MOLECULAR CHARACTERIZATION OF 33K PROTEIN OF BOVINE ADENOVIRUS TYPE 3

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ABSTRACT

Bovine adenovirus type 3 (BAdV-3) is a non-enveloped icosahedral particle which contains a double stranded DNA genome. The genome of BAdV-3 is organized into early, intermediate and late regions. The late region is organized into seven regions L1-L7 (Reddy et.al., 1998). The L6 region of late transcription unit of BAdV-3 encodes one of the non structural protein named 33K protein. The objective of the present study was to characterize the 33K protein and to identify the viral/cellular proteins involved in the interaction with 33K protein.

The RT-PCR analysis revealed the presence of spliced and unsliced mRNAs encoding 33K and 22K proteins respectively in BAdV-3 infected cells. The 33K and 22K proteins share a N-terminus region of 138 amino acids. To determine the specificity of these two proteins, rabbit polyclonal antiserum was raised against peptides representing unique C- terminal regions of the proteins. Anti-33Kp serum detected two major proteins of 42 kDa and 22 kDa and five minor proteins of 39kDa, 35kDa, 29kDa, 25kDa and 19kDa in BAdV-3 infected cells or 33K transfected cells. Similarly, anti-22Kp serum detected three proteins of 41kDa, 39kDa and 37kDa in BAdV-3 infected cells. However, a protein of 39kDa and 37kDa was detected in 22K (having splice sites removed) transfected cells. The 33K protein is predominantly localized to the nucleus of BAdV-3 infected cells and is involved in stimulating the transcription from major late promoter. Analysis of mutant 33K proteins demonstrated that amino acids 201-240 and amino acid 204-231 are required for nuclear localization and MLP transactivation.

The adenovirus 33K protein appears to be a multifunctional protein performing different role in viral infection. Earlier study has shown that the 33K protein plays a role in viral capsid assembly and efficient capsid DNA interaction in BAdV-3 (Kulshreshtha et.al., 2004). The involvement of 33K protein in different steps of adenovirus replication may require protein protein interaction. Using 33K protein as bait in yeast two hybrid system, open reading frames (ORFs) of BAdV-3 were screened for the potential interactions with 33K protein. The 33K protein showed specific interactions with two late viral proteins- 100K and protein V (pV). The yeast two hybrid findings were validated by

in vitro binding using *in vitro* synthesized transcription-translation products. It was demonstrated that the interaction of 33K with 100K and pV takes place during BAdV-3 infection. The stretch of amino acids 81-120 and 161-200 in 33K protein were involved in the interaction with pV and 100K protein.

For screening the cellular interactions, the 33K protein was used as a bait to screen bovine retina cDNA library. The yeast two hybrid screening revealed that the 33K protein appears to interact with bovine presenilin-1-associated protein / mitochondrial carrier homolog 1 (BoPSAP / BoMtch1) and bovine microtubule associated protein (BoMAP). However, subsequent analysis by various *in vitro* and *in vivo* assays could only confirm the interaction between 33K protein and BoPSAP/BoMtch1. In addition, the 33K protein was also shown to be colocalized with BoPSAP in mitochondria. Based on these observations, it may be possible that 33K protein may play an anti-apoptotic by interacting with BoPSAP since the human homolog of PSAP has been known to induce apoptosis.

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ABBREVIATIONS USED IN THIS WORK

AD	Activation domain
ADP	Adenovirus death protein
AdVP	Adenoviral protease
BAdV	Bovine adenovirus
BoMAP	Bovine microtubule associated protein
BoPSAP	Bovine presenilin-1-associated protein
Bp	Base pair
CAdV	Canine adenovirus
CAR	Coxsackie virus-adenovirus receptors
CR	Conserved regions
CtBP	C-terminal-binding protein
DAPI	4'-6-Diamidino-2-phenylindole
DBP	DNA binding protein
DISC	Death-inducing signaling complex
DSBR	Double-strand break repair
EAdV	Equine adenovirus
EGFR	Epidermal growth factor receptor
EYFP	Enhanced yellow fluorescent protein
FADD	Fas-associated death domain
FAdV	Fowl adenovirus
FBS	Fetal bovine serum
FalAdV	Falcon adenovirus
FCA	Freunds Complete Adjuvant
FIA	Freunds Incomplete Adjuvant
FrAdV	Frog adenovirus
GFP	Green fluorescent protein
GON	Group of nine
GrB	Granzyme B

GST	Glutathione-S-transferase
HAdV	Human adenovirus
HVR	Hypervariable regions
iNOS	Inducible nitric oxide synthase
IPTG	Isopropyl-β-D-thiogalactopyranoside
ITR	Inverted terminal repeat
Kb	Kilobases
KCL	Potassium chloride
kDa	Kilodalton
KLH	Keyhole limpet hemocyanin
LiAc	Lithium acetate
LPS	Lipopolysaccarides
MCMV	Mouse cytomegalovirus
MDBK	Madin-Darby bovine kidney
MEM	Minimal essential medium
MHC	Major Histocompatibility complex
MLP	Major late promoter
MLTU	Major late transcription unit
NF	Nuclear factor
NES	Nuclear export sequences
NLS	Nuclear localization sequences
OAdV	Ovine adenovirus
ORF	Open reading frame
Ori	Origin of replication
PABP	poly A-binding protein
PAdV	Porcine adenovirus
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly ADP-ribose polymerase
PCR	Polymerase chain reaction
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase

PML	Promyelocytic leukemia
POD	PML oncogenic domains
Pol	Polymerase
pTP	Pre-terminal protein
RB	Retinoblastoma
RGD	Arginine glycine asparagine
RID	Receptor internalization and degradation
RLU	Relative luciferase units
RS	Arginine serine
SDS	Sodium dodecyl sulfate
TAdV	Turkey adenovirus
TAF	Template activating factor
TP	Terminal protein
TPL	Tripartite leader
TLR	Toll-like receptor
TRADD	TNF receptor associated death domain
TRAIL	TNF-related apoptosis-inducing ligand
TuAdV	Tupaia adenovirus
YPDA	Yeast peptone dextrose adenine

1. LITERATURE REVIEW

The review of literature is divided into three sections: Adenovirus classification, Adenovirus biology, protein-protein interactions.

1.1 Adenoviruses

Adenoviruses were first identified as viral agents by various investigators while searching for the etiology of respiratory infections in human beings. These viruses were first isolated in human adenoids (tonsils), from which the name is derived. The adenoviruses infect membranes of the respiratory tract, intestines and urinary tract of humans/animals. Recently, adenoviruses have been shown to be prevalent in coastal waters, swimming pool waters, and drinking water (Jiang, 2006). Adenovirus infections affect young children and infants more frequently than adults.

1.1.1 Adenovirus classification

Adenoviruses, members of *Adenoviridae* family are classified into five genera, namely Atadenovirus, Aviadenovirus, Mastadenovirus, Siadenovirus, and unnamed genera of adenovirus based on the infecting host range (Benko et al., 2002). Members of Atadenovirus genus including bovine adenovirus type (BAdV) -4, ovine adenovirus type (OAdV) -7 and egg drop syndrome virus contain AT rich genome. The members of Aviadenovirus genus are serologically distinct from the members of other adenovirus genera and can only infect birds. Two aviadenovirus, fowl adenovirus type 1 (FAdV)-1 and (FAdV) -9 genomes represent the longest adenoviral DNA molecules. Recently, falcon adenovirus (FalAdV) has been added as a new member to the aviadenovirus genus (Oaks et al., 2005). The Mastadenovirus genus which infect mammals includes human adenovirus (HAdV) -2 and -5, bovine adenovirus (BAdV) -3, porcine adenovirus (PAdV)- 3, equine adenovirus (EAdV)- 2, canine adenovirus (CAdV) -2 and tupaia adenovirus (TuAdV). Members of Siadenovirus genus possess a putative sialidase gene and comprise of Frog adenovirus (FrAdV) -1 and Turkey adenovirus (TAdV) -3 (Benko et al., 2002). Members within each genus show antigenic crossreactivity due to the presence of conserved epitopes on the hexons of virus capsids. The DNA sequencing and

phylogenetic analysis of moderately conserved region(s) of the hexon gene helps in determining adenovirus species and serotype identification (Casas et al., 2005).

1.1.2. Adenovirus biology

The adenovirus research has contributed to the basic knowledge about DNA replication, transcription and translation processes in eukaryotic cells, and has led to the discovery of RNA splicing (Thomas and Mathews, 1980). In recent years, interest has also been focused on its potential use as a vector for vaccination and gene therapy (Russell, 2000).

1.1.2.1 Genome

Adenoviruses contain a non-enveloped, double-stranded DNA genome of 26 Kb to 45 Kb in length with a G+C content of 33-63% (Davison et al, 2000). The strand of DNA transcribed towards right is called the "r" strand and the strand transcribed towards left is called the "l" strand. The genome contains short inverted terminal repeats (ITRs), whose length varies among adenoviruses. The ITRs play a major role in adenovirus replication. Adenovirus genome contains two identical origins of DNA replication, which are localized within the two terminal repeats. The genome also contains ***cis***-acting packaging sequences located within several hundred base pairs to the left end. These sequences are required for interaction of viral DNA with proteins involved in encapsidation (Grable and Hearing, 1992). Adenoviruses are more resistant to UV irradiation than RNA viruses since adenoviruses contain double stranded DNA, an undamaged DNA strand in adenoviruses can serve as a template for repair by host (Fong and Lipp, 2005).

1.1.2.2 Structural proteins

The structural proteins are mainly part of the capsid and are essential structural components of the virus. The adenovirus genome is surrounded by an icosahedral capsid composed of major (hexon, penton, fiber) and minor (pIIIa, pVI, pVIII and pIX) structural proteins (Russell, 2000). In addition to the capsid proteins, there are core proteins (V, VII, mu and terminal protein [TP]), which directly bind the viral DNA.

1.1.2.3 Non structural proteins

Non structural proteins (100K, 33K/22K and 52K/55K) are not the integral part of the mature virions (Davison et al., 2000). These proteins are involved in processing of precursor virion proteins, and transport and assembly of virion proteins necessary for the maturation of viruses.

1.1.3 Virus host interactions

As a first step in the virus-host interactions, adenovirus requires attachment to the cells which is mediated by different cellular receptors. The HAdV-5 attaches the cells via coxsackie virus-adenovirus (CAR) receptors (Russell, 2000; Arnberg et al, 2002; Zhang and Bergelson, 2005). The HAdV-5 fiber-CAR receptor interaction enables the virus to attach to the host cells but is not sufficient for the virus internalization into the cells. The entry of the virus into the cells requires penton base interaction with the integrins $\alpha v\beta 3$ or $\alpha v\beta 5$ (Nemerow, 2000). Following these interactions, the HAdV-5 is internalized by means of receptor mediated endocytosis in clathrin coated pits and endocytic vesicles known as endosomes. The low pH of the endosomes, mainly due to the acidification action of proton pumps, leads to the confirmational change in the virus structure and subsequent exposure of the hydrophobic domains, which in turn penetrate the endosomal membrane and allows the viral particles to escape to the host cytosol. Studies have suggested that HAdV-5 entry also requires dynamin (100-kDa cytosolic GTPase, which selectively regulates clathrin-mediated endocytosis) as well as activation of several signaling molecules including phosphatidylinositol-3-OH kinase (Wang et al., 1998; Meier et al., 2002). These signaling molecules promote endosomal leakage and viral exit to the host cytosol. During this stage, the viral capsid is still intact. The HAdV-5 capsids then interact with microtubules and dynein motors followed by docking at nuclear pore complexes and finally undergo dismantling of the capsid (Kelkar et al., 2004; 2006). The capsid dissociation and nuclear import depends on the ability of L3 protease to degrade internal protein VI. The protein pVI acts as cement between the core and outer capsid and is shown to shuttle between the cytoplasm and the nucleus (Honkavuori et al., 2004). The HAdV-5 capsid then looses pVIII protein, which is also one of the cementing proteins. After complete dismantling, viral DNA along with proteins V and VII enter the

nucleus and genes from the early regions (E1, E2, E3, and E4) are transcribed (Matthews and Russell, 1998).

