B) The results were analyzed by using Odyssey Infrared Imaging System. Values represent averages from two independent repeats and error bars indicate the standard deviations. C) DNA replication. Viral DNAs were extracted at indicated times post-infection with BAV304a (lane 2,4,6) or BAV.dV (lanes3, 5, 7) and digested with BmtI. The specific bands are indicated by white arrows. The sizes of markers (M) are depicted on left and right of the panel.
5.3.7 Analysis of protein incorporation in BAV.dV viral particles

To determine the incorporation of pV in the progeny virions, proteins from purified virions were separated by 10% SDS-PAGE, transferred to nitrocellulose and probed in Western blot using anti-pV sera. As seen in Fig 5.5A, anti-pV detected a protein of 55 kDa in purified BAV304a grown in CRL cells (lane 1). A protein band of similar molecular weight could be detected in purified BAV.dV grown in CRL.pV cells (lane 3). However, no such protein could be detected in BAV.dV grown in CRL cells (lane 2). Moreover, there was no detectable difference in the incorporation of the viral proteins in purified BAV304a or BAV.dV virions (grown in CRL cells or CRL.pV cells). As seen in Fig 5.5A, hexon and fiber proteins were efficiently incorporated in purified BAV304a grown in CRL cells (lane 1), purified BAV.dV grown in CRL cells (lane 2) or purified BAV.dV grown in CRL.pV cells (lane 3).

Anti-pVII serum detected both precursor and cleaved form of pVII in BAV304a infected cells (Fig 5.5B, lane 2) or BAV.dV infected CRL cells (Fig 5.5B, lane 4) or CRL.pV cells (Fig 5.5B, lane 6). As expected, a protein consistent with the cleaved form of pVII could be detected in purified BAV304a grown in CRL cells (Fig 5.5B, lane 1), purified BAV.dV grown in CRL cells (Fig 5.5B, lane 3) or grown in CRL.pV cells (Fig 5.5B, lane 5). Similarly, a cleaved form of pVIII is incorporated in purified BAV304a grown in CRL cells (Fig 5.5A, lane 1), BAV.dV grown in CRL cells (Fig 5.5A, lane 2) or BAV.dV grown in CRL.pV cells (Fig 5.5A, lane 3).
Figure 5.5 Analysis of viral protein incorporation in purified virions. A) Proteins from the purified BAV304a grown in CRL cells (lane 1), BAV.dV grown in CRL cells (lane 2), BAV.dV grown in CRL.pV cells were separated by 10% SDS-PAGE, transferred to nitrocellulose and probed in Western blot using protein specific antisera. B) Proteins from purified BAV304a grown in CRL cells (lanes 1), BAV.dV grown in CRL cells (lane 3), BAV.dV grown in CRL.pV cells (lane 6) or proteins from the lysates of CRL cells (lane 2, 4) infected with BAV304a (lane 2), BAV.dV (lane 4) and CRL.pV cells infected with BAV.dV (lane 6) were separated by 10% SDS-PAGE, transferred to nitrocellulose and probed in Western blot by anti-pVII serum (Paterson, 2010). Purified virus (PV); infected cell (IC).
5.3.8 Analysis of BAV.dV by transmission electron microscopy

To examine if the deletion of pV affects the formation of BAdV-3 particles, CRL cells were infected with BAV304a or BAV.dV at an MOI of 2. At 24 hrs post infection, the cells were collected, processed and analyzed by TEM. As seen in Fig 5.6A, BAV304a (panel 3,4) appeared to produce more viral particles than BAV.dV (panel 5,6) in infected CRL cells. Moreover, BAV304a particles were uniform and loosely arranged (panel 3). In contrast, BAdV.dV particles appeared to be clustered together and appeared tightly organized in rows (panel 5). Analysis of the enlargement of selected areas of TEM images suggested that BAV304a are clearly of typical icosahedral in shape (panel 4). However, BAV.dV showed less clear morphology and did not possess clear icosahedral shape (panel 6). No such virions could be detected in mock-infected CRL cells (Fig 5.6A, panel 1,2)

Next, we analyzed the CsCl purified BAV304a or BAV.dV (grown in CRL cells) by TEM. The analysis of mature BAV304a virions detected intact capsids with typical icosahedral shape (Fig 5.6B, panel 1, 2). In contrast, most of the BAV.dV particles appeared circular in shape with partially degraded capsids (Fig 5.6B, panel 3, 4)

5.3.9 Thermostability of BAdV.dV

To determine if the deletion of pV alters viral thermostability, purified viral particles in PBS containing 10% glycerol were incubated at different temperatures (-80°C, -20°C, 4°C, 25°C and 37°C) for 3 days or incubated at different temperatures (-80°C, 4°C and 37°C) for 0, 1, 3 and 7 days. Finally, the infectivity was measured by TCID$_{50}$ assay. As seen in Fig 5.7A, there appeared no difference in the thermostability or
dynamics of viral inactivation of BAV304a or BAV.dV grown in CRL.pV cells. In contrast, both thermostability and dynamics of viral inactivation of BAV.dV grown in CRL cells appeared significantly different from BAV304a (Fig 5.7B).
Figure 5.6 Electron microscopic analysis. A) Uninfected (panel 1), BAV304a infected (panel 3) or BAV.dV infected (panel 5) CRL cells. The arrows depicted the enlargement of selected boxed (red) region of panel 1 (panel 2), panel 3 (panel 4) and panel 5 (panel 6). B) Purified BAV304a (panel 1) or BAV.dV (panel 2). The arrows depicted the enlargement of selected boxed (red) region of panel 1 (panel 2) and panel 3 (panel 4).
Figure 5.7 Thermostability of BAdV-3. A) Viruses grown in CRL.pV cells. Purified virions ($10^5$ TCID$_{50}$) grown in CRL.pV cells were incubated at various temperatures for 3 days (panel 1) and the residual viral infectivity was determined by titration on CRL.pV cells. Purified BAV304a (panel 2) or BAV.dV (panel 3) ($10^5$ TCID$_{50}$) grown in CRL.pV cells were incubated at different temperatures for indicated periods of time and the residual viral infectivity was determined by titration on CRL.pV cells. B) Viruses grown in CRL cells. Purified virions ($10^4$ TCID$_{50}$) grown in CRL cells were incubated at various temperatures for 3 days (panel 1) and the residual viral infectivity was determined by titration on CRL.pV cells. Purified BAV304a (panel 2) or BAV.dV (panel 3) ($10^4$ TCID$_{50}$) grown in CRL cells were incubated at different temperatures for indicated periods of time and the residual viral infectivity was determined by titration on CRL.pV cells.
5.4 DISCUSSION

The members of *Mastadenovirus* genus encode genus-specific unique proteins including pIX and pV (Davison et al., 2003). Earlier work has suggested that HAdV-5 pV protein is essential for virus replication in primary cells and may participate in virus assembly (Ugai et al., 2012). Moreover, pV may act as a bridge between the core and the capsid proteins of HAdV-5 (Chatterjee et al., 1985; Lehmberg et al., 1999; Matthews and Russell, 1998a; Vayda et al., 1983). The L2 region of BAdV-3 encodes pV, which shows 28%-41% homology to pV encoded by other members of *Mastadenovirus* genus (Reddy et al., 1998). Since recent reports suggest that positional homologs of proteins encoded by human and animal adenoviruses of *Mastadenovirus* genus may differ in their structure and function (Li et al., 2009), we sought to determine the function of pV in BAdV-3 replication.

The pV appears essential for the replication of BAV304a as production of viable infectious BAV.dV required the isolation of helper cell line providing the pV protein *in trans*. Further analysis of BAV.dV demonstrated no significant difference in the infectivity and DNA replication of mutant BAV.dV and BAV304a. Moreover, no affect was observed in the early gene expression in BAV.dV infected cells. Despite down regulation of some late protein expression, the capsid formation and the virus assembly appeared to occur in BAV.dV infected cells suggesting that pV may not be essential for virion assembly. Earlier report suggested that pV is essential for the replication of HAdV-5 in primary cells but not in cancer cells (Ugai et al., 2012). However, BAdV-3 pV appears essential for replication in primary CRL cells and continuous MDBK cells.
Although, BAV.dV does not appear to produce infectious progeny virions in CRL cells, the capsid formation and the virus assembly appears to occur in BAV.dV infected CRL cells as CsCl gradient analysis of BAV.dV infected CRL cells produced virions banding at CsCl gradient density consistent with the formation of mature virions. Moreover, the deletion of pV does not significantly affect the incorporation of other structural proteins. However, the analysis of these mature virions by TEM revealed that, compared to BAV304a, the capsids of BAV.dV did not appear icosahedral in shape and most of the capsids did not appear to be intact. These results suggest that deletion of BAdV-3 pV may not significantly alter the virus assembly, but instead make virion capsid more fragile leading to the detectable changes in virion morphology and infectivity.

The deletion of pV did not affect the expression of early gene product namely DBP. However, the expression of late proteins particularly 100K, pX and pVII appeared down regulated in BAV.dV infected cells, suggesting that pV may be involved in the regulation of late gene expression probably by acting on major late promoter (Leong et al., 1990). Similar results have been earlier reported for HAdV-5 pV (Ugai et al., 2007).

The production of infectious progeny adenovirus requires a maturation step involving the cleavage of capsid and core proteins by adenovirus protease (Anderson et al., 1973). However, the significance of cleavage of each precursor protein in determining the infectivity is not clear (Mangel and San Martín, 2014). Analysis of viral protein expression in BAV.dV infected cells revealed that deletion of pV did not significantly inhibits the cleavage of pVII. Similarly, analysis of purified BAV.dV demonstrated that
mainly the cleaved form of pVII or pVIII could be detected in purified mature BAV.dV virions.

Unlike primary cells (Ugai et al., 2012), HAdV-5 pV is not required for virus replication and formation of infectious virus particles in cancer cells (Ugai et al., 2012). This is due to apparent thermostable mutations (G13E and R17I) in the less conserved region of core protein X/Mu, which compensate for the lack of pV (Ugai et al., 2007). These mutations increase the expression of X/Mu, which helps to stabilize the capsid thus compensating the absence of pV. Moreover, analysis of CsCl gradient purified pV deleted HAdV-5 grown in cancer cells show increased incorporation of protein X/Mu in mature virions. In contrast, pV appears essential for the replication of BAdV-3 CRL or MDBK cells. Despite conservation of arginine residue at amino acid 20 of BAdV-3 pV (Ugai et al., 2007), analysis of DNA sequence of different clones of BAV.dV grown (different passages) in CRL or MDBK cells did not reveal any mutation in the core proteins X/Mu or pVII (data not shown). Because of unavailability of reagents, the incorporation of the X/Mu could not be analyzed in CsCl gradient purified BAV.dV grown in CRL cell. Our results suggest that deletion of pV does not introduce compensatory mutations in core proteins X/Mu or pVII.

In summary, we have demonstrated that BAdV-3 pV is essential for the replication of BAdV-3 in CRL (primary) and MDBK (continuous) cells. Analysis of BAV.dV suggested that pV appears to be required for maintaining the integrity of the capsid structure and helps in stability of BAdV-3 capsid. However, lack of pV did not introduce any compensatory mutations in other core proteins X/Mu or pVII. Moreover, pV may have a role in the proteolytic cleavage of pVII.
6.0 TRANSITION FROM SECTION 5.0 TO SECTION 7.0

Viruses have relatively small genomes that cannot encode enough proteins for their replication; thereby they hijack many different cellular machineries, such as transcription and translation systems. They also have to evade the host immune system to finish their life cycles. To fulfill these processes, various cellular proteins are recruited for viral replication via protein-protein interaction. In the previous studies, pV has been proved to locate to nucleolus, regulate viral protein expression and mediate viral assembly. And all of these functions are probably realized by interaction of pV with cellular/viral proteins. To figure out which cellular/viral proteins can interact with pV and what biological significances these interaction have, the following chapter was performed.
7.0 INTERACTION OF pV WITH CELLULAR/VIRAL PROTEINS AND THEIR BIOLOGICAL SIGNIFICANCE

7.1 INTRODUCTION

BAdV-3 belongs to *Mastadenovirus* genus in *Adenoviridae* family, and has been developed as a vector for vaccination in animals (Reddy et al., 1999b). It is also a promising vaccine vector for human, because the pre-existing immunity of human adenoviruses influences the efficiency of human adenovirus-based vectored vaccines significantly (Zakhartchouk et al., 2007). Like the other members of *Adenoviridae* family, BAdV-3 is a non-enveloped icosahedral particle with a double-stranded DNA genome, which is organized into early, intermediate and late regions (Reddy et al., 1998). Early and intermediate proteins encoded by early and intermediate regions, respectively, are required for cell cycle modulation, DNA replication, and host immune response evasion. In contrast, late proteins are all encoded by the major late transcription unit (MLTU), which is activated after the onset of viral DNA replication and controlled by the major late promoter (MLP).

Protein-protein interaction plays an indispensable role in all stages of adenovirus life cycle to realize different purposes. To enter host cells, adenovirus fiber knob firstly interacts with cellular receptors, which followed by the Penton RGD motif interaction with integrins (Arnberg, 2009). Adenovirus modulates cell cycle via E1A protein interaction with cellular regulatory proteins and transcription factors (Avvakumov et al., 2002), and inhibits apoptosis by E1B proteins interaction with tumor suppressor p53 (Ben-Israel and Kleinberger, 2002) or pro-apoptotic protein BAK and BAX (Cuconati et al., 2003). The interaction between DBP, pTP and DNA pol are required for the initiation,
elongation and termination of viral DNA replication (van Breukelen et al., 2003).

Adenovirus late proteins also interact with other viral or cellular proteins to facilitate viral assembly. Many interactions of adenovirus late proteins have been reported, such as 52/55K and IVa2 or 33K (Ahi et al., 2015), 52/55K and pVII (Zhang and Arcos, 2005), pVI and hexon (Wodrich et al., 2003), 33K with 100K or pV (Kulshreshtha and Tikoo, 2008), etc.

The protein V (pV) is the only protein encoded by BAdV-3 L2 region (Reddy et al., 1998). It is 423 amino acids in length and with 150-180 copies in each viral particle (Puntener et al., 2011; Ugai et al., 2012). As a structural protein, pV bridges the capsid and viral DNA to stabilize the viral particle by interacting capsid protein pVI (Matthews and Russell, 1998b) and core protein pVII (Harpst et al., 1977). Besides, pV has been proved to play other functions in adenovirus replication. Human adenovirus pV interacts with a mitochondria-nucleus shuttle protein p32, and this interaction may be involved in viral particle transport from the cytoplasm to the nucleus after the release from the endosome (Matthews and Russell, 1998a). In another study, pV has been proved to interact with a nucleolar protein B23, and this interaction may be associated with the redistribution of nucleolin and B23 from nucleolus to cytoplasm and nucleoplasm (Samad et al., 2007).

Nucleolin, which is predominantly located in the nucleolus, is one of the most abundant nucleolar proteins in the cell (Tajrishi et al., 2011). It is organized into three different domains: N-terminus, central and C-terminus (Tajrishi et al., 2011), and they are featured with different functional domains (Storck et al., 2009) and undergoes different post-translational modifications (Durut and Saez-Vasquez, 2015). As a multifunctional
protein, nucleolin is associated not only with ribosome biosynthesis (Ginisty et al., 1998; Rickards et al., 2007) but also with other cellular processes, such as regulation of RNA pol II based transcription (Mongelard and Bouvet, 2007), post-transcription translation (Jiang et al., 2014), DNA repair (Kobayashi et al., 2012; Lim et al., 2015), cell cycle regulation (Ugrinova et al., 2007; Wang et al., 2014a). Moreover, nucleolin functions in different viruses replication by interacting with viral proteins, such as rabies virus P3 (Oksayan et al., 2015), hepatitis C virus NA5B (Shimakami et al., 2006), herpes simplex virus ICP8 (Calle et al., 2008), feline calicivirus NS6 and NS7 (Cancio-Lonches et al., 2011), and for other viruses, nucleolin can facilitate their cellular entry by serving as a cellular receptor, including human respiratory syncytial virus (Tayyari et al., 2011), human parainfluenza virus type 3 (Bose et al., 2004) and enterovirus 71 (Su et al., 2015).

Here, we report the interaction of BAdV-3 pV with viral and cellular proteins, confirm the interaction between pV and nucleolin and elucidate what functions this interaction may play in BAdV-3 replication.

7.2 MATERIALS AND METHODS

7.2.1 Cell lines and viruses

Madin-Darby bovine kidney (MDBK) cells and VIDO DT1 (cotton rat lung fibroblast expressing endonuclease I-SceI [CRL-I-SceI]) cells (Du and Tikoo, 2010) were propagated in minimum essential medium (MEM) (Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). VERO cells and HEK293T cells were cultured in Dulbecco’s modified eagle’s medium (DMEM) supplemented with 5% FBS. Wild-type
BAdV-3 and BAdV-3 expressing strep-tagged pV (BAV.ST) were grown in MDBK cells in MEM supplemented with 2% FBS (Invitrogen).

7.2.2 Antibodies

Production and characterization of BAdV-3 protein specific sera, namely anti-pV serum (Zhao and Tikoo, 2016, manuscript in preparation), anti-DBP serum (Kulshreshtha et al., 2004), anti-hexon serum (Kulshreshtha et al., 2004), anti-fiber serum (Wu and Tikoo, 2004), anti-pVII serum (Paterson et al., 2012) and anti-pX have been described. Anti-β-actin monoclonal antibody (Sigma-Aldrich), C23 antibody (MS-3) (Santa Cruz Biotechnology), alkaline phosphates (AP)-conjugated goat anti-rabbit antibody (Jackson Immunoresearch), Alexa Fluor 647-conjugated goat anti-rabbit IgG, Alexa Fluor 680 conjugated goat anti-rabbit antibody (Invitrogen), and IRDye800 Conjugated goat anti-mouse antibody (Rockland) were purchased.