1.1.3.1 Early gene expression

The HAdV-5 E1A gene is the first viral gene transcribed after the viral DNA reaches the nucleus. The E1A transcripts appear within one hour of adenoviral infection. The primary E1A transcript is processed by splicing to yield five different messages coding for 289R, 243R, 217R, 171R and 55R proteins (Russell, 2000). The E1A proteins, especially, 289R and 243R regulate the transcription of viral and cellular genes in the adenovirus infected cells. The 289R has additional 46 amino acid region termed as "unique region". Comparison of E1A sequences from various adenovirus serotypes identified four conserved regions: CR1, CR2, CR3 (Kimelman et al., 1985) and CR4 (Avvakumov et al., 2004). Out of all these regions, CR1 is the most conserved region among the currently known protein sequences (Avvakumov et al., 2004). The E1A proteins reprogram the host cell gene expression and force the quiescent cells to enter the S phase of cell cycle (Frisch and Mymryk, 2002; Berk, 2005; Turnell and Mymryk, 2006; Ferrari et al., 2008). These proteins also play a role in transcriptional activation, transcriptional repression and productive viral infection in the adenovirus-infected cells (Berk, 2005). The E1A alone has been shown to trigger apoptosis (Frisch and Mymryk, 2002; Berk, 2005) and transform the rodent cells in cooperation with adenovirus E1B gene (Berk, 2005; Turnell and Mymryk, 2006). In addition, E1A has been shown to repress nitric oxide (an antiviral effector of the innate immune system; Higashimoto et al., 2006) production through transcriptional control of the inducible nitric oxide synthase (iNOS) gene, allowing the virus to survive in human tissue.

The HAdV-5 E1B region encodes five proteins (Sieber and Bobner, 2007), which are expressed shortly after the E1A protein expression. These proteins inhibit apoptosis and thereby sustain the DNA transformation. The E1B-19K protein is expressed early in infection and is localized to the membrane of the nuclear envelope (Rao et al., 1997). A large amount of E1B-19K protein is shown to be localized in mitochondria and suppress the adenovirus induced apoptosis using p53 dependant and p53 independent pathways (Lomonosova et al., 2005). E1B-19K is a homologue of Bcl-2 since these proteins show -

40 % amino-acid sequence homology (Chiou et al., 1994). The E1B-55K protein regulates the selective export of viral mRNAs from the nucleus to the cytoplasm and translation of viral mRNAs (Dobbelstein et al., 1997; Gonzalez et al., 2006).

The E2 region is one of the most important regions for successful completion of HAdV-5 replication as it encodes the proteins essential for adenoviral DNA replication. Two major types of transcripts, E2A and E2B arise from the E2 region. The mRNA coding for DNA-binding protein (DBP) arises from the E2A region (Stillman et al., 1981). DBP is a 72 kDa protein that contains a highly phosphorylated N-terminal protein and highly conserved non-phosphorylated C-terminal domains (Linne and Philipson, 1980). The N-terminal domain of DBP has phosphorylated serine and threonine residues, and is essential for viral DNA replication. The C-terminal domain of DBP is involved in DNA binding, initiation and elongation phases of DNA replication, and transcriptional control of the major late promoter (MLP). DBP enhances the rate of initiation of adenoviral DNA replication by stimulating the binding of viral DNA polymerase to double stranded DNA (Van Breukelen et al., 2003). The mRNAs coding for the precursor terminal protein (pTP) and DNA polymerase (pol) arise from the E2B region. The pTP is a 75 kDa protein, which forms a heterodimer with the 140 kDa DNA polymerase. The pTP-pol complex is capable of initiating viral DNA replication and is enhanced by the host proteins like Oct-1 (de Jong et al., 2003; Mysiak et al., 2004). This complex shows reduced polymerase and exonuclease activity as compared to free adenovirus polymerase, which may be due to the binding of pTP at the primer binding groove of the adenovirus polymerase (Brenkman et al., 2002). This heterodimer is transported to the nucleus of infected cells due to the presence of a nuclear localization signal in pTP and attaches to the nuclear matrix (Fredman and Engler, 1993).

The HAdV-5 E3 region transcribes nine mRNAs generated by alternative splicing of the common transcript that initiates from the E3 promoter. The E3 promoter has two binding sites for NF-kB, which drives most of the E3 transcription in activated T cells (Williams et al., 1990). The E3 promoter has been shown to be upregulated in activated T-cells in an E1A independent manner for the efficient production of anti-apoptotic proteins (Mahr et al., 2003). The E3 region encodes gp19, 14.7K, 14.5K, 12.5K, 10.4K, 11.6K and 6.7K (Tollefson et al.,1996). The E3 proteins are also called as stealth proteins

since these proteins appear to reduce the recognition of the adenovirus infected cells by innate and adaptive arms of the immune system (Toth et al., 2005).

The gp19 protein is the predominant protein in the E3 region and is retained in the rough endoplasmic reticulum. It binds to major histocompatibility (MHC) class I antigens in the rough endoplasmic reticulum, thereby prevent their transport to the cell surface and cell lysis by adenovirus - specific cytotoxic T lymphocytes. (Rawle et al., 1989). This protein also decreases the amount of peptides presented on MHC class I due to inhibition of peptides processing by tapasin (Horwitz, 2004) but does not block the binding of peptides to MHC class I (Liu et al., 2005). Tapasin is a transmembrane glycoprotein located in the endoplasmic reticulum. The E3-14.7K protein inhibits TNF-mediated apoptosis (Horton et al., 1991). The TNF induced apoptosis is initiated by ligand-induced recruitment of TNF receptor associated death domain (TRADD) and Fas-associated death domain (FADD) to the death domain of TNF receptor 1 (TNFR1), forming death-inducing signaling complex (DISC). The 14.7K protein inhibits apoptosis by inhibiting TNF induced death-inducing signaling complex (DISC) (Schneider-Brachert et al., 2006). In addition, the 14.7K protein inhibits the antiviral immunity and inflammation by inhibiting the NF-KB transcriptional activity caused by TNF mediated receptor signaling (Carmody et al., 2006). The E3 11.6K protein or ADP (adenovirus death protein) promotes the killing and lysis of adenovirus infected cells by caspase dependant and caspase independent mechanisms (Zou et al., 2004), thereby helps in the spread of virus (Tollefson et al., 1996; Doronin et al., 2003). Currently this property of ADP is being exploited in designing oncolytic adenovirus vectors used in cancer gene therapy (Toth et al., 2004; Yun et al., 2005). The role ADP can be compensated by abolishing the function of E1B-19K since ADP plays an opposite role to that of E1B-19K, which inhibits apoptosis (Subramanian et al., 2006). The E3-10.4K (RIDalpha) and E3-14.5K (RIDbeta) proteins form a heterotrimer known as the RID (receptor internalization and degradation) complex that localizes to the plasma membrane (Stewart et al., 1995). These proteins are transmembrane proteins and are post-translationally modified. The adenovirus RID complex modulates the immune response by decreasing the expression of surface levels of tumour necrosis factor receptor 1 (TNFR1), inhibiting the activation of NF-kB, thereby creating a environment favorable for the survival of the virus (Fessler

et al., 2004; Chin and Horwitz, 2006). In addition, RID complex inhibits the LPS mediated inflammation by inhibiting IL-1R and TLR4 (Toll-like receptor 4) signaling and activation of NF-kB (Delgado-Lopez and Horwitz, 2006). The E3-6.7K protein is a small integral membrane protein having three N-glycosylation sites. Of these three sites, one site is modified with high-mannose oligosaccharides, which suggest that this protein is localized to the endoplasmic reticulum (Wilson-Rawls and Wold, 1993; Moise et al., 2004). The E3-6.7K protein along with RID complex downregulates TNF-related apoptosis-inducing ligand (TRAIL) receptor 2 thereby shows the anti-apoptotic property (Lichtenstein et al., 2004). The E3-12.5K protein is synthesized in early stages of infection and its function is not known (Hawkins and Wold, 1992).

The E4 transcription unit is localized at the right end of the genome. The HAdV-5 E4 promoter generates a primary transcript, which undergoes alternative splicing to produce at least 18 distinct mRNAs encoding seven different polypeptide products. Six of these polypeptides are known to exist in the adenovirus infected cells (Weitzman, 2005). The E4 gene products play a role in viral DNA replication, late protein synthesis, early to late switch in adenoviral infection and target regulators of cell signaling contributing to the cell transformation and oncogenicity (Tauber and Dobner, 2001).

The E4 ORF1 is conserved among all HAdVs and contributes to the oncogenecity of adenovirus (Latorre et al., 2005). The E4 ORF1 mRNA accumulates in the cytoplasm following the adenovirus infection due to formation of E1B-55K/E4ORF6 complex (Dix and Leppard, 1993). The E4 ORF3 is a highly conserved protein, which is associated with nuclear matrix causing alterations in the architecture of nucleus (Doucas et al., 1996). It is involved in viral DNA replication, late viral protein synthesis, host protein synthesis shut off, accumulation of misfolded protein in the aggresomes (cytoplasmic structures) and inactivation of MRN complex preventing the concatemer formation resulting in viral DNA replication (Araujo et al., 2005; Hart et al., 2006). The MRN complexes comprise of cellular proteins MRE11, RAD50, and NBS1 required for DNA double-strand break repair and for binding to DNA ends. The E4 ORF4 is also highly conserved among all the adenoviruses and is not essential for the virus growth (Halbert et al., 1985). It plays an important role in regulating viral replication and splicing of late adenoviral mRNAs (Bridge et al., 1993; Kanopka et al., 1998). The E4 ORF6 encodes a

34 kDa protein, which has several cysteine and histidine residues that form a functional zinc binding domain (Boyer and Ketner, 2000). The E4 ORF6 shows oncogenic potential in cooperation with E1B and blocks p53 mediated transcriptional activity (Dobner et al., 1996). In addition, it plays an important role in viral DNA replication, host protein synthesis shutdown and nucleocytoplasmic transport of late viral mRNA.