7.2.3 Plasmids construction

The construction of plasmids pUC304A+ (contains insertion of EYFP in E3 region of pFBAV302) (Baxi et al., 2000), pUC304A.dV containing deletion of pV Orf (nucleotide 15068 to 16340) (Zhao and Tikoo, 2016, manuscript in preparation), pMCS.pV containing pV Orf and flanking sequences have been described (Zhao and Tikoo, 2016, manuscript in preparation). Plasmid pDsRed.NCL and pDsRed.B23, a gift from Dr. Denis Archambault, encode fusion protein DsRed with nucleolin and B23, respectively (Gomez Corredor and Archambault, 2012). Plasmid pLV-DDX3i#3, a gift
from Dr. Kato Nobuyuki, encodes shRNA of DDX3 (Ariumi et al., 2007). Plasmid pSUPER was purchased from Oligoengine.

**a) pUC304A.pVStrep.** A 529-bp DNA fragment amplified by PCR using primers dV-F1-F and pV-ST-F1-R (Table 7.1), and plasmid pUC304A+ DNA as a template. A 1787-bp DNA fragment amplified by PCR using primers pV-ST-F2-F and dV-F2-R (Table 7.1), and plasmid pUC304A+ DNA as a template. In the third PCR, these two PCR fragments were annealed and used to amplify a 2270-bp fragment with a Strep-tag II encoding sequence (Schmidt and Skerra, 2007) by overlapping PCR using primers dV-F1-F and dV-F1-F (Table 7.1). An 1190-bp EcoRI-SacI fragment of the 2270-bp PCR fragment was ligated to EcoRI-SacI digested plasmid pMCS.pV to create plasmid pMCS.pV-ST. Finally, the SphI digested plasmid pUC304.dV was recombined with a 6.2-kb EcoRV-Bst1107I fragment of pMCS.pV-ST to generate plasmid pUC304A.pVStrep.

**b) pEY-pV.** A 1291-bp DNA fragment was amplified by PCR using primers pV-EYXhoI-F and pV-EYBamHI-R (Table 7.1), and plasmid pUC304A+ DNA as a template. A 1277-bp BamHI-XhoI fragment of the 1291-bp PCR fragment was ligated to BamHI-XhoI digested plasmid pEYFP-N1 (Clontech) to create plasmid pEY-pV.

**c) pRDI292-1117, pRDI292-1298 and pRDI292-1453.** Sense and antisense oligonucleotide primer pairs targeting nucleolin, Bncl1117-S and Bncl1117-AS; Bncl1298-S and Bncl1298-AS; Bncl1453-S and Bncl1453-AS, were annealed to generate 68-bp HindIII-BglII double-stranded DNA fragments and ligated to pSUPER (Oligoengine) to construct plasmids pSUPER-1117, pSUPER-1298 and pSUPER-1453, respectively. A 322-bp BamHI-SalI fragment of plasmids pSUPER-1117, pSUPER-1298
or pSUPER-1453 was individually ligated to *Hind*III-*Bgl*II digested pLV-DDX3i#3 to create plasmids pRDI292-1117, pRDI292-1298 and pRDI292-1453, respectively.

### 7.2.4 Isolation of BAV.ST

Monolayers of VIDO-DT1 cells in six-well plate were transfected with 5-7.5 ug of plasmid pUC304A.pVstrep with Lipofectamine 2000 (Invitrogen). At 4-hour post-transfection, the medium was replaced with fresh MEM containing 2% FBS and cells were observed for fluorescence expression and cytopathic effects. The transfected cells showing cytopathic effect (CPE) were harvested, freeze-thawed three times and propagated in MDBK cells.

### 7.2.5 Western blotting

MDBK cells (mock infected or virus infected) or purified viruses were analysed by Western blot using protein-specific antisera and alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (Sigma) or Alexa Fluor 680-conjugated goat anti-rabbit antibody (Invitrogen) or IRDye800-conjugated goat anti-mouse antibody (Rockland). Finally, the membranes were imagined and analyzed using the Odyssey infrared imaging system (Licor).

### 7.2.6 Immunofluorescence microscopy

MDBK cells infected with BAV304a or BAV.ST or VERO cells transfected with indicated plasmid DNA were fixed with 3.7% paraformaldehyde for 15 min and permeabilized with PBS supplied with 0.5% Triton X-100. The cells were then blocked
### Table 7.1 List of primers used for PCR.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<tr>
<td>dV-F1-F</td>
<td>5’-TGATCCGGTGCCGCAACACATCGAG-3’</td>
</tr>
<tr>
<td>pV-ST-F1-R</td>
<td>5’-TGCGCTTGTAGCTCTTCTCGAATTTGTGGTGACCAC-3’</td>
</tr>
<tr>
<td></td>
<td>AGGCAGCAACGATGTCTAAAC-3’</td>
</tr>
<tr>
<td>pV-ST-F2-F</td>
<td>5’-GCCGCTGAGTGGTCACACCCCAATTCTCAGAAGATCTAC</td>
</tr>
<tr>
<td></td>
<td>AAGCGCAAACCGGCCCAG-3’</td>
</tr>
<tr>
<td>dV-F2-R</td>
<td>5’-GTCCCATGGCGTGTTACAAAGCTGTG-3’</td>
</tr>
<tr>
<td>pV-EYXhoI-F</td>
<td>5’-TCGACTCGAGATGGCCTCTCTCGGTGATTTAAAG-3’</td>
</tr>
<tr>
<td>pV-EYBamHI-R</td>
<td>5’-TATAGGATCCAAACGGGAGCCCGGTTACTGTG-3’</td>
</tr>
<tr>
<td>Bncl1117-S</td>
<td>5’-GATCCCCGCTTGGAAACTCAGTTATTCAAGAGATAA</td>
</tr>
<tr>
<td></td>
<td>ACCAGTGAAGTTCAAGGCTTTTTGGAAA-3’</td>
</tr>
<tr>
<td>Bncl1117-AS</td>
<td>5’-AGCTTTTCTTGGAAACTCAGTTATTCAAGAGATAA</td>
</tr>
<tr>
<td></td>
<td>GAATTTAAACCAGTGAAGTTCAAGGCTTTTTGGAAA-3’</td>
</tr>
<tr>
<td>Bncl1298-S</td>
<td>5’-GATCCCCGCAAGGATGGAAAGATGAAAGTTCAAGAGACT</td>
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<td></td>
<td>TTACTCTTTTCTTTCATTTGCTTTTTGGAAA-3’</td>
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<tr>
<td>Bncl1298-AS</td>
<td>5’-AGCTTTTCTTGGAAACTCAGTTATTCAAGAGATAA</td>
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<td>GAATTTAAACCAGTGAAGTTCAAGGCTTTTTGGAAA-3’</td>
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<tr>
<td>Bncl1453-S</td>
<td>5’-GATCCCCGCTTGGAAAGAACAGCATTGCTTCAAGAGAC</td>
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<tr>
<td>Bncl1453-AS</td>
<td>5’-AGCTTTTCTTGGAAACTCAGTTATTCAAGAGATAA</td>
</tr>
<tr>
<td></td>
<td>GAATTTAAACCAGTGAAGTTCAAGGCTTTTTGGAAA-3’</td>
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with 5% goat serum, and then incubated with rabbit anti-serum and Alexa Fluor 647-conjugated goat anti-rabbit antibody (Jackson Immunoresearch). Finally, the cells were mounted using mounting buffer (Vector Laboratories Inc.) with DAPI and imagined under the confocal microscope TCS SP5 (Leica).

7.2.7 CsCl gradient centrifugation

Monolayers of MDBK cells in T-150 Flasks were infected with BAV304a or BAV.ST at a multiplicity of infection of 5. At 48 h post-infection, the cells were collected, freeze-thawed three times and the cell lysates was subjected to CsCl density gradient centrifugation at 35 000 rpm for one hour at 4°C. The bands containing viruses were collected and subjected to a second centrifugation at 35 000 rpm for overnight at 4°C. Finally, the virus band was collected, dialyzed three times to remove the trace amount of CsCl and stored in small aliquots at -80°C.

7.2.8 Virus single-cycle growth curve

Monolayers of MDBK cells in 24-well plate were infected with BAV304a or BAV.ST at a MOI of 1. At indicated times post-infection, the infected cells were harvested, lysed by freeze-thawing three times to released the virus into medium and used to determine virus titers by TCID$_{50}$ as described (Kulshreshtha et al., 2004).

7.2.9 Analysis of protein expression

Monolayers of MDBK in six-well plate were infected with BAV304a or recombinant BAV.ST at a MOI of 1. At 24h post-infection, the cells were harvested and
probed by Western blot using protein-specific rabbit antisera or mouse anti-β-actin (Sigma) as primary antibodies, Alexa Fluor 680 goat anti-rabbit (Rockland) or IRDye 800 goat anti-mouse (Invitrogen) as secondary antibodies. Finally, the membranes were imaged and analyzed by using the Odyssey infrared imaging system (Licor).

### 7.2.10 Purification and identification of Strep-tagged protein complexes

MDBK cells were infected with BAV304a or BAV.ST at a MOI of 5. At 24 hour post-infection, the cells were washed with PBS and lysed with cell lysis buffer (Cell signaling technologies) supplemented with phenylmethylsulfonyl fluoride (PMSF). The cell lysates were centrifuged at 14,000×g for 15 min at 4 °C. Supernatant was collected and subjected to the strep-tagged protein complex purification by using Strep-tactin sepharose according to the manufacturer’s instruction with minor modifications. Briefly, the Strep-tactin sepharose was washed extensively with buffer W (100 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA), followed by the incubation of the supernatant and the prewashed sepharose at 4 °C. After overnight incubation, the lysate-sepharose mixture was transferred to a polypropylene column. After non-specific binding proteins were washed off thoroughly with buffer W, proteins attached to the sepharose were eluted with buffer E (buffer W containing 2.5 mM desthiobiotin). Finally, the protein samples were concentrated with an MWCO 10 kDa centrifugal filter (Millipore) and sent for mass spectrometry by LC-MS/MS.
7.2.11 Co-immunoprecipitation

MDBK cells were infected with mutant BAdV-3s at MOI of 2. At 24 hrs post-infection, the cells were washed with PBS and lysed with cell lysis buffer supplemented with PMSF. After centrifugation at 14,000×g for 15 min at 4 °C, the supernatant was incubated with anti-pV serum and protein A sepharose beads (GE Healthcare) overnight at 4 °C. After extensive washes to remove the non-specific binding, the proteins attached to the beads were eluted, and subjected to SDS-PAGE and detected by Western blot using C23 antibody (MS-3) (Santa Cruz Biotechnology) and IRDye800 Conjugated goat anti-mouse antibody (Rockland).

7.2.12 Nucleolin knockdown

To knockdown the expression of nucleolin, the second generation replication defective lentivirus system was used (Du and Tikoo, 2010). Briefly, HEK293T cell were co-transfected with plasmids pRDI292-1117+pSPAX2+pMD2G DNAs, plasmids pRDI292-1298+pSPAX2+pMD2G DNAs or plasmids pRDI292-1453 + pSPAX2 + pMD2G DNAs. At 48 hrs post-transfection, the lentivirus in media was collected and used to transduce CRL cells in the presence of 8ug/ul polybrene. At 24 hrs post-transduction, the transduced cells were transferred to 10 cm² dishes. After 24 hrs, the transduced cells were cultivated in medium containing 5ug/ml of puromycin. The puromycin-resistant cell clones were picked, propagated in puromycin-containing media and tested by Western blot using C23 antibody (MS-3) (Santa Cruz Biotechnology).
7.3 RESULTS

7.3.1 Construction of BAdV-3 with strep-tagged pV

To determine if pV interacts with other viral or cellular proteins during viral infection, we attempted to isolate recombinant BAdV-3 containing Strep-Tag II motif (WSHPQFEK) inserted in pV Orf. Since deletion of amino acid 20-50 of pV did not alter the biological properties of BAdV-3 (Zhao and Tikoo, 2016, manuscript in preparation), we constructed a plasmid pUC304A.pVstrep containing full length genomic DNA of BAV304a with insertion of Strep-tag motif between amino acid 1819 of pV Orf. The transfection of VIDO DT1 cells with 5-7.5ug of pUC304A.pVstrep DNA produced cytopathic effects in 9 days (Fig 7.1). The cells showing cytopathic effects were collected; freeze-thawed and recombinant virus named BAV.ST was propagated in MDBK cells.

The presence of Strep-tag in pV was confirmed by DNA sequencing and restriction endonuclease (RE) analysis of BAV.ST genomic DNA. The DNAs isolated from MDBK cells infected with BAV.ST or BAV304a were digested with BglII (since an additional BglII recognition site was introduced into the viral genome of BAV.ST) and analyzed by agarose gel electrophoresis. As shown in Fig 7.2A, two DNA fragments of 6.1 kb and 1 kb were detected in BAV304a genome. In contrast, two DNA fragments of 5.1 kb and 1 kb were detected in BAV.ST (Fig 7.2A).

The expression of recombinant protein in infected cells was analyzed by Western blot analysis using anti-pV serum. As seen in Fig. 7.2B, a 55 kDa protein was detected in both BAV304a and BAV.ST infected cells.
7.3.2 Characterization of BAV.ST

To determine if insertion of Strep-tag in BAdV-3 pV affects the replication of BAV.ST, we performed single step growth assay. Briefly, monolayers of MDBK cells were infected with BAV304a or BAV.ST at a MOI of 2. At indicated times post-infection, the infected cells were collected, freeze-thawed three times, and virus titers were determined in MDBK cells using TCID$_{50}$ assay. As seen in Fig 7.3 BAV304a or BAV.ST exhibited similar growth characteristics.

Since alterations in pV expression can affect the expression of other viral proteins in infected cells without affecting the incorporation of the viral proteins in progeny virions (Zhao and Tikoo, 2016, manuscript in preparation), we determined the effect of the Strep-tag insertion in pV on the expression of early and late viral proteins in BAV304a or BAV.ST infected MDBK cells by Western blot using protein specific sera. As seen in Fig 7.4, the expression of DNA binding protein, an early protein appeared similar in BAV304a or BAV.ST infected cells. Hexon, Fiber, pV and pX in BAV.ST infected cell was slightly lower in BAV304a infected cells by approximately 10%-25%. However, expression of 100K and pVII was significantly decreased by 70% and 80%, respectively.

The subcellular localization of pV-ST was examined by confocal microscopy. CRL cells were firstly transfected with pDsRed-B23, which expresses a fusion protein composed of a red fluorescent protein DsRed and a nucleolar marker B23. At 48 hours post-transfection, cells were infected with BAV304a or BAV.ST at a MOI of 1. At 24 hours post-infection, cells were fixed with 3.7% paraformaldehyde, and then pV or pV-ST were stained with anti-pV serum and Alexa Fluor 647-conjugated goat anti-rabbit IgG,
Figure 7.1 Isolation of pV deleted BAdV-3. A) Schematic diagram of indicated plasmid DNA. Thick black box represents BAdV-3 genomic DNA. Red box represents pV with Strep-tag. Thin lines represent plasmid DNA. The human cytomegalovirus immediate early promoter (CMV), enhanced yellow fluorescent protein gene (EYFP), ampicillin resistance gene (Amp), early region 3 (E3) and pV location is depicted. B) Direct fluorescence. Monolayer of VIDO DT1 cells (Du and Tikoo, 2010) were transfected with 7.5µg indicated plasmid DNAs and visualized for the expression of EYFP and development of cytopathic effects using fluorescent microscope TCS SP5 (Leica).
Figure 7.2 Identification of BAV.ST. A) Restriction enzyme analysis of BAV304a or BAV.ST genomes. The viral DNAs were extracted from VIDO DT1 cells infected with BAV304a (lane 1) or BAV.ST (lane 2), digested with BglII and analyzed by agarose gel electrophoresis. Lane M, GeneRuler 1kb DNA ladder (Thermo Fisher Scientific) was used for sizing the viral DNA fragments. Diagnostic bands are indicated with white arrows. Sizes of markers are shown on the right of the panel. B) Western blot. Proteins from the lysates of BAV304a infected VIDO DT1 cells (lane 1), BAV.ST infected VIDO DT1 cells (lane 2) or uninfected VIDO DT1 cells (lane 3) were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed in Western blot by using rabbit anti-pV anti-serum and AP-conjugated goat anti-rabbit antibody (Jackson Immunoresearch). The position of the molecular weight in kDa is shown on the left of the panel. The molecular weight in kDa of the observed protein is shown on the right of the panel.
Figure 7.3 Virus titers. Monolayers of MDBK cells were infected with BAV304a or BAV.ST. At different time points post infection, infected cells were collected, freeze-thawed and titrated on MDBK cells as described. Values represent averages of two independent repeats and error bars indicate the standard deviations.
**Figure 7.4 Analysis of gene expression in BAV.ST infected cells.** A) Proteins from lysates of MDBK cells infected with BAV304a (Lane 1) or BAV.ST (Lane 2) were separated by 10% SDS-PAGE, transferred to nitrocellulose and probed with protein specific antisera and Alexa Fluor 680 conjugated goat anti-rabbit antibody (Invitrogen). β-actin was used as a loading control and was detected using anti-β-actin monoclonal antibody (Sigma-Aldrich) and IRDye800 Conjugated goat anti-mouse antibody (Rockland). Protein names are depicted on the right of the panel. E (Early), L (Late), DBP (DNA binding protein). B) The values were analyzed by using Odyssey® CLx Imaging System (LI-COR). Values represent averages of two independent repeats and error bars indicate the standard deviations.
followed by analysis of confocal microscopy. As seen in Fig 7.5, pV-ST was still predominantly located in nucleolus after the introduction of strep tag, and there was no detectable difference between pV and pV-ST cellular localization.