1.1.3.2 DNA replication

The HAdV-5 genome contains cis-acting DNA sequence within ITRs, which is known as origin (Ori) of DNA replication (Coenjaerts et al., 1994). In addition, there are auxillary regions, which enhance the rate of DNA replication up to 200 fold *in vivo* and *in vitro*. The replication initiation occurs by a protein priming mechanism in which pTP binds the first nucleotide of the nascent strand, a dCMP residue, covalently through a Ser-dCMP phosphodiester bond. The pTP, pol and DBP together with the core origin initiate a low level of replication (de Jong et al., 2003; Mysiak et al., 2004). After the formation of the pTP-dCMP complex, elongation starts by strand displacement mechanism. Elongation is rapid and requires DBP and polymerase. Later, the displaced strand can duplicate either by the formation of a panhandle structure, which regenerates at the origin or by the intermolecular renaturation of opposite strands originating from the use of the origins at both ends (Wang and Pearson, 1985). The cellular factors NF1 and NFIII/Oct1 along with these viral proteins form a stabilized nucleoprotein structure at the origin resulting in enhancing the rate of the reaction (Wang and Pearson, 1985).

1.1.3.3 Intermediate gene expression

Intermediate genes are expressed at the time of viral DNA synthesis. The two genes encoding pIX and IVa2 proteins form the structural components of the HAdV-5 virion and have been shown to activate the major late promoter (Lutz et al., 1997). The pIX is a 140 amino acid protein which contain a conserved (among adenovirus pIX) amino-terminal domain, a central alanine-rich domain, and a conserved carboxy-terminal domain (Rosa-Calatrava et al., 2001). Each virion capsid has 240 molecules of pIX (Parks, 2005). Deletion of pIX results in the production of heat labile, adenovirus virions suggesting that pIX confers stability to viral capsid (Vellinga et al., 2005). The C-

terminal domain of pIX contains a leucine repeat region, which is involved in trimerization. The trimerization of pIX is not essential for its inclusion in the adenoviral capsid (Rosa-Calatrava et al., 2001). The C-terminal domain of pIX acts as a transcriptional activator and can reorganize the nuclear proteins (ND10 domains; Parks, 2005). The C-terminus of pIX is exposed on the surface of the mature virion and can be used for the addition of targeting ligands. By exploiting this property of pIX, adenoviruses are able to infect many cell types which are not normally infected by wild - type adenovirus (Dmitriev et al., 2002; Li et al., 2005).

The HAdV-5 IVa2 is a 450 amino acid protein localized in the nucleoplasm and nucleolus of virus infected cells (Lutz et al., 1996). It acts as a transcriptional activator of the major late promoter (Tribouley et al., 1997; Pardo-Mateos and Young, 2004) and plays a role in viral DNA packaging (Zhang et al., 2001; Ostapchuk et al., 2005), and virus assembly (Zhang and Imperiale, 2003; Perez-Romero et al., 2005; Christensen et al., 2008). The cellular protein IVa2-RF binds specifically to IVa2 promoter and represses the transcription from the IVa2 promoter (Iftode and Flint, 2004). Creation of mutations in the IVa2 promoter impairs the binding of IVa2-RF factor to the promoter, resulting in more efficient IVa2 transcription and efficient viral DNA synthesis (Iftode and Flint, 2004).

1.1.3.4 Late gene expression

Following the onset of viral DNA replication, the transcription pattern changes from early to late phase. The late phase is characterized by the establishment of late transcription program, reduction in the early gene expression, inhibition of host cell cap-dependent translation and commencement of viral assembly. All these events depend on the expression of the major late transcription unit (MLTU), which is controlled by the major late promoter (MLP) (Thomas and Mathews, 1980). In HAdV-5, the MLTU generates a primary transcript that is subsequently processed into more than 20 different mRNAs, which are further divided into five families L1-L5 (Thomas and Mathews, 1980). Each family consists of multiple alternatively spliced mRNAs with a common poly A site. Each mature mRNA contains a common set of three short 5` leader segments called tripartite leader (TPL) sequences (Chow et al., 1977). The TPL enhances the

translation of viral mRNA in infected and transfected cells (Huang and Flint, 1998).

The L1 region of HAdV-5 encodes two proteins 52K/55K and IIIa, which have a common polyA signal. The 52K/55K proteins are differentially phosphorylated forms of a single 48kDa polypeptide, which are localized to the nucleus of the infected cells (Lucher et al., 1986). Both of these isoforms have been shown to be present in the virus assembly intermediates but not within the mature virions. 52/55-kDa regulates transcription from the major late promoter (Gustin et al., 1996). In addition, it plays an important role in the viral DNA encapsidation and assembly (Hasson et al., 1989; Perez-Romero et al., 2005, 2006; Wohl and Hearing, 2008), as it is required for the stable association between viral DNA and empty capsid (Gustin and Imperiale, 1998). These proteins are localized to the regions of the nucleus different from viral DNA replication centers, suggesting that replication and assembly of adenovirus occur in separate nuclear compartments (Hasson et al, 1992). Another L1 protein, IIIa protein, is a 64 kDa viral phosphoprotein, which is generated by the cleavage of pIIIa at the N-terminus during maturation of the virus (Vellinga et al., 2005). It is shown to be located underneath the vertex region (San Martin et al., 2008). The protein IIIa is involved in the virus assembly (Chroboczek et al., 1986; San Martin et al., 2008) and is capable of enhancing its own synthesis using an autostimulatory system by reducing the 52K/55K expression (Molin et al., 2002; Tormanen et al., 2006; Vellinga et al., 2005).

The L2 region encodes pIII, pV, pVII and mu proteins. The pIII protein is a penton base protein, which plays an important role in the entry of the adenovirus by binding of RGD (Arg-Gly-Asp) motifs present in the penton base to the cell surface integrins thus stimulating receptor-mediated endocytosis and endosomal penetration (Vellinga et al., 2005; Rentsendorj et al., 2006). Deletion of penton base RGD motifs affects the efficiency of internalization and the endosomal escape of viral particles (Shayakhmetov et al., 2005). The pV protein is a 50 kDa protein and forms a link between the viral DNA-protein complex and inside of the adenovirus capsid (Matthews and Russell, 1998). This protein plays a role in the delivery of viral DNA to the host cell during the infection process and associates with infected cell nucleoli during the virus life cycle. It contains multiple nuclear and nucleolar targeting signals and is capable of redistributing the major nucleolar protein, nucleolin from nucleus to cytoplasm

(Matthews, 2001). It is not essential for adenovirus viability and plays a role in the correct assembly of the infectious viral particles (Ugai et al., 2006). The pVII protein is a 19.2 kDa major core protein accounting for 10% of the protein mass of the virion. The protein VII is localized to the nucleus of the infected cells, which is mediated by the cellular nuclear import receptors like importin alpha, importin beta (Wodrich et al., 2006). It is associated with the viral DNA during the early phase of infection and mediates transcriptional repression in the nucleus before E1A expression. Following the expression of E1A, there is a decrease in protein VII mediated repression, resulting in the onset of transcription (Johnson et al., 2004). The pVII plays a role in remodeling of the adenovirus chromatin and stimulates transcription and replication of adenovirus *in vitro* (Gyurcsik et al., 2006). It also plays a role in packaging of viral DNA (Zhang and Arcos, 2005). The protein Mu is an 11 kDa protein, which binds to viral DNA and plays a role in viral chromosome condensation (Anderson et al., 1989; Keller et al., 2002). The precursor form of Mu is localized to the nucleolus and modulates the expression of E2 proteins (Lee et al., 2004). The proteolytic processing of pre Mu at amino acid positions 31 and 50 by adenovirus protease generates three fragments (Lee et al., 2004). The central polypeptide fragment is subsequently incorporated in the virions. The C-terminal pre-Mu is essential for the repression of the pVI (late protein) expression in adenovirus-infected cells while the function of the precursor's N-terminal peptide in infected cells is not clear (Lee et al., 2004).

The L3 region encodes three proteins namely protein VI, hexon, and a 23kd proteinase. The VI is a 22 kDa protein generated by cleavage from its precursor pVI and is located in the capsid adjacent to the hexon (Greber et al., 1993). The pVI contains two nuclear-localization sequences (NLS) and two nuclear-export sequences (NES), which are removed upon proteolysis during virus maturation (Vellinga et al., 2005). It is responsible for the transport of hexon capsomeres to the nucleus (Matthews and Russell, 1995) and helps the virus particle to escape from the endosome (Wiethoff et al., 2005). The 23 kDa proteinase is a protein needed for proteolytic cleavage of some adenovirus proteins during maturation of the virus. This protein has also been shown to possess deubiquitinating activity, which interferes with various cellular processes like transcription and intracellular signaling, and conferring the advantage to the adenovirus

replication (Balakriev et al., 2002). Another L3 protein, hexon, is the most abundant capsid protein present in adenoviruses. The hexon is a trimeric protein, which comprises of three identical tightly associated molecules (Rux and Burnett, 2000). Structural and genetic analysis revealed that the hexon protein contains two loop structures, seven hypervariable regions (HVR) and type-specific residues. The loop 1 of the hexon protein contains HVR-1 to HVR-6 and loop 2 contains HVR-7, which is associated with type specific determinants (Wu et al., 2005). The hexon surface loops show the highest antigenic variability among adenoviruses and contain most of the type specific epitopes. (Rux and Burnett, 2000). Significant differences in the epitopes are present in the hexon hypervariable regions between chimpanzee adenovirus and other known adenoviruses (Farina et al., 2001). Modification of the hexon by mutating asparagine residues to leucine has been shown to increase the adenovirus under different conditions (Blanche et al., 2001). As hexon is involved in the nuclear delivery of infectious virus, it can be exploited for enhancing the nuclear targeting of synthetic systems (Carlisle, 2002). In addition, the hexon protein has been shown to have an adjuvant effect, which is exerted by the disrupted adenovirus particles in response to the immunogen (Molinier et al., 2002).

The L4 region encodes three non-structural proteins- pVIII, 100K and 33K/22K polypeptides. The pVIII a 15 kDa protein, is one of the hexon-associated proteins, which connects the core with the inner surface of the adenovirus capsid (Liu et al., 1985). It is found in empty capsids and not in purified virus preparations (Vellekamp et al., 2001). It plays a role in the virions structural stability (Liu et al., 1985).

The 100K protein plays an important role in various aspects of the adenovirus life cycle. It has been shown to be involved in the transport of newly synthesized hexon monomers from the cytoplasm to the nucleus and trimerization of hexon monomers (Cepko and Sharp, 1983; Gambke and Deppert, 1981; Hong et al., 2005). The arginine methylation in 100K protein contributes to its localization to nucleus and plays an important role in productive infection (Iacovides et al., 2007). This protein appears to act as a scaffolding protein required for the assembly of adenoviral capsids (Morin and Boulanger, 1986). Deletion of the gene encoding the 100K protein results in reduction of hepatotoxicity caused by adenoviral vectors (Hodges et al., 2001). In addition, the 100K

protein interacts with a number of RNA transcripts preferentially allowing the translation of adenovirus derived late transcripts (Riley and Flint, 1993). The 100K protein initiates the translation of adenoviral mRNAs by ribosome shunting (Cuesta et al, 2004; Xi et al., 2004) in a tyrosine phosphorylation-dependent manner (Xi et al., 2005). The 100K protein has also been shown to be a Granzyme B (GrB) substrate that prevents cytotoxic lymphocyte granule-induced apoptosis in infected target cells by potently inhibiting GrB (Andrade et al., 2001, 2007). Granzyme B is a cytotoxic lymphocyte granule protease that plays a critical role in mediating cytotoxicity.