To determine the incorporation of structural proteins in the progeny virions, proteins from purified virions were separated by 10% SDS-PAGE, transferred to nitrocellulose and probed in Western blot using protein specific sera. As seen in Fig 7.6, there was no detectable difference in the incorporation of the viral proteins in purified BAV304a or BAV.ST virions.

7.3.4 Purification and identification of cellular/viral proteins interacted with pV

After construction and characterization of recombinant BAV.ST, Strep-tag introduction did not influence viral replication significantly. Therefore, the newly constructed BAV.ST was used to determine cellular/viral proteins interacting with pV. To purify cellular/viral proteins interacted with pV, MDBK cells were infected with BAV304a or BAV.ST at the MOI of 1. At 24 hour post-infection, infected cells were lysed and cell lysates were subjected to protein purification using Strep-tactin sepharose beads. Proteins in the purified samples were separated by SDS-PAGE followed by silver staining of the gel. As shown in Fig 7.7, protein bands were detected after the silver staining in lanes with 10, 20 and 40 μl of the protein samples purified from BAV.ST infected MDBK; while no band was found in lanes of protein samples purified from BAV304a infected MDBK cells.

To identify these cellular/viral proteins purified from BAV.ST infected MDBK cells, protein complexes were sent to University of Victoria-Genome BC Proteomics
Figure 7.5 Confocal microscopy. VIDO DT1 cells were transfected with plasmid pDsRed.B23 DNA and infected with indicated BAdV-3s. Infected cells were fixed at 48 hrs post-infection. The DsRed.B23 was visualized by direct fluorescence microscopy (panels b, f). BAdV-3 pV (panels c, g) was visualized by indirect immunofluorescence microscopy using anti-pV serum and Alexa Fluor 647-conjugated goat anti-rabbit IgG. The nuclei were stained with DAPI.
**Figure 7.6 Structural protein incorporation assay.** Structural proteins from the purified BAV304a (lane 1) or BAV.ST (lane 2) grown in MDBK cells were separated by 10% SDS-PAGE, transferred to nitrocellulose and probed by Western blot using protein specific antisera. Protein bands were visualized by Odyssey® CLx Imaging System (LI-COR). Protein names are depicted on the right of the panel.
Centre for LC-MS/MS. Cellular or viral proteins with different functions and cellular localizations were detected from these protein complexes. Seventeen cellular proteins (ribosomal proteins excluded), whose scores were higher than 200, were listed in Table 7.2. In these identified proteins, the most abundance ones were carboxylase and proteins implicated with gene expression, like Y box binding protein, poly A binding protein, DDX21 and UPF1, which suggesting pV may play a function in cellular/viral transcription regulation. Three nucleolar proteins were detected: nucleolin, DDX21 and LYAR, suggesting pV may play a function in nucleolus or ribosome biosynthesis regulation. Trypsinogen, drebrin 1, histone H2A, Uracial-DNA glycosylase superfam and MYH9 are also presented in the protein sample. In Table 7.3, there were 16 ribosomal proteins whose scores were more than 200 were identified form the protein complexes, suggesting pV may be involved in ribosome biosynthesis or translation regulation. Including pV, six viral proteins, whose scores were higher than 100, were detected and listed in Table 7.4.

7.3.5 Interaction between nucleolin and pV

Among the identified cellular or viral proteins, we are particular interested in nucleolin. Since nucleolin has been identified from the protein complexes purified form BAV.ST infected MDBK cells with a high score, we suspected that pV can interact with nucleolin and this interaction may play a function in viral replication. To confirm this interaction, MDBK cells infected with BAV304a or BAV.dV were harvested at 24 hours post-infection, and the cell lysates were subjected immunoprecipitation with anti-pV serum and Western blot with anti-NCL antibody (C23 antibody, Santa Cruz Biotech). As
Figure 7.7 Silver staining of proteins interacting with pV. Proteins were purified from the BAV304a (Lane 3, 5, 7) or BAV.ST (Lane 2, 4, 6) infected MDBK cells by streptactin sepharose beads (Ginisty et al.). 10 μl (Lane 2, 3), 20 μl (Lane 4, 5) or 40 μl (Lane 6, 7) of purified protein samples were separated by 10% SDS-PAGE, and the gel was stained with silver staining. Lane 1, PageRuler Prestained Protein Ladder (Thermo Fisher Scientific) was used for sizing the protein bands. The position of the molecular weight in kDa is shown on the left of the panel.
### Table 7.2 Cellular proteins identified from LC-MS/MS

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### Table 7.3 Ribosomal proteins identified from LC-MS/MS

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Table 7.4 Viral proteins identified from LC-MS/MS

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shown in Fig 7.8A, a band indicating nucleolin was detected with anti-NCL antibody in BAV304a infected MDBK sample immunoprecipitated with rabbit anti-pV serum, while no such band detected neither in BAV304a infected MDBK sample immunoprecipitated with pre-bleed rabbit serum nor in BAV.dV infected MDBK sample immunoprecipitated with rabbit anti-pV serum.

To examine if pV and nucleolin share the same cellular localization, plasmid pEY-pV (expressing fluorescent protein EYFP fused with pV) and plasmid pDsRed.B23 (expressing fluorescent protein DsRed fused with nucleolin) were used individually/together to transfect/co-transfect Vero cells. Transfected cells were fixed at 24-hour post-transfection and followed by imagined under the confocal microscope TCS SP5 (Leica). As shown in Fig 7.8B, both pV and nucleolin were localized in the nucleolus in single plasmid transfected and double plasmids co-transfected cells.

7.3.6 Knockdown of Nucleolin expression

In previous reports, nucleolin knockdown was used to study its role in viral replication (Oksayan et al., 2015; Su et al., 2015); herein we also tried to knockdown nucleolin to investigate the functions of nucleolin in BAdV-3 replication. Lentiviruses expressing shRNAs targeting bovine nucleolin mRNA were produced in transfected HEK293T cells and used to transduce MDBK cells. The difference of nucleolin expression between MDBK and shRNA expressing lentivirus transduced MDBK was detected by Western blot. As shown in Fig 7.9, compare to MDBK cells, no significant decrease of nucleolin expression was detected in lentiviruses transduced MDBK cells.
Figure 7.8 The interaction between pV and nucleolin. A) Co-IP. Lysates of MDBK cells infected with BAV304a (Lane 1, 2, 3) or BAV.dV (Lane 4, 5, 6) were immunoprecipitated with anti-pV serum (Lane 1, 4) or pre-bleed serum (Lane 2, 5), followed by Western blot using C23 antibody (MS-3) (Santa Cruz Biotechnology) and AP-conjugated goat anti-rabbit antibody (Jackson Immunoresearch). B) Confocal microscopy. Vero cells were transfected with indicated plasmid DNAs. Transfected cells were fixed at 48 hrs post-transfection. DsRed-NCL and EYFP-pV were visualized by direct fluorescence microscopy. The nuclei were stained with DAPI.
**Figure 7.9 Nucleolin knockdown assay.** Proteins of MDBK or lentivirus transduced MDBK were separated by 10% SDS-PAGE, transferred to nitrocellulose and probed by Western blot using C23 antibody (MS-3) (Santa Cruz Biotechnology) and IRDye800 Conjugated goat anti-mouse antibody (Rockland). β-actin was used as a loading control and was detected using anti-β-actin monoclonal antibody (Sigma-Aldrich) and IRDye800 Conjugated goat anti-mouse antibody (Rockland). Protein bands were visualized by Odyssey® CLx Imaging System (LI-COR). Protein names are depicted on the right of the panel. Lentiviruses used to transduce MDBK are depicted on the left of the panel.
7.3.7 Interactions between nucleolin and pV mutants

Accumulation of nucleolar proteins in the nucleolus results from their interactions with nucleolar components, such as ribosome RNA, DNA, and proteins (Carmo-Fonseca et al., 2000). The nucleolar localization signal, or called nucleolar retention signal, is responsible for nucleolar retention of nucleolar proteins (Musinova et al., 2011). To investigate if the pV nucleolar localization is because of its interaction with nucleolin, interactions between nucleolin and pV mutants were detected by co-immunoprecipitation. Briefly, MDBK cells were infected with recombinant BAdV-3s, and infected cell lysates were immunoprecipitated with anti-pV serum, and followed by Western blotting using anti-NCL antibody (C23 antibody, Santa Cruz Biotech). As shown in Fig 7.10, like BAV304a, anti-NCL antibody detected bands around 100 kDa representing nucleolin in all three recombinant BAdV-3s infected MDBK cells when anti-pV serum was used for immune-precipitation. However, no such band was detected in lanes when pre-bleed serum was used for immunoprecipitation.
Figure 7.10 The interaction between NoLSs deleted pV and nucleolin. Lysates of MDBK cells infected with BAV304a, BAV.pVd1, BAV.pVd2 or BAV.pVd1d2 were immunoprecipitated with anti-pV serum (Lane 1) or pre-bleed serum (Lane 2), followed by Western blot using C23 antibody (MS-3) (Santa Cruz Biotechnology) and IRDye800 Conjugated goat anti-mouse antibody (Rockland).
7.4 DISCUSSION

BAdV-3 pV is a structural protein and bridges the viral capsid and nucleic acid core (Russell, 2000), but its function in adenovirus replication is still poorly understood since only few studies regarding pV functions have been reported (Matthews and Russell, 1998a, b; Samad et al., 2007). In addition, adenovirus replicates and assembles in nucleus while pV has been demonstrated to be predominantly in nucleolus by our previous study (section 3.3) and others (Matthews and Russell, 1998a, b; Puntener et al., 2011; Ugai et al., 2012), also suggesting pV may play a role in nucleolus to optimize BAdV-3 replication rather than just being a structural protein.

To purify and identify proteins interacting with pV, a Strep-tag II was introduced into pV Orf to construct a recombinant BAdV-3. Strep-tag II is a peptide of eight amino acids (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) reversibly interacts with a variant streptavidin Strep-tactin (Schmidt and Skerra, 2007; Voss and Skerra, 1997), thus makes it is easy to pull-down Strep-tag II by Strep-tactin sepharose beads. The Strep-tag is often used as a tag for protein purification because it does not influence protein folding or secretion as well as protein functions (Schmidt and Skerra, 2007). Compare to His-tag or Flag-tag, Strep-tag II is suitable for purification and analysis of functional protein (Lin et al., 2012). Therefore, after the introduction of Strep-tag into pV Orf, a recombinant BAdV-3 expressing strep-tagged pV was rescued from VIDO DT1 cells and the cellular localization of Strep-tagged pV was the same as the wild-type pV in infected cells. Moreover, the introduction of Strep-tag just slightly decreased the expression of late proteins, and viral replication characteristics were reduced gently after the introduction of Strep-tag in pV Orf. The Strep-tag II was inserted between pV amino acids 18EI19. The
first reason for this position is that it locates in a hydrophilic region (data not shown), which ensures the Strep-tag exposed on the surface of pV and accessible to its ligand Strep-tactin, thereby allowing the purification of protein complexes containing strep-tagged pV from the infected cell lysates, and it is also because the amino acids 21-50 are dispensable for BAdV-3 replication (Section 3.3) so that the BAdV-3 replication will not be influenced by the introduction of Strep-tag II.

LC-MS/MS result revealed many cellular/viral proteins that may associate with pV. In those five viral proteins identified from the Strep-tactin purified protein complexes, two proteins, protein 33K and pVII, were detected to interact with pV. This is in agreement with the previously studies of pV in BAdV-3 or HAdV-5 (Harpst et al., 1977; Kulshreshtha et al., 2004). 100K was the most abundant viral protein identified form the protein complexes. Although there was no study reported its interaction with pV, interactions of 33K with 100K or pV have been proved by our previous study (Kulshreshtha et al., 2004), suggesting that these three viral proteins may form a complex in the process of BAdV-3 replication. Mass spectrometry identified IVa2 is associated with pV for the first time. Several possibilities regarding this interaction can be expected. IVa2 may indirectly interact with pV via other viral proteins since both 33K (Ahi et al., 2015) and pVII (Zhang and Arcos, 2005) have been proved to interact with both pV and IVa2. Another possibility for this interaction is that it depends on viral DNA since pV binds to viral DNA in a sequence-independent manner in encapsidation (Matthews and Russell, 1998a, b), while IVa2 binds to viral DNA for its packaging (Zhang and Imperiale, 2003) and major late promoter activation (Tribouley et al., 1994). Moreover, both pV and IVa2 are localized in the nucleolus (Lutz et al., 1996; Matthews and Russell,
These findings suggest that they may form a complex in the nucleolus or indirectly interact with each other via nucleolar components. Another viral DNA binding protein, pTP, was detected to interact with pV, which may also be because of its binding to viral DNA (Mysiak et al., 2004).

Several proteins involved in gene expression regulation were identified by mass spectrometry. Nuclease-sensitive element-binding protein 1, or Y box binding protein 1 (YBX-1), was identified to interact with pV. YBX-1 is a multifunctional protein (Eliseeva et al., 2011). It has been proved essential for oncolytic adenovirus in tumor control (Mantwill et al., 2013), and to enhance E2 gene expression (Holm et al., 2002). However, the function of the interaction between pV and YBX-1 is still unclear. Another protein, dead box helicase 21 (DDX21), also intrigued us because of its function in promoting AP-1 activity in innate immunity (Zhang et al., 2014) and inhibiting virus replication (Chen et al., 2014), which suggest pV may be involved in inhibiting host innate immunity. Indeed, pV has been proved to inhibit the expression of type I interferon and phosphorylation of interferon regulatory factor 3 (Data not shown). In addition, some proteins, such as carboxylase, poly A binding protein, UPF1, LYAR and some ribosomal proteins, were firstly detected to interact possibly with pV, but the functions of these interactions remain to be determined. Although B23 interacts with HAdV-5 pV in infected cells (Samad et al., 2007), BAdV-3 pV does not appear to be interacting with B23. This may explain why B23 is not redistributed in pV expressing cells.

Nucleolin is one of the most abundant nucleolar proteins and plays multiple functions in different cellular processes, such as ribosome synthesis (Rickards et al., 2007), transcription (Grinstein et al., 2007), DNA metabolism (Jiang et al., 2014; Lim et
al., 2015; Wang et al., 2014a), and cell cycle regulation (Jiang et al., 2014; Lim et al., 2015; Wang et al., 2014a). In the ribosome synthesis, nucleolin is involved in both rDNA transcription (Rickards et al., 2007) and pre-rRNA processing (Ginisty et al., 1998). Nucleolin has been proved to interact with both the UCGA motif of the pre-rRNA (Ginisty et al., 2000) and the U3 small nucleolar ribonucleoprotein (Kass et al., 1990), which is essential for the first cleavage of the pre-rRNA processing. Therefore, the pre-rRNA may recruit the U3 small ribonucleoprotein via its interaction with nucleolin and the interaction of nucleolin with U3. Recently, nucleolin has been found to facilitate the replication of some viruses (Cancio-Lonches et al., 2011; Oksayan et al., 2015; Su et al., 2015; Tayyari et al., 2011). Both HAdV-5 (Lawler et al., 1989) and BAdV-3 (Paterson, 2010) infection can inhibit the pre-rRNA processing, while the 52K, a BAdV-3 nonstructural protein, is not involved in inhibition of pre-rRNA processing (Paterson, 2010).

BAdV-3 pV, a nucleolus localized protein, interact with nucleolar protein nucleolin as determined by the mass spectrometry data and co-immunoprecipitation. Unlike HAdV-5 (Matthews, 2001), the interaction and localization of both pV and nucleolin to nucleolus does not lead to redistribution of nucleolin to the cytoplasm. A possible explanation for this is that pV and nucleolin may competitively bind to the same nucleolar component in HAdV-5, but not BAdV-3, infected cells. Nucleolar localization signals deleted pV can interact with nucleolin in infected cells, suggesting that pV motif interacting with nucleolin is different from its nucleolar localization signals and the nucleolar localization of pV is not via its interaction with nucleolin. Since both pV and
nucleolin are also localized in the nucleoplasm (Matthews, 2001), their interaction may occur, at least partially, in the nucleoplasm.
8.0 GENERAL DISCUSSION AND CONCLUSION

The inherent property of viruses to efficiently deliver genetic material to eukaryotic cells has led to the development of viruses as gene delivery vectors (Choi and Chang, 2013; Draper and Heeney, 2010). Based on the successful development of human adenoviruses as a vector for humans (Draper and Heeney, 2010; Wold and Toth, 2013), animal species-specific adenoviruses are being evaluated as gene delivery vehicles for animals (Hammond et al., 2001; Mittal et al., 1995; Singh et al., 2008; Wright et al., 2013). Our laboratory is evaluating bovine adenovirus-3 as a vaccine delivery vehicle for vaccination of cattle (Baxi et al., 2000; Brownlie et al., 2015; Reddy et al., 1999b; Zakhartchouk et al., 1998) and have demonstrated the potential of using replication-competent bovine adenovirus-3 as vaccine delivery vector in cattle (Baxi et al., 2000; Brownlie et al., 2015; Reddy et al., 1999b; Zakhartchouk et al., 1998). However, use of replication competent BAdV-3 vectors may raise certain regulatory concerns in future for registration of BAdV-3 based vaccines for use in the field. Thus, to exploit the potential of using BAdV-3 as vaccine delivery vehicle in cattle, safer and more efficient vaccine vector based on BAdV-3 has to be developed. One way to achieve this is by developing disabled infectious single cycle (DISC) BAdV-3 as vector. DISC virus vectors are safer than replication competent virus vectors and more efficacious than replication defective vectors due to the expression of antigen gene. To develop a safe and efficient DISC vaccine vector based on BAdV-3, elucidation of structure and function of structural and non-structural proteins of BAdV-3 is essential (Boursnell et al., 1998; Rees et al., 2002). Earlier, analysis of BAdV-3 DNA sequence predicted to encode a protein of 423 amino acids, which showed variable degree of sequence identity (28%-41%) to pV encoded by
other members of *Mastadenovirus* (Reddy et al., 1998). The main objective of the present work was to elucidate the function of BAdV-3 core protein pV in virus replication, including a) characterizing the protein and its distributions in transfected infected cell; b) analyzing the effect of pV deletion on viral replication; and c) determining the interaction of pV with other viral\cellular proteins.