The 33K protein is a phosphoprotein predominantly localized in the nucleus of infected cells (Gambke and Deppert, 1981; Oosterom-Dragon and Anderson, 1983). The gene encoding the 33K protein is unique among the genes transcribed from the MLP in that it contains a 202-nt intron (Oosterom-Dragon and Anderson, 1983). The 33K protein contains a long stretch of acidic amino acids (aa 15 to 34) at the N- terminus, splicing enhancer domain at the C-terminus and a central region with two alanine stretches serving as hinge region separating two protein domains (Tormanen et al, 2006). The 33K protein has a molecular weight of 39 kDa, which is higher than expected due to presence of high proline and glutamic acid content (Oosterom-Dragon and Anderson, 1983). The 33K protein has been shown to switch from early to late mRNA transcription from MLP (Farley et al, 2004). Studies have suggested that the 33K protein plays a role in viral assembly (Fessler and Young, 1999; Finnen et al., 2001). In addition, this protein acts as a virus encoded RNA splicing factor, which selectively enhances the splicing of transcripts with weak 3' splice site (Tormanen et al., 2006; Akusjarvi et al., 2008). It is maximally expressed at the beginning of the late phase of infection and binds in the presence of IVa2 protein to intragenic sequences of the MLP essential for late phase of transcription (Ali et al., 2007). Another L4 protein, the 22K protein shares N-terminal 105 amino acids with 33K protein and plays a role in adenoviral DNA encapsidation (Ostapchuk et al., 2006).

The L5 region encodes the structural protein fiber (Ruigrok et al., 1990). The fiber is a long trimeric structure extending from twelve vertices of the adenovirus capsid. It is comprised of conserved N-terminal tail, which is embedded in the penton base, a shaft that is made of repeating units and a C-terminal head that is involved in the initial

attachment of the virus to the cellular receptor. The shaft provides rigidity to the fiber and is composed of a number of 15 amino-acid repeats, which determine the length of the shaft (Ruigrok et al., 1990). These repeats are rich in proline and glycine residues, which are pivotal for the proper folding of the shaft. The length of the shaft plays an important role in determining the adenovirus tropism (Ambriovic-Ristov et al., 2003). The C-terminal head forms the knob region, which starts from the conserved motif TWLT (Chroboczek et al., 1995) and mediates the attachment to the cellular receptor (Devaux et al., 1990). This variability is responsible for the antigenic diversity among the adenovirus serotypes. The knob contains a stabilization element for fiber trimerization and plays an important role in fiber protein synthesis (Henning et al., 2006; Li et al., 2006)

1.1.3.5 Virus assembly and release

During the late phase of infection, adenovirus structural and non-structural proteins are synthesized. These proteins are rapidly transported to the nucleus where the assembly process begins. The first step in the adenoviral assembly is formation of capsomers, which are the major structural units of the capsid assembled from monomeric polypeptide chains. This process requires 100K protein, which acts as a scaffolding protein (Hodges et al., 2001). During viral assembly, intermediate stages (light and heavy intermediate) are observed, which contains some viral proteins which are normally absent in mature virions. The light intermediates become heavy intermediates after the release of the scaffolding proteins including the 100K and 33K proteins, and association with viral DNA (Hodges et al., 2001; Finnen et al., 2001). The assembly begins with formation of empty capsids and subsequent entry of the viral DNA into the preformed capsids. A cis-acting DNA sequence located within the left end of viral DNA, mediates DNA-capsid interaction (Russell, 2000). Therefore, the encapsidation starts from the left end of the viral DNA. It is anticipated that one or more proteins bind at the packaging sequence, thereby mediating the interaction of viral DNA with the capsid. The next step of maturation involves the formation of young virions where several polypeptides like pIIIa, pTP, pVI, pVII, pVIII are in precursor form and are cleaved during the maturation phase by proteinase to generate mature IIIa, TP, VI, VII, VIII proteins (Russell, 2000). This process of cleavage stabilizes the structure of the particle and renders it infectious. The

mature virions remain in the infected cells and are released upon cell lysis.

1.1.4 Protein protein interactions

Proteins play an important role in carrying out the biological activities of the cell. Because of their different structures, proteins interact with other proteins in complicated ways. Thus, protein-protein interactions refer to the association of the proteins and their study with a biochemical point of view. These interactions can be either permanent or transient. The permanent interactions exist in complex forms and are stable. The transient interactions are not stable as in these interactions proteins associate and dissociates *in vivo*. A few interactions are transient *in vivo* but later on become permanent due to changes in cellular conditions (Nooren and Thornton, 2003). In the life cycle of adenoviruses, protein-protein interactions play an important role in viral entry, DNA replication, transcription and cell cycle control.

Adenoviruses are exploited as a model to study the virus-host interactions and to identify the cellular molecular mechanisms (Russell et al., 2004; O'Shea, 2005). During the adenovirus infection, there is a reprogramming of host cells to establish conditions favorable for replication to ensure efficient production of progeny virus. For achieving this goal, adenovirus encodes several proteins, which interact with other viral and cellular proteins resulting in inactivating the cellular DNA machinery.

As a part of the virus-host cell interaction, multiple receptor binding is required for gaining the entry of virus to the cells. In HAdV-5, the fiber and penton base interacts with CAR receptors and integrins respectively, leading to the entry of virus into cells (Nemerow, 2000). The flexibility of fiber is important for the efficient interaction of fiber with CAR receptors (Wu et al., 2003). In addition, the interaction of adenovirus fiber knob with cellular receptors determines the intracellular trafficking route of the adenoviruses (Shayakhmetov et al., 2003). The intracellular trafficking of HAdV-5 and HAdV-5/35 viruses (HAdV-5 based vector containing sequences encoding the HAdV-35 fiber knob domain instead of the HAdV-5 knob) varies since HAdV-5/35 virus is found in late endosomes/lysosomes upto 4 hours after infection, whereas HAdV-5 escapes to the cytosol and is not colocalized with late endosomes/lysosomes (Shayakhmetov et al., 2003).

1.1.4.1 Interactions during early gene expression

The E1A proteins of adenoviruses reprogram cellular gene expression by interacting with various regulatory proteins (Frisch and Mymryk, 2002). These proteins exhibit a wide range of biological interactions involving transcriptional activation and transcriptional repression. Since the E1A proteins do not bind to DNA with any sequence specificity, they trans-activate by binding to the factors regulating transcription. So far conserved regions named critical region (CR) 1 to (CR) 4 have been identified in E1A protein of HAdV-5 (Avvakumov et al., 2004).

The CR3 region of E1A interacts with both basal factors (e.g. TATA-binding protein [TBP]) as well as transcription factors (e.g. ATF, c-Jun, Sp1, upstream stimulatory factor [USF], CCAAT box binding factor, and Oct 4) (Berk, 2005). The CR3 region consists of C- terminal domain and N- terminal domain. The C- terminal domain of CR3 region interacts with DNA binding domain of transcription factors that binds to adenovirus early promoters (Berk, 2005). The N- terminal domain of CR3 (functions as the activation domain) region, binds to full form of TBP (TBP) and activates the transcription by stimulating the formation of a multiprotein transcription initiation complex by RNA polymerase II (Berk, 2005). The transactivation capacity of CR3 region is directly proportional to the binding ability of E1A to MED23, a component of transcriptional mediator (Berk, 2005).

The interaction of HAdV-5 E1A protein (through Leu-X-Cys-X-Glu motif) with the pocket domain of RB proteins (Felsani et al., 2006), activates cyclin kinases (Cdk4 and Cdk6), which triggers pRB phosphorylation, leading to the release of E2F. The E2F, in turn, functions as a transcriptional activator and induces a large number of cellular genes. This displacement of RB proteins from E2Fs drive the cells from G0 phase to S phase of cell cycle leading to the immortalization of the primary cells. In addition, the E1A stabilizes the expression of p53 by interacting with Mdm4, a p53-binding protein (Li et al., 2004).

The interaction of adenovirus E1A protein with some cellular proteins helps to repress the transcription of many genes involved in the regulation of growth and differentiation. The N-terminus of HAdV-5 E1A protein binds to the protein complex

p300/CBP involved in the chromatin structure (Loewenstein et al., 2006). The p300/CBP complex possesses histone acetyltransferase activity (HAT), which promotes the destabilization of histone-DNA interactions, resulting in the increased accessibility of the chromatin to the transcription factors. The binding of E1A to the p300/CBP complex inhibits the function of histone acetyltransferase activity and thereby represses the transcriptional activity (Frisch and Mymryk, 2002). The E1A-p300 interaction also modulates the expression of cellular genes promoting apoptosis and reduces the tumor cell metastasis (Miura et al., 2006).

The HAdV-5 E1A also interacts with the cellular proteins like p400 and TRRAP (factors regulating gene transcription by affecting chromatin organization) and causes the epidermal growth factor receptor (EGFR) suppression resulting in apoptosis (Fuchs et al., 2001; Flinterman et al., 2007). The interaction of E1A proteins with corepressors like C-terminal-binding protein (CtBP) is required for the optimal infectivity of adenovirus as the E1A-CtBP complex represses the cellular genes by interacting with their promoter regions. (Chinnadurai, 2002; Zhao et al., 2006; Bruton et al., 2008). In the absence of E1A-CtBP interaction, the expression of early viral proteins is decreased (Zhao et al., 2006).

The HAdV-5 E1B-19K protein protect the cells from TNF-induced apoptosis during viral infection (Chiou et al., 1994). It interacts with pro-apoptotic cellular proteins namely Bax and Bak, and blocks the apoptosis by inhibiting mitochondrial pore formation and caspase activation (Sundararajan et al., 2001; Sundararajan and White, 2001; Berk, 2005). The E1B 156R protein interacts with cellular apoptosos regulator protein Daxx suggesting its role in cell transformation (Sieber and Dobner, 2007).

The HAdV-5 E1B-55K protein counteracts the apoptotic functions of E1A by interacting with N-terminus of p53 (Kao et al., 1990), resulting in SUMOylation of p53 (Muller and Dobner, 2008). These interactions maintain the cells in transformed stage (Martin and Berk, 1998; Liu et al., 2000; Roth and Dobbelstein, 2003; Schwartz et al., 2008). In addition, the E1B-55K also interacts with E4-ORF6 resulting in the selective export of viral mRNAs from the nucleus and their translation in cytoplasm (Dobbelstein et al., 1997; Gonzalez et al., 2006). Another E1B protein, E1B 156R, promotes the cell transformation independent of repression of p53 mediated transcriptional activation. It

contains a transcriptional repression domain which interacts with the cellular apoptosis regulator Daxx, suggesting its role in cell transformation (Sieber and Dobner, 2007).

The HAdV-5 E2 proteins play a role in adenoviral replication by interacting with other proteins. The DBP interacts with SrCap (potent activator of transcription mediated by CBP and CREB) and inhibits the transcription mediated by the carboxyl-terminal region of SrCap (Xu et al., 2003). Another E2 protein, pTP forms a complex with pol (DNA polymerase) and attaches to core region of adenoviral ITR to initiate the viral replication. The viral replication is augmented by the interaction of cellular factors like Nuclear Factor 1(NF1) and Oct1 with the auxillary region of adenoviral ITR (Coenjaerts et al., 1994).