Like other BAdV-3 late proteins (Kulshreshtha et al., 2004; Makadiya et al., 2015; Wu and Tikoo, 2004; Zhou et al., 2001), anti-pV sera raised against peptides representing amino acids \(^{1}\text{MASSRLIKEEMLDIVAPEIYKRKR}^{24}\) and \(^{180}\text{SRKRGVGKVEPTIQVLA SKKRRMA}^{212}\) detected a protein of 55 kDa at late times post infection. Analysis of the intracellular distribution of protein demonstrated that pV localized predominantly in the nucleolus of the transfected and BAdV-3 infected cells. Deletion analysis identified redundant nucleolar localization signals residing in amino acid 21-50 and 380-389 and redundant NLS residing in amino acids 80-120, 190-210 and 380-389. Although the presences of both separate and overlapping NLS and NoLs have been reported in other proteins, including viral proteins (Li et al., 2011a; Mori et al., 2005), their individual contribution has not been investigated. It will be interesting to determine the individual contribution of NoLS motifs by constructing and analyzing recombinant BAdV-3 expressing pV containing mutations in identified NoLS.

Unlike primary cell, pV is not essential for the replication of HAdV-5 in cancer cells, due to the introduction of compensatory mutations in another protein pX\Mu protein (Ugai et al., 2007). In contrast, BAdV-3 pV appears essential for proper assembly of progeny virus in primary and continuous cell lines. This could be due to the difference in protein structure and possible function of pV or pX\Mu of HAdV-5 and
BAdV-3. The amino acid sequence of pV of BAdV-3 and HAdV-5 show low (41%) homology (Reddy et al., 1998). Moreover, amino acids involved in introducing compensatory mutations show only 50% homology between pX\Mu of BAdV-3 and HAdV-5 (Reddy et al., 1998). Alternatively, this could be due to difference in the cell lines use for the rescue and propagation of pV deleted BAdV-3 and HAdV-5.

Earlier, proteins encoded by adenovirus late genes have been shown to be able to regulate expression of other late genes (Morris and Leppard, 2009; Wright et al., 2015; Wu et al., 2013) by acting on major late promoter (Wu et al., 2013) tripartite leader sequence or processing of viral mRNAs. Like HAdV-5 (Ugai et al., 2007), BAdV-3 pV appears to be involved in the regulation of selected late gene expression. Further studies should explore the mechanism involved in regulating the expression of selected late genes.

Localization of pV predominantly in the nucleus \nucleolus suggests that pV uses active import pathways. Unlike BAdV-3 33K (Kulshreshtha et al., 2014) which uses both classical Imp α\β and transportin dependent nuclear import pathway, the nuclear transport of pV appears to be mediated only by classical Imp α\β heterodimer dependent pathway by interacting with Imp α3. The active nuclear transport of pV mediated by Imp α3 requires at least one of the three identified NLS motifs of pV. Interestingly, the nuclear import of BAdV-3 52K (Paterson et al., 2012) and pVIII (Ayalew et al., 2014) also appears to be mediated by interacting with Imp α3 of classical Imp α\β dependent pathway.

Earlier reports demonstrated the relationship between nucleolar localization of a viral protein and viral replication (Li et al., 2011a). Depending on the virus, nucleolar
localization of a viral protein may (Li et al., 2011a) or may not be (Boyn and Whitehouse, 2006; Lee et al., 2006b; Li et al., 2011a) essential for viral replication. Our results demonstrate that nucleolar localization of pV appears essential for production of infectious BAdV-3. Analysis of the mutant BAdV-3 viruses revealed alteration in the expression of some late viral proteins particularly 100K and hexon, and changes in virus thermostability. It is possible that decrease in the expression of 100K may lead to less production of hexon (Hayes et al., 1990) or impaired transport of hexon trimerization and nuclear transport to the nucleus (Hong et al., 2005), thus leading to formation of reduced amount of capsids (Fig 3.9A). Secondly, decreased thermostability of mutant viruses, particularly BAVd1d3, appears to produce fragile capsids prone to disintegration and leading to the production of non-infectious progeny virions.

Protein-protein interaction appears to play important functions in adenovirus replication (Arnberg, 2012; Gustin et al., 1996; Matthews and Russell, 1994; Ostapchuk et al., 2005) including BAdV-3 (Ayalew, 2014; Kulshreshtha and Tikoo, 2008). Previous report suggested that 100K and 33K interact with pV in BAdV-3 infected cells (Kulshreshtha and Tikoo, 2008). Mass spectrophotometry analysis of proteins purified from recombinant BAV.ST infected cells using Strep-actin sepharose beads detected a number of cellular and viral proteins that may have the potential of interacting with BAdV-3 pV. The validity of our approach was confirmed by demonstrating the interaction of one of the identified proteins named nucleolin with pV in BAdV-3 infected cells.

Nucleolin is involved in different steps of virus replication (Calle et al., 2008; Cancio-Lonches et al., 2011; Oksayan et al., 2015; Shimakami et al., 2006) and cellular
processes including ribosomal RNA processing (Ginisty et al., 1998). Earlier, we have demonstrated that ribosomal RNA processing is altered at late times post infection of BAdV-3 infected cells (Paterson, 2010). We postulate that interaction of BAdV-3 pV with nucleolin may play a role. As such future work will elucidate the role of pV-nucleolin interaction in ribosomal RNA processing in BAdV-3 infected cells.
9.0 REFERENCES


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APPENDIX A PLASMIDS USED IN SECTION 3.0

Plasmid pDsRed.B23 (expression a fusion protein DsRed and nucleolar marker B23) was a gift from Dr Denis Archambault.

Plasmid pCMVGbeta expresses a fusion protein of GFP/β-galactosidase

a) Plasmid pcV

A 1292-bp DNA fragment was amplified by PCR using primers pV-EcoRI-F and pV-XhoI-R (Table A1), and pUC304A+ as a DNA template. A 1272-bp EcoRI-XhoI fragment of the 1292-bp DNA fragment was ligated to a 5413-bp EcoRI-XhoI DNA fragment of plasmid pcDNA3 to create pcV.

b) Plasmid pcV.d2

A 599-bp DNA fragment was amplified by PCR using primers pV-EcoRI-F and d(190-210)-F1-R (Table A1), and plasmid pcV as a template. A 657-bp DNA fragment was amplified by PCR using primers d(190-210)-F2-F and pV-XhoI-R (Table A1), and plasmid pcV as a template. In the third PCR, two PCR fragments were annealed and used to amplify a 1299-bp DNA fragment by overlapping PCR using primers pV-EcoRI-F and pV-XhoI-R. A 1225-bp EcoRI-XhoI DNA fragment of the 1299-bp fragment was ligated to EcoRI-XhoI digested plasmid pcDNA3 to create plasmid pcV.d2.

c) Plasmid pcV.d3

A 1171-bp fragment was amplified by PCR using primers pV-EcoRI-F and d(380-389) F1-R (Table A1), and plasmid pcV as a template. A 592-bp fragment was amplified by PCR using primers d(380-389) F2-F and d(380-389)-F2-R (Table A1), and plasmid pcV as a template. In the third PCR, two PCR fragments were annealed and used to amplify a 1713-bp DNA fragment by overlapping PCR using primers EcoRI-F and
A 1247-bp EcoRI-XhoI fragment of 1713 bp fragment was ligated to EcoRI-XhoI digested plasmid pcDNA3 to create plasmid pcV.d3.

d) Plasmid pcV.d1d2

A 761-bp SnaBI band of pcV.d1 was isolated and ligated to SnaBI digested pcVd2 to create pcV.d1d2.

e) Plasmid pcV.d1d3, pcV.d2d3 and pcV.d1d2d3

A 552-bp AgeI-XhoI fragment of pcV.d3 was isolated and ligated to AgeI-XhoI digested pcV.d1, pcV.d2 and pcV.d1d2 to construct pcV.d1d3, pcV.d2d3 and pcV.d1d2d3, respectively.

f) Plasmid pcV.m1d3

A 984-bp DNA fragment was amplified by PCR using primers M-F and m1-F1-R (Table A1), and plasmid pcV.d3 as a template. An 1102-bp DNA fragment was amplified by PCR using primers m1-F2-F and pV-XhoI-R (Table A1), and plasmid pcV.d3 as a template. In the third PCR, two PCR fragments were annealed and used to amplify a 2043-bp DNA fragment by overlapping PCR using primers M-F and pV-XhoI-R. An 1144-bp EcoRI-XhoI DNA fragment of the 2043-bp fragment was ligated to EcoRI-XhoI digested plasmid pcDNA3 to create plasmid pcV.m1d3

g) Plasmid pcV.m2d3

A 996-bp DNA fragment was amplified by PCR using primers M-F and m2-F1-R (Table A1), and plasmid pcV.d3 as a template. A 1087-bp DNA fragment was amplified by PCR using primers m2-F2-F and pV-XhoI-R (Table A1), and plasmid pcV.d3 as a template. In the third PCR, two PCR fragments were annealed and used to amplify a 2043-bp DNA fragment by overlapping PCR using primers pV-M-F and pV-XhoI-R. An
1144-bp EcoRI-XhoI DNA fragment of the 2043-bp fragment was ligated to EcoRI-XhoI digested plasmid pcDNA3 to create plasmid pcV.m2d3.

**h) Plasmid pcV.m12d3**

A 987-bp DNA fragment was amplified by PCR using primers M-F and m12-F1-R (Table A1), and plasmid pcV.d3 as a template. A 1088-bp DNA fragment was amplified by PCR using primers m12-F2-F and pV-XhoI-R (Table A1), and plasmid pcV.d3 as a template. In the third PCR, two PCR fragments were annealed and used to amplify a 2043-bp DNA fragment by overlapping PCR using primers pV-M-F and pV-XhoI-R. An 1144-bp EcoRI-XhoI DNA fragment of the 2043-bp fragment was ligated to EcoRI-XhoI digested plasmid pcDNA3 to create plasmid pcV.m12d3.

**i) Plasmid pcV.m3d3, pcV.m13d3, pcV.m23d3 and pcV.m123d3**

Four 1060-bp DNA fragments were amplified by PCR using primers pV-M-F and m3-F1-R (Table A1), and plasmid pcVd380-380, pcVm1d380-380, pcVm2d380-380 or pcVm12d380-380 as templates. A 1024-bp DNA fragment was amplified by PCR using primers m3-F2-F and pV-XhoI-R (Table A1), and plasmid pcV.d3 as a template. In the third PCR, each fragment from the first PCR and the fragment from the second PCR were annealed and used to amplify four 2043-bp DNA fragments by overlapping PCR using primers pV-M-F and pV-XhoI-R. Each 1144-bp EcoRI-XhoI DNA fragments of these four 2043-bp fragments were ligated to EcoRI-XhoI digested plasmid pcDNA3 to create plasmid pcV.m3d3, pcV.m13d3, pcV.m23d3 and pcV.m123d3, respectively.

**j) Plasmid pcV.d4**

A 1157-bp DNA fragment was amplified by PCR using primers pV-EcoRI-F and d(380-423)XhoI-R (Table A1), and pcV as a template. A 1143-bp EcoRI-XhoI fragment
of the 1157-bp fragment was isolated and ligated to EcoRI-XhoI digested pcDNA3 to construct pcV.d4.

**k) Plasmid pcV.d5 and pcV.d6**

995-bp and 842-bp DNA fragments were amplified by PCR using primers pV-EcoRI-F and d(323-423)XhoI-R (Table A1), and pcV and pcV.d1d2 as template, respectively. 883-bp and 828-bp EcoRI-XhoI DNA fragments of the 995-bp and 842-bp DNA fragment, respectively, were ligated to the EcoRI-XhoI digested pcDNA3 to create pcV.d5 and pcV.d6, respectively.

**l) Plasmid pcV.d21-50/380-423, pcV.d18 and pcV.d12**

A 445-bp AgeI-XhoI fragment of pcV.d4 was isolated and ligated to AgeI-XhoI digested pcV.d1, pcV.d2 and pcV.d1d2 to create pcV.d21-50/380-423, pcVd18 and pcV.d12, respectively.

**m) Plasmid pcV.d10**

A 905-bp DNA fragment was amplified by PCR using primers d(1-100)EcoRI-F and pV-XhoI-R (Table A1), and pcV.d18as template. A 891-bp EcoRI-XhoI band of the 905-bp fragment was ligated to EcoRI-XhoI digested pcDNA3-HA to construct pcV.d10.

**n) Plasmid pcV.d9**

A 1130-bp DNA fragment was amplified by PCR using primers pV-M-F and d(101-210)-F1-R (Table A1), and plasmid pcV.d21-50/380-423 as a template. A 648-bp DNA fragment was amplified by PCR using primers d(101-210)-F2-F and d(380-423)XhoI-R (Table A1), and plasmid pcVd21-50/380-423 as template. In the third PCR, two PCR fragments were annealed and used to amplify a 1733-bp DNA fragment without overlapping PCR using primers pV-M-F and d(380-423)XhoI-R. An 834-bp EcoRI-
XhoI DNA fragment of the 1733-bp fragment was ligated to EcoRI-XhoI digested plasmid pcDNA3 to create plasmid pcV.d11.

**o) Plasmid pcV.d7 and pcV.d8**

590-bp and 500-bp fragments were amplified by PCR using primers pV-EcoRI-F and d(190-423)XhoI-R (Table A1), and pcV and pcV.d1, respectively, as templates. 576-bp and 486-bp EcoRI-XhoI bands of the 590-bp and 500-bp fragments, respectively, were ligated to EcoRI-XhoI digested pcDNA3 to construct pcV.d7 and pcV.d8, respectively.

**p) Plasmid pcV.d15 and pcV.d11**

1156-bp and 1066-bp DNA fragments were purified by PCR using primers M-F and d(81-120)-F1-R (Table A1), and pcV and pcV.d1, respectively, as templates. An 853-bp DNA fragment was amplified by PCR using primers d(81-120)-F2-F and pV-d(380-423)XhoI-R (Table A1), and plasmid pcV.d18 as a template. In the third PCR, each fragment from the first PCR and the fragment from the second PCR were annealed and used to amplify 1967-bp and 1877-bp fragments by overlapping PCR using primers pV-M-F and pV-d(380-423)XhoI-R. 1099-bp and 1009-bp BamHI-XhoI DNA fragments of the 1967-bp and 1877-bp fragments, respectively, were ligated to BamHI-XhoI digested plasmid pcDNA3 to create plasmid pcV.d15 and pcVd11, respectively.

**q) Plasmid pcV.d13 and pcV.d14**

A 641-bp SnaBI DNA fragment from plasmid pcV.d11 was isolated and ligated to SnaBI digested plasmid pcV.d4 and pcV.d2 to create pcV.d13 and pcVd14, respectively.

**r) Plasmid pcV.d19**

A 582-bp AgeI-XhoI DNA fragment from plasmid pcV.d15 was isolated and ligated to AgeI-XhoI digested pcV to create pcV.d15.
s) **Plasmid pcV.d17**

A 731-bp *SnaBI* fragment of plasmid pcV.d15 was isolated and ligated to *SnaBI* digested pcV.d4 to construct pcV.d17.

t) **Plasmid pcV.d16**

A 1895-bp DNA fragment was amplified by PCR using primers M-F and d(390-423)*XhoI*-R, and plasmid pcV.d19 as a template. A 1027-bp *BamHI*-*XhoI* band of the 1895-bp fragment was ligated to *BamHI*-*XhoI* digested pcDNA3 to create pcV.d16.

u) **Plasmid pNoLS1.EY**

A 112-bp DNA fragment was purified by PCR using primers NoLS1-EY-F and NoLS1-EY-R (Table A1), and pcV as a template. A 98-bp *BamHI*-SalI band of the 112-bp fragment was ligated to *BamHI*-SalI digested pEYFP-N1 to construct pNoLS1.EY.

v) **Plasmid pNoLS2.EY**

A 1237-bp DNA fragment was amplified by PCR using primers NoLS2-EY-F and NoLS2-EY-R (Table A1), and plasmid pEYFP-N1 as a template. A 772-bp *BamHI*-SalI band of the 1237-bp fragment was ligated to *BamHI*-SalI digested pEYFP-N1 to create pNoLS2.EY.

w) **Plasmid pNoLS1-GFPβGal**

A 116-bp DNA fragment was amplified by PCR using primers GB-NoLS1-BamHI-F and GB-NoLS1-NheI-R (Table A1), and plasmid pcV as a template. A 102-bp *BamHI*-NheI band of the 116-bp fragment was isolated and ligated to *BamHI*-NheI digested pCMVβg to create pNoLS1-GFPβGal.
### Table A1 List of primers used for PCR

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<th>Name</th>
<th>Sequence</th>
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<td>pV-EcoRI-F</td>
<td>5'- GGAGCCGAAATTTCATGGGCTCCTCTCTCGGTTGATTTAAGAAGG</td>
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<td>M-F</td>
<td>5'- TCTGCTCTGTA TGCCGCATAGTGAAGAC</td>
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<td>d(21-50)-F1-R</td>
<td>5'- CGGTTTTCTAGAGCCGGGTAATATCTCAGGGCCACAGA</td>
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<td>F1-R</td>
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