The HAdV-5 E3 proteins are known to evade host antiviral immunity. The E3-14.7K protein inhibits antiviral immunity and inflammation by inhibiting NF-kB activity. It interacts with the p50 homodimer (NF-κB family of transcription factor) and blocks the p50 homodimer DNA binding resulting in an anti-inflammatory action (Carmody et al., 2006). Another E3 11.6K protein, known as adenovirus death protein (ADP), promotes the lysis of adenovirus infected cells, thereby helps in the spread of virus (Tollefson et al., 1996). The ADP interacts with MAD2B resulting in the cell lysis and release of the virus (Ying and Wold, 2003). The MAD2B is a member of MAD (mitotic arrest deficiency) family, involved in the mitotic checkpoint control mechanism (Ying and Wold, 2003).

The HAdV-5 E4 proteins interact with regulators of cell signaling thereby contributing to the cell transformation by adenovirus. The E4 ORF1 targets a family of cellular proteins which play a role in cell signaling and signal transduction (Tauber and Dobner, 2001). The E4 ORF1 has a PDZ domain binding motif through which it interacts with the PDZ cellular proteins like DLG, MAGI-1 and disrupts the tight junction between cells. These interactions contribute to the oncogenecity of adenovirus since the disruption of tight junctions and loss of apicobasal polarity are common features of the epithelial cancer cells (Latorre et al., 2005). In addition, domain 2 motif (consisting of seven amino acids) of E4 ORF1, which binds to a cellular phosphoprotein, phosphatidylinositol 3-kinase (PI3K) and activates PI3K (Chung et al., 2007). The PI3K plays an important role in signaling pathway regulating cellular metabolism, survival and proliferation, and has been shown to be commonly activated in many types of cancer (Mills et al., 2001).

The E4 ORF3 binds to E1B-55kDa protein and induces the reorganization of nuclear bodies called as ND10 domains or PML oncogenic domains (PODs) (Hoppe et al., 2006). The ND10 domains are granular nuclear bodies present in the nucleus and contribute many cellular functions like transformation, DNA repair and apoptosis. The disruption of ND10 domains favors the adenoviral DNA replication (Weitzman, 2005). The PML proteins belong to tripartite motif (TRIM) family of proteins, which contain a RBCC domain (Nisole et al., 2005). The RBCC domain present in transcriptional regulator TIF1α (TRIM protein) is considered to mediate the protein-protein interactions between the E4 ORF3 and TIF1α (Yondola and Hearing, 2007). The functional significance of this interaction is not known. In addition, E4 ORF3 interacts with double-strand break repair (DSBR) protein kinase (DNA-PK) resulting in regulating the subnuclear localization of CBP (transcriptional regulator) and preventing the concatemer formation resulting in viral DNA replication (Araujo et al., 2005; Hart et al., 2006).

The interaction of E4 ORF4 with Src kinases triggers the p53 independent cell killing, which subsequently deregulates the Src signaling complex (Lavoie et al., 2000; Champagne et al., 2004). Hence, it plays a role in the induction of the cytoplasmic death pathway.

The E4 ORF6 shows oncogenic potential in cooperation with E1B-55K and blocks p53 mediated transcriptional activity (Dobner et al., 1996; Goodrum and Ornelles, 1999; Orlando and Ornelles, 2002). During the adenovirus infection, the E4 ORF6 interacts with E1B-55K and other cellular proteins (e.g. elongins B, cullin5) to form an E3 ubiquitin ligase complex that polyubiquitinates MRE11-RAD50-NBS1 (MRN) complex, resulting in its degradation by proteosomes. (Liu et al., 2005). The MRN complex enables exonucleolytic and endonucleolytic activities, which help in nonhomologous end joining.

1.1.4.2 Interactions during intermediate gene expression

During the intermediate gene expression, pIX and IVa2 genes are expressed. The HAdV-5 pIX protein interacts with group of nine (GON) hexons, confering the stability to viral capsid (Vellinga et al., 2005). The pIX deleted HAdV-5 virions are heat labile and fail to produce GON structures upon capsid dissociation (Vellinga et al., 2005).

In addition, the pIX interact with ND10 domains and disrupt the ND10 domains providing an environment favorable for the replication of HAdV-5 (Rosa-Calatrava et al., 2003). The C-terminus leucine repeats of pIX are responsible for the interaction of pIX with other proteins (Rosa-Calatrava et al., 2001).

The HAdV-5 IVa2 protein interacts with 52/55K protein and AT rich region in viral DNA packaging sequences, suggesting a role in viral assembly (Zhang and Imperiale, 2003) and DNA packaging (Zhang et al., 2001; Ostapchuk et al., 2005). These interactions are essential in viral assembly since the lack of either protein result in the absence of progeny virus formation (Perez-Romero et al., 2006). It also interacts with VII and 52/55K proteins, which is thought to be important for viral DNA packaging (Zhang and Arcos, 2005). Recently, it has been shown to interact with ATP and subsequently hydrolyze ATP (Ostapchuck and Hearing, 2008), which may be required for the viral DNA packaging during adenovirus assembly. The interaction of IVa2 protein with 52/55K protein has also been shown to regulate the transcriptional activation of the major late promoter (Gustin et al., 1996; Perez-Romero et al., 2006; Tribouley et al., 1997; Pardo-Mateos and Young, 2004).

1.1.4.3 Interactions during late gene expression

During the late phase of adenovirus life cycle, there is reduction in the early gene expression followed by viral assembly. The adenovirus assembly requires scaffolding proteins assisted assembly pathways, which are dependant on protein localization as well as protein-protein interactions. The scaffolding proteins mediate proper assembly of precursor capsids (procapsids) during assembly. After assembly, scaffolding proteins are removed followed by viral DNA packaging (D'Halluin et al., 1978).

The HAdV-5 L1 protein 52K/55K plays an important role in the late phase of adenovirus life cycle. The 52/55-kDa (N-terminal 173 amino acids) protein interacts with IVa2 protein specifically during the adenovirus infection, thereby regulating transcription from the major late promoter (Gustin et al., 1996; Perez-Romero et al., 2006). The interaction of N-terminal 331 amino acids of 52/55-kDa protein with IVa2 protein helps to bind to packaging sequences *in vivo* (Perez-Romero et al., 2005, 2006). Its interaction

with VII and IVa2 plays an important role in packaging of viral DNA (Zhang and Arcos, 2005).

The HAdV-5 L2 protein VII strongly interacts with cellular nuclear import receptors like importin alpha, importin beta and transportin, resulting in the entry of protein VII to the nucleus of the cells (Wodrich et al., 2006). In addition, it also interacts with histone chaperone like template activating factor (TAF), which helps in remodeling of the adenovirus chromatin and stimulates the transcription and replication of adenovirus *in vitro* (Gyurcsik et al., 2006). It also interacts with IVa2 and L1 52K/55K protein and plays a role in packaging of viral DNA (Zhang and Arcos, 2005).

The HAdV-5 L3 pVI protein interacts with hexon and is responsible for the transport of hexon capsomeres to the nucleus (Matthews and Russell, 1995). In addition, the C-terminus of pVI interacts with adenoviral protease (AdVP) for maximizing enzymatic activity of AdVP (Bajpayee et al., 2005).

The HAdV-5 L4 encodes 100K and 33/22K proteins. The 100K protein plays an important role in various aspects of the adenovirus life cycle. It binds the hexon protein via its globular domain, transports newly synthesized hexon monomers from the cytoplasm to the nucleus and helps in the trimerization of hexon monomers (Hodges et al., 2001; Hong et al., 2005). It has the selective binding element for the tripartite leader which forms a complex with initiation factor eIF4G and poly (A)-binding protein (PABP).

The HAdV-5 33K protein, forms a complex with IVa2 protein and stimulates late phase transcription by binding to the major late promoter (Ali et al., 2007). The 22K form of the protein along with IVa2 protein bind the adenovirus packaging domain suggesting a role of 22K protein in adenoviral DNA encapsidation (Ostapchuk et al., 2006).

1.2 Bovine adenovirus

Bovine adenovirus (BAdV) has been isolated from cattle, African buffalo, sheep and deer (Darbyshire, 1965). There are ten reported serotypes of bovine adenovirus (Reddy et al., 1998). Depending upon different characteristics including genome organization and A+T rich content of genome, these serotypes are classified in two genera *Mastadenovirus* (BAdV-1, BAdV-3, BAdV-10, BAdV-9 and BAdV-2) and

Atadenovirus (BAdV-4, BAdV-5, BAdV-8, BAdV-6 and BAdV-7 ; Davison et al., 2003). Different serotypes belonging to *Mastadenovirus* genus is further classified into five species (Davison et al., 2003) namely BAdV A (BAdV-1), BAdV B (BAdV-3), BAdV C (BAdV-10), HAdV C (BAdV-9) and OAdV A (BAdV-2). Similarly serotypes belonging to *Atadenovirus* genus are further classified into three species (Davison et al., 2003) namely BAdV D (BAdV-4,-5,-8), BAdV E (BAdV-6) and BAdV F (BAdV7). BAdV type -1, -2, -3, and -9 grow relatively well in established bovine cell lines and induce a single nuclear inclusion of irregular shape. BAdV type 4, 5, 6, 7, 8, and 10) can be propagated in low-passage cultures of calf testicular or thyroid cells and usually form multiple inclusions of circular shape with sharp outlines (Bartha, 1969).The calves infected with BAdV-1, BAdV-2 or BAdV-3 show no clinical symptoms while calves infected BAdV-4 and BAdV-5 have been shown to cause bronchopneumonia (Mohanty, 1971). BAdV-7 was first isolated from a cow having a febrile illness (Inaba et al., 1968). Previous study has shown that bovine adenoviruses can induce tumors in rodents and transform non-permissive cells in tissue culture (Darbyshire et al., 1966). Of these, BAdV-3 is best characterized and has been shown to be the best candidate as a vaccine vector (Baxi et al., 2001; Reddy et al., 1999; Zakhartchouk et al., 1999; 2004) because of its low virulence and the ability of the virus to grow to high titers in cell culture (Lehmkuhl et al., 1975).

BAdV-3 was first isolated from the conjunctiva of an apparently healthy cow in Great Britain (Darbyshire et al., 1965). BAdV-3 is a non-enveloped icosahedral particle with a diameter of 75-nm and contains a double stranded DNA genome of 34,446 base pairs (Reddy et al., 1999) flanked by 195 bp inverted terminal repeats (Shinagawa et al., 1987). The cis-acting packaging sequences are localized within 224-250 nucleotides on the left end of the BAdV-3 genome (Xing et al., 2003). The transcription map of BAdV-3 is shown in Fig. 1.1. Like HAdVs, the BAdV-3 genome is also organized into early, intermediate and late regions. The early region (E) comprises four transcriptional units-E1, E2, E3 and E4. However, the late region is organized into seven regions L1-L7 (Reddy et al., 1998).

1.2.1 Early gene expression

Molecular characterization of the early region of BAdV-3 has been reported (Reddy et al., 1998). As in HAdV, the E1 transcriptional unit of BAdV-3 is located at the left end of the genome and has ORFs coding for the proteins homologous to the E1A and E1B region of HAdV-5 (Reddy et al., 1998). The conserved regions of E1A promoter of BAdV-3 are located within the ITRs (Xing and Tikoo, 2006).The mRNAs of E1A and E1B share same poly A signal suggesting that the E1A and E1B transcripts of BAdV-3 are 3` co-terminal. (Reddy et al., 1999). Within the E1A region of BAdV-3, several mRNAs are produced as a result of alternative splicing, which encodes 211R, 115R and 100R proteins (Reddy et al., 1999a). The E1A protein sequences do not show the consensus nuclear localization signal (NLS). It appears that E1A interaction with cellular proteins that help E1A proteins localize to the nucleus (Reddy et al., 1999a). The E1A region proteins of BAdV-3 are essential for virus replication and transactivation of early viral genes (Zhou et al., 2001).

The E1B region of BAdV-3 encodes two major overlapping mRNAs (Reddy et al., 1999). The $E1B^{small}$ mRNA encodes the 157R protein, which is the homolog of the HAdV-5 19K protein (Zheng et al., 1994). Interestingly, $E1B^{small}$ appears essential for BAdV-3 replication in fibroblast cells (Zhou et al., 2001). The $E1B^{large}$ mRNA is translated into a 420R protein, which is a homologue of HAdV-5 55K protein. Mutational analysis of the E1B region has shown that $E1B^{large}$ is essential for BAdV-3 replication (Zakhartchouk et al., 2001; Zhou et al., 2001).

The E2 region of BAdV-3 is transcribed leftward on the “l” strand producing several mRNAs by differential splicing of the primary transcript. The E2 region encodes the DNA replication proteins and is further divided into the E2A region and E2B region (Reddy et al., 1998). The DBP encoded by the E2A region of BAdV-3 shows little homology with the DBP of other adenoviruses. The DBP of BAdV-3 contains a variable N-terminal domain and a conserved C- terminal domain (Reddy et al., 1998). There are number of serine and threonine residues at the N-terminus of DBP, which may serve as the potential phosphorylation sites (Reddy et al., 1998). DBP is expressed abundantly during the early and late phase of BAdV-3 replication (Zhou et al., 2001a). The E2B region of BAdV-3 codes for the DNA polymerase (DNA pol) and pre-terminal protein

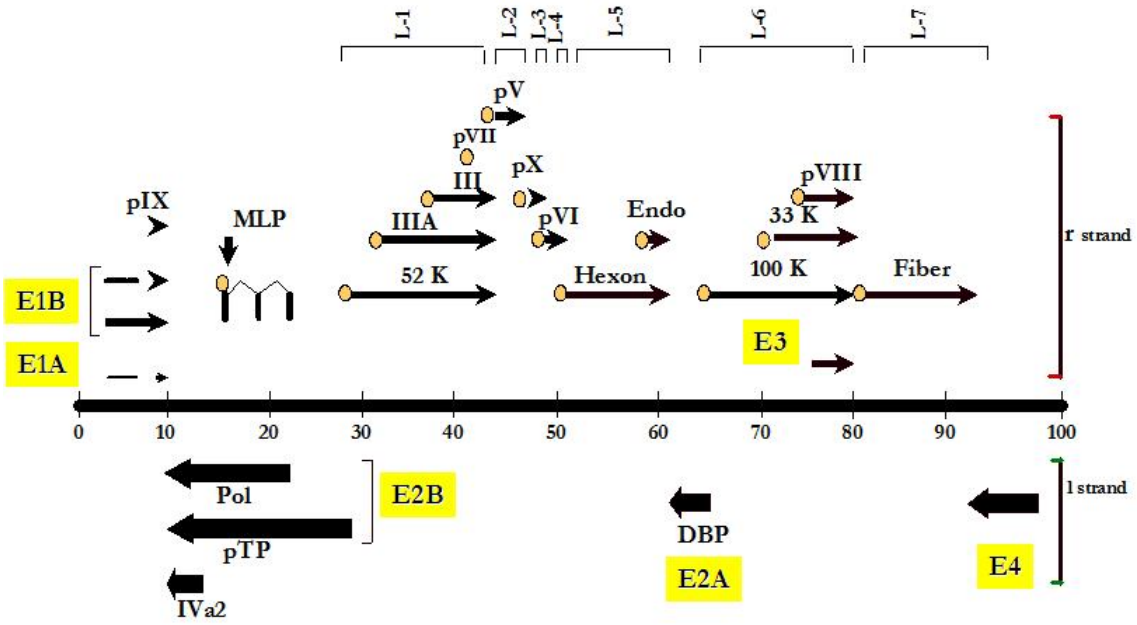


Fig 1.1: **Transcription map and genome organization of BAdV-3**. The schematic diagram showing BAdV-3 genome (34,446 bp; GenBank Acession #AF030154). The arrows represent the mRNAs from the "r" and "l" strands, respectively. The solid lines represent sequences found in mRNA, broken lines indicate introns, and arrowheads represent poly(A) sites and show the direction of transcription. E1 to E4 are early regions. (Adapted from Reddy et al., 1998).

(pTP). The transcripts of DNA pol and pTP are 3` coterminal (Reddy et al., 1998).

The E3 region of BAdV-3 is 1.5 kb in length and is transcribed left to right on "r" strand of the genome. Four mRNAs transcribed from the E3 promoter are 5' and 3'coterminal while one mRNA transcribed from the MLP share the 3' end (Idamakanti et al., 1999). These five transcripts encode four proteins 284R, 121R, 86R and 82R (Idamakanti et al., 1999). The protein 284R is a glycoprotein (Idamakanti et al., 1999), which shows no homology to proteins encoded by the E3 region of other adenoviruses (Reddy et al., 1998). It is a post translationally modified protein and contains N-terminal signal sequence, a hydrophobic transmembrane anchor sequence near C- terminus and potential sites for the addition of N-linked oligosaccarides (Idamakanti et al., 1999). The 121R protein shows little homology to the 14.7K protein encoded by the E3 region of HAdV-5 (Reddy et al., 1998) and is not post-translationally modified (Idamakanti et al., 1999). It inhibits the cytolysis of mouse cells by human tumor necrosis factor (Zakhartchouk et al., 2001). The isolation of replication competent E3-deleted BAdV-3 suggests that the E3 region of BAdV-3 is non-essential for virus replication in vitro (Zakhartchouk et al., 1998) or in vivo (Zakhartchouk et al., 1999). Hence, the foreign genes under the control of promoters like mouse cytomegalovirus (MCMV) immediate early promoter are efficiently expressed in E3 region of BAdV-3 with the aim of using the BAdV-3 as vectors for the development of vaccines (Zhang et al., 2005).

The E4 region of BAdV-3 is located on the right side of the BAdV-3 genome and is transcribed right to left from the "l" strand of the genome. The BAdV-3 E4 region is 3 kb in length. This region is transcriptionally active at early and late times post infection. The E4 region of BAdV-3 produces seven transcripts, which can encode at least five unique proteins namely 143R (ORF1), 69R (ORF2), 286R (ORF3), 143R2 (ORF4) and 219R (ORF5) (Baxi et al., 1999). The proteins encoded by the E4 ORF3 and E4 ORF5 of BAdV-3 show partial homology to the 34 kDa protein of HAdV-2 (Baxi et al., 1999). BAdV-3 E4 deletion studies have shown that none of the individual ORFs (ORF1 to ORF5) appear to be essential for the viral replication (Baxi et al., 2001)

1.2.2 Intermediate gene expression

Two genes, pIX and IVa2 comprise the intermediate region. The pIX protein of

BAdV-3 is a 14 kDa protein comprising 125 amino acids and is a component of BAdV-3 capsid (Reddy et al., 1999a). The BAdV-3 protein pIX shows less than 30% homology with pIX proteins of other HAdVs. The BAdV-3 pIX transcript is 3' coterminal with those of E1A and E1B. The C-terminus of pIX is exposed on the surface of BAdV-3 and hence is exploited as a site for the incorporation of targeting ligands (small or longer polypeptides) with the aim of developing BAdV-3 as vector for gene therapy (Zakhartchouk et al., 2004). Another intermediate protein, IVa2 protein of BAdV-3 is 376 amino acids in length, which is shorter than IVa2 proteins of other adenoviruses.

1.2.3 Late gene expression

Like in HAdVs (Chow et al., 1977), the mRNAs transcribed from the MLP of BAdV-3 contain a 5' non-coding segment which is known as the tripartite leader (TPL) (Reddy et al., 1998) which is 205 nucleotides in length. The first leader of the TPL is 40 nucleotides in length and is adjacent to the MLP. The second leader is 78 nucleotides in length and is located within the gene encoding DNA polymerase. The third leader is 87 nucleotides in length and is located within gene encoding pTP (Reddy et al., 1998). The late region of BAdV-3 produces several mRNAs, which are grouped into seven families of mRNAs (L1 to L7) (Reddy et al., 1998).

The L1 region codes four genes- namely 52k, pIIIa, pIII and pVIII, whose mRNAs have a common poly A signal. The 52k protein is 331 amino acids in length and is smaller than 52K of other HAdVs due to a large C- terminal deletion (Reddy et al., 1998). The pIIIa protein is 568 amino acids long. The BAdV-3 pIII protein is a penton base protein comprising of 482 amino acids and contains a MDV motif instead of the RGD motif found in HAdV-5 (Reddy et al., 1998). The BAdV-3 pVII protein (171 amino acids) is the major coat protein, which has protease cleavage sites at the amino terminus of the protein (Reddy et al., 1998).

The L2 region encodes pV protein, which is 410 residues in length and is rich in basic amino acids, especially in the central domain. Like in HAdVs, this protein contains a bipartite nuclear localization sequence (NLS) located in the central region of the protein (Reddy et al., 1998).

The L3 region of BAdV-3 encodes the pX protein, which is 80 amino acids in

length and is rich in basic amino acids (Reddy et al., 1998). The BAdV-3 pX protein contains two protease cleavage sites suggesting that the pX protein of BAdV-3 is cleaved by viral proteases.

The L4 region of BAdV-3 encodes a 282 amino acid pVI protein, which is longer than pVI of other HAdVs (Sussenbach and Van der Vliet, 1984). The mature VI protein is a minor component of the virion. In BAdV-3, the pVI protein contains two sequence motifs near the N- terminus and C- terminus, which are cleaved by endoproteases (Reddy et al., 1998).

The L5 region of BAdV-3 encodes the hexon protein and a proteinase. The proteinase of BAdV-3 is 204 amino acids in length and shows homology to proteinase of other adenoviruses (Reddy et al., 1998). The hexon protein of BAdV-3 is 910 amino acids in length and shows significant homology (45% to 70%) to the hexons of other adenoviruses (Reddy et al., 1998). The hexon proteins of different adenoviruses can be differentiated by differences in the external loops L1 and L2 (Athappilly et al., 1994). In BAdV-3, the L1 loop is shorter than that of HAdVs due to the absence of the region rich in acidic amino acids, which is normally present in HAdVs (Athappilly et al., 1994). Unlike in other adenoviruses, there is no overlapping of ORFs of BAdV-3 hexon and endoprotease (Reddy et al., 1998).

The L6 region of BAdV-3 encodes two non-structural proteins namely 100K and 33K, and one structural protein pVIII. The 100K protein is 850 amino acids in length and is longer than that of the 100K protein of other HAdVs. The 33K protein of BAdV-3 is predicted to code for a protein of 274 amino acids in length in the absence of splicing and 279 amino acids in length in the presence of splicing (Reddy et al., 1998). In BAdV-3, the 100K ORF slightly overlaps the 33K ORF. The 33K protein of BAdV-3 is expressed as 42, 38, and 33 kDa isoforms in BAdV-3 infected cells (Kulshreshtha et al., 2004). The 33K protein has also been shown to be involved in capsid assembly and efficient DNA capsid interaction (Kulshreshtha et al., 2004). The BAdV-3 pVIII protein is 216 amino acid residues long and contains two protease cleavage sites (Reddy et al., 1998).

The L7 region of BAdV-3 encodes fiber protein. Like in HAdV-5, the fiber of BAdV-3 is comprised of a shaft, head and the tail region. The tail and head region of BAdV-3 is almost similar in size to that of HAdVs. However, the shaft of BAdV-3 fiber

protein is comparatively larger (976 amino acids) than that of HAdVs due to the presence of 46.5 repeats of 15-residue repeats (Ruigrok et al., 1994). The fiber of BAdV-3 appears to be bent at one or more places, which have not been obeserved in any other adenoviruses (Ruigrok et al., 1994). An amino terminus hydrophobic sequence motif [FNPVYPY (D/E)] of HAdV-5 fiber, involved in the specific interaction between the fiber and penton base proteins (Caillet-Boudin, 1989) is strongly conserved in BAdV-3 fiber (Reddy et al., 1998). Like HAdV-5 (Chroboczek et al., 1995), the carboxy-terminal domain or head of the BAdV-3 fiber protein begins at the well-conserved TLWT motif (Reddy et al., 1998). The fiber of BAdV-3 is expressed as 102 kDa glycoprotein and is localized to the nucleus of the infected cells (Wu et al., 2004).

2.0 OBJECTIVES

Although advances in recombinant DNA technology have led to the identification of potent vaccine antigens, one of the impediments in the development of effective vaccines has been the appropriate delivery of vaccine. Currently, a number of viruses are being evaluated as vaccine delivery vehicles (Robert-Guroff, 2007). The development of efficient live viral vector systems requires a comprehensive knowledge of the interplay between virus and host cell.

Since BAdV-3 is non pathogenic and grows to high titers, our laboratory has been carrying out molecular characterization of bovine adenovirus (BAdV) -3 (Tikoo, 2001) with the aim of developing BAdV-3 as an efficient vector for gene delivery in humans and animals. Although our laboratory has developed the first generation of E3 deleted BAdV-3 vectors (Zakhartchouk et al., 1998), they replicate and excreate progeny virus thus transmitting virus to other farm animals. Moreover, large dose of vector is needed to produce protective immune responses.

Since the assembly of mature adenovirus is a multi-step process involving protein-protein (homotypic / heterotypic) and protein-DNA interactions, the involvement of the non structural proteins in various steps of adenovirus virus replication and assembly may require the direct protein-protein interactions. By determining the structure, function and importance of interaction of other viral and cellular proteins with non structural proteins, we may be able to develop BAdV-3 vectors (expressing vaccine antigens inserted in E3 region), which can replicate the recombinant BAdV-3 genome and thus amplify transgene expression without the production of infectious virus. Moreover, detailed knowledge of these protein-interactions is critical for understanding the biology of virus.

Most adenoviruses contain a collinear homolog of a non structural protein designated 33K. Since the 33K protein appears to be involved in viral assembly, we hypothesize that 33K protein may interact with other viral and/or cellular proteins during assembly. The present study is directed towards the molecular characterization and interactions of 33K protein of BAdV-3.

The primary objective is achieved with the following aims:

1. Characterization of 33K protein of BAdV-3.
2. Interaction of BAdV-3 33K protein with other viral proteins.
3. Interaction of BAdV-3 33K protein with cellular proteins.

3.0 CHARACTERIZATION OF 33K PROTEIN OF BAdV-3

3.1 Introduction

Adenoviruses contain a non-enveloped, double-stranded DNA genome of 26kb to 45kb with a G+C content of 33-63% (Davison et al., 2000). The adenovirus genome is organized into complex transcriptional units comprising of early, intermediate and late regions. Expression of the early and intermediate transcription units precedes the onset of viral DNA replication. However, expression of the late transcription units is dependent on the initiation of viral DNA replication and expression of the major late transcription unit (MLTU), which is controlled by the major late promoter (MLP) (Thomas and Mathews, 1980). The late transcriptional units encode structural and nonstructural proteins involved in the formation of progeny virions.

Bovine adenovirus (BAdV) - 3 is a member of subgroup I of BAdVs and is being developed as a potential vector for animal vaccines (Baxi et al., 2001 and Zakhartchouk et al., 1999; 2004) and human gene delivery (Rasmussen et al., 1999). Complete DNA sequence of the BAdV-3 genome has already been reported (Reddy et al., 1998). Like human adenovirus (HAdV) -5, the BAdV-3 genome is organized into early, intermediate, and late regions (Reddy et al., 1998). Unlike HAdV-5, the late region of BAdV-3 genome is organized into seven regions, L1–L7 (Reddy et al., 1998). The L6 region of the late transcription unit of BAdV-3 encodes 33K and 22K protein (Reddy et al., 1998; Kulshreshtha et al, 2004). Earlier, using anti-33K serum we detected three proteins of 42 kDa, 38kDa and 33kDa (Kulshrestha et al., 2004). The L6 33K protein is a product of a spliced transcript, while 22K protein is translated from the unspliced form of this transcript (Reddy et al, 1998). The 33K and 22K proteins share a N-terminus region of 138 amino acids.

Recently, adenovirus 33K protein has been shown to act as an alternative RNA splicing factor (Tormanen et al., 2006), a transcriptional activator (Ali et al., 2007) and to induce early to late switch of major late transcription unit expression (Farley et al., 2004). Moreover, adenovirus 22K has been shown to be involved in the packaging of adenovirus genome (Ewing et al., 2007; Ostapchuk et al., 2006). Here, we report the structure and functions of BAdV-3 33K protein.

3.2. Materials and methods

3.2.1. Cell lines and virus

Madin-Darby bovine kidney (MDBK) cells and 293T cells were cultured in minimal essential medium (MEM) (Gibco-BRL) containing 10% fetal bovine serum (FBS). HeLa cells were cultured in Dulbecco's modified MEM containing 10% FBS. The wild-type BAdV-3 was cultivated in MDBK cells as described (Reddy et. al, 1999).

3.2.2. RT-PCR analysis

RNA was extracted from BAdV-3 infected MDBK cells at 48h post infection using RNeasy kit (Qiagen). The first strand cDNA was synthesized using superscript II reverse transcriptase and oligo (dT) primers (Invitrogen). The cDNA was amplified by performing PCR using the L6 region specific primers (5'- GGAATTCTG A TGAAACCCCGCAGCATGTC-3' and 5'-GTAATCTTGACTGGCACCTG-3'). The PCR product(s) was ligated to *SmaI* digested pGEX-5X-1 (Pharmacia). The plasmid DNA was used to determine the DNA sequence of PCR product (s). The plasmids containing unspliced 22K and spliced 33K were designated as pGEX.22K and pGEX.33K respectively.

3.2.3 Plasmid construction

a) Construction of pEY.33K, pEY.33Kg2, pEY.33Kg3

i) A 845 bp fragment containing 33K ORF (Spliced) was amplified by PCR [using primers P1 and P4 (Table 3.1), and plasmid pGEX.33K DNA as a template], digested with *Eco*RI-*Bam*HI and ligated to *Eco*RI-*Bam*HI digested plasmid pEYFP-N1 (Clontech) creating plasmid pEY.33K.

ii) A 825bp fragment containing 33K ORF (spliced) initiating from 2nd ATG was amplified by PCR [using primers P2 and P4 (Table 3.1), and plasmid pGEX.33Ks DNA as template], digested with *Eco*RI-*Bam*HI and ligated to *Eco*RI-*Bam*HI digested pEYFP-N1 creating plasmid pEY.33Kg2.

iii) A 525bp fragment containing 33K ORF (spliced) initiating from 3rd ATG was amplified by PCR [using primers P3 and P4 (Table 3.1) and plasmid pGEX.33Ks DNA

as template], digested with *Eco*RI-*Bam*HI and ligated to *Eco*RI-*Bam*HI digested pEYFP-N1 creating plasmid pEY.33Kg3.

b) Construction of pC.33K, pC.33Kg2 and pC.33Kg3

i) A 845 bp *NdeI-EcoRI* fragment of plasmid pGEX.33K was isolated, blunt end repaired with T4 polymerase and ligated to *Hind*III digested (blunt end repaired with T4 polymerase) pCDNA3 (Invitrogen), creating the plasmid pC.33K.

ii) A 862 bp *NdeI-AscI* fragment of plasmid pEY.33Kg2 was isolated and ligated to 5.4 kb *Nde*I-*Asc*I digested plasmid pC.33K creating plasmid pC.33Kg2.

iii) A 649 bp *Aat*II-*Eco*RI fragment of plasmid pC.33K was isolated, blunt end repaired with T4 polymerase and ligated to *Hind*III digested (blunt end repaired with T4 polymerase) plasmid pcDNA3, creating plasmid pC.33Kg3.

c) Construction of pC.22K, pC.22Kss, pC.22KS1, pC.22KssS2

i) pC.22K. A 835 bp fragment was amplified by PCR using primers P20 and P37 and plasmid DNA pSM12 (Kulshrestha et al., 2004) DNA as a template. The PCR fragment was digested with *Sac*II-*Eco*RI and ligated to 5.4 kb *Sac*II-*Eco*RI fragment of plasmid pC.33K creating plasmid pC.22K.

ii) pC.22Kss. A 425 bp fragment was amplified by PCR using the primers P20 and P35 and plasmid pC.22K DNA as a template. Similarly, a 600 bp fragment was amplified from by PCR using the primers P22 and P36 (Table 3.1), and plasmid pC.22K DNA as a template. In a third PCR reaction, both amplified fragments were annealed and external primers P20 and P37 (Table 3.1), were used to PCR across to give a 840 bp amplicon. This PCR product was digested with *Sac*II-*Eco*RI and ligated to *Sac*II-*Eco*RI digested pC.33K creating plasmid pC22Ks (splice donor site GT removed to GC).

A 580 bp fragment was amplified from by PCR using the primers P20 and P38 (Table 3.1), and plasmid pC.22Ks DNA as a template. Similarly, a 427 bp fragment was amplified from by PCR using the primers P22 and P39 (Table 3.1), and plasmid pC.22Ks as a template. In a third PCR reaction, both amplified fragments were annealed and external primers P20 and P37 (Table 3.1) were used to PCR across to give a 840 bp amplicon. This PCR product was digested with *Sac*II-*Eco*RI and ligated to *Sac*II-*Eco*RI

Table 3.1 Sequence of primers for the construction of 33K deletion plasmids

Primer	Sequence
P1	5’-GGAATTCTGATGAAACCCCGCAGCATGTC-3’
P2	5’-GGAATTCTGATGTCGGCAGCCGGGCCT-3’
P3	5’-GGAATTCTGATGATTTCGATACCCCGCGA-3’
P4	5’-CGGGATCCCGGGCGGGTCCGGATTCGTC-3’
P5	5’-GGATTTCCAAGTCTCCAC-3’
P6	5’-CATCATATGCAGGTCCTCCTC-3’
P7	5’-GCTTCTCTGAATCCCACCGC-3’
P8	5’-GGAATTCTGAATGGCTTCTCTGAATCCCAC-3’
P9	5’-AGGGCGAGGCTTGCCCTGGCCCTTCTTG-3’
P10	5’-CAGGGCAAGCCTCGCCCTCCTCCTCTT-3’
P11	5’-GAGAGTGGGCGCGCTGGCACTCCGC-3’
P12	5’-GCCAGCGCGCCCACTCTCTATGCCATAT-3’
P13	5’-GTCCTCGATGTTGTGGCGG-3’
P14	5’-GGTTCGCTGAAAGATCAGCTCCCGAAGCTT-3’
P15	5’-CTGATCTTTCAGCGAACCCTAGCAGACT-3’
P16	5’-GGCGGGTCCGAGCTGTTCCTCCCTTGTTGT-3’
P17	5’-GAACAGCTCGGACCCGCCTAAGAATTCC-3’
P18	5’-GGTTCGCTGGCGCGTCA G TGAACGTA AG-3’
P19	5’-CTGACGCGCCAGCGAACCCTA GCAGAC T-3’
P20	5’-GGAATTCCATATGATGAAACCCCGCAGCATGTCG-3’
P21	5’-CCGCTGAGCGATCA GCTCCCGAAGCTTG-3’
P22	5’-GAAGGCACAGTCGAGGCT-3’
P23	5’-GAGCTGATCGCTCAGCGGTGTCACCTC-3’
P24	5’-GGAATTCCTTAGGCGGGTCCGGATTCG-3’
P25	5’-GTGGTAGAGGAGAG TGGGAAAGATCAGCT-3’
P26	5’-CCCACTCTCCTCTACCACAACAAGGAGGAA-3’
P27	5’-TTTCACTTTGGCG TGACACCGC-3’
P28	5’-CGGTGTCACGCCAAAGTGAAAAAT-3’
P29	5’-CAGTGAACGTGCGGATCTATTTTTC-3’
P30	5’-AATA GATCCGCACGTTCACTGACG-3’
P31	5’-GTTGTGGTAGGC GCAGCTGCG-3’
P32	5’-CGCAGCTGCGCCTACCACAAC-3’
P33	5’-GGTTCGCTGGGCCT GTTCCTC-3’
P34	5’-GAGGAACAGGCCCAGCGAACC-3’
P35	5’-GCTCCTCTTGCCTCTAGCGCC-3’
P36	5’-GCTAGAGGCAAGAGGAGCTAC-3’
P37	5’-GGAATTCCTATTTTTCACTTTGAGGTG-3’
P38	5’-TTCGGGTCGGCAGTAAGAATT-3’
P39	5’-TCTTACTGCCGACCCGAAGC-3’

digested pC.22K creating plasmid pC.22Kss (splice donor and splice acceptor site removed).

iii) *pC.22KS1.* A 835 bp *Nde*I-*Eco*RI fragment amplified by PCR using primers P20 and P37 (Table 3.1), and plasmid pSM12 (Kulshrestha et al., 2004) DNA as a template and ligated to 7.2kb *Nde*I-*Eco*RI digested plasmid pGBKT7 (Clontech) creating plasmid pGBK-22. A 8.1 kb *Nco*I fragment (blunt end repaired by T4 polymerase) of pGBKT7.22K was ligated to a *Xba*I linker (containing three way stop codon) creating the plasmid pGBKT7.22Ks. A 310 bp *Psh*AI-*Asc*I fragment of plasmid pGBKT7.22Ks and ligated to 5.9 kb *Psh*AI-*Asc*I fragment of pC.22K creating the plasmid pC.22KS1.

iv) *pC.22KssS2.* A 425 bp fragment was amplified by PCR using the primers P20 and P35 (Table 3.1), and plasmid pC.22KS1 as a template. Similarly, a 600 bp fragment was amplified from by PCR using the primers P22 and P36 (Table 3.1),and plasmid pC.22KS1 DNA as a template. In a third PCR reaction, both amplified fragments were annealed and external primers P20 and 37 (Table 3.1), were used to PCR across to give a 840 bp amplicon. This PCR product was digested with *Sac*II-*Eco*RI and ligated to *Sac*II-*Eco*RI digested pC.33K creating plasmid pC22Ks (splice donor site GT removed to GC)

A 580 bp fragment was amplified from by PCR using the primers P20 and P38 (Table 3.1), and plasmid pC.22KS1 as a template. Similarly, a 427 bp fragment was amplified from by PCR using the primers P22 and P39 (Table 3.1), and plasmid pC.22KS1 as a template. In a third PCR reaction, both amplified fragments were annealed and external primers P20 and P37 (Table 3.1), were used to PCR across to give a 840 bp amplicon. This PCR product was digested with *Sac*II-*Eco*RI and ligated to *Sac*II-*Eco*RI digested pC.22K creating plasmid pC.22KssS2 (splice donor and splice acceptor site removed).

d) Construction of pEY.33Kd1. A 220 bp fragment was amplified from by PCR using the primers P5 and P6 (Table 3.1) and plasmid EY.33K DNA as a template. Similarly, a 725 bp fragment was amplified by PCR using the primers P4 and P7 (Table 3.1) and plasmid pEY.33K DNA as a template. In a third PCR reaction, two PCR fragments that have 9 bp of internal overlap were annealed and external primers P4 and P8 (Table 3.1) were used to PCR across to give a 765 bp amplicon. This PCR product was digested with *Eco*RI-*Bam*HI and ligated to *Eco*RI-*Bam*HI digested pEYFP-N1 creating plasmid pEY.33Kd1

(2-40 amino acid deletion)

e) Construction of pEY.33Kd2. A 440 bp *Sac*II-*Asc*I fragment of plasmid p33Kd2 (Chapter 4.2.3) containing deletion of amino acid 41-80 was isolated and ligated to 5.4 kb *Sac*II-*Asc*I fragment of plasmid pEY.33K creating plasmid pEY.33Kd2 (41-80 amino acid deletion).

f) Construction of pEY.33Kd3. A 440 bp *Sac*II-*Asc*I fragment of plasmid p33Kd3 (Chapter 4.2.3) containing deletion of amino acid 81-120 was isolated and ligated to 5.4 kb *Sac*II-*Asc*I fragment of plasmid pEY.33K creating plasmid pEY.33Kd3 (81-120 amino acid deletion).

g) *Construction of pEY.33Kd4*. A 360 bp fragment was amplified from by PCR using the primers P1 and P9 (Table 3.1), and plasmid EY.33K DNA as a template. Similarly, a 375 bp fragment was amplified by PCR using the primers P4and P10 (Table 3.1), and plasmid pEY.33K DNA as a template. In a third PCR reaction, two PCR fragments that have 9 bp of internal overlap were annealed and external primers P1 and P4 (Table 3.1) were used to PCR across to give a 765 bp amplicon. This PCR product was digested with *Eco*RI-*Bam*HI and ligated to *Eco*RI-*Bam*HI digested plasmid pEYFP-N1 creating plasmid pEY.33Kd4 (121-160 amino acid deletion).

h) *Construction of pEY.33Kd5.* A 485 bp fragment was amplified by PCR using the primers P1 and P11 (Table 3.1), and plasmid pEY.33K DNA as a template. Similarly, a 905 bp fragment was amplified by PCR using the primers P12 and P13 (Table 3.1), and plasmid pEY.33K DNA as a template. In a third PCR reaction, two PCR fragments that have 9 bp of internal overlap were annealed and external primers P1 and P4 (Table 3.1) were used to PCR across to give a 765 bp amplicon. This PCR product was digested with *Eco*RI-*Bam*HI and ligated to *Eco*RI-*Bam*HI digested plasmid pEYFP-N1 creating plasmid pEY.33Kd5 (161-200 amino acid deletion).

i) *Construction of pEY.33Kd6*. A 610 bp fragment was amplified by PCR using the primers P1 and P14 (Table 3.1), and plasmid EY.33K DNA as a template. Similarly, a 660 bp fragment was amplified by PCR using the primers P15 and P13 (Table 3.1), and plasmid pEY.33K DNA as a template. In a third PCR reaction, two PCR fragments that have 9 bp of internal overlap were annealed and external primers P1 and P4 (Table 3.1) were used to PCR across to give a 765 bp amplicon. This PCR product was digested with

*Eco*RI-*Bam*HI and ligated to *Eco*RI-*Bam*HI digested plasmid pEYFP-N1 creating plasmid pEY.33Kd6 (201-240 amino acid deletion).

j) Construction of pEY.33Kd7. A 730 bp fragment was amplified by PCR using the primers P1 and P16 (Table 3.1), and plasmid EY.33K DNA as a template. Similarly, a 525 bp fragment was amplified from by PCR using the primers P17 and P13 (Table 3.1), and plasmid EY.33K DNA as a template. In a third PCR reaction, two PCR fragments that have 9 bp of internal overlap were annealed and external primers P1 and P18 (Table 3.1) were used to PCR across to give a 765 bp amplicon. This PCR product was digested with *Eco*RI-*Bam*HI and ligated to *Eco*RI-*Bam*HI digested plasmid pEYFP-N1 creating plasmid pEY.33Kd7 (241-275 amino acid deletion).

k) *Construction of pEY.33Kd6a, pEY.33Kd6b, pEY.33Kd6c*

i) A 597 bp *Sac*II-*Blp*I fragment of plasmid pC.33Kd6a to 4.9 kb *Sac*II-*Blp*I fragment of pEY.33K plasmid creating plasmid pEY.33Kd6a (200-211 amino acid deletion).

ii) A 764 bp fragment was amplified from by PCR using the primers P1 and P4 (Table 3.1), and plasmid pC.33Kd6b DNA as a template. This PCR product was digested with *Eco*RI-*Bam*HI and ligated to *Eco*RI-*Bam*HI digested EYFP-N1 plasmid creating the plasmid pEY.33Kd6b (204-231 amino acid deletion)

iii) A 686 bp fragment was amplified from by PCR using the primers P1 and P18 (Table 3.1), and plasmid pEY.33K DNA as a template. Similarly, a 660 bp fragment was amplified from by PCR using the primers P13 and P19 (Table 3.1), and plasmid pEY.33K DNA as a template. In a third PCR reaction, both amplified fragments were annealed and external primers P1 and P13 (Table 3.1) were used to PCR across to give a 1.3 kb amplicon. This PCR product was digested with *Eco*RI-*Bam*HI and ligated to *Eco*RI-*Bam*HI digested EYFP-N1 plasmid creating the plasmid pEY.33Kd6c (230-240 amino acid deletion).

l) *Construction of pC.33Kd6, pC.33Kd6a, pC.33Kd6b and pC.33Kd6c.*

i) A 683 bp *Sac*II-*Eco*RI fragment of plasmid pEY.33Kd6 was ligated to 5.4 Kb *Sac*II-*Eco*RI fragment of plasmid pC.33K creating plasmid pC.33Kd6 (201-240 amino acid deletion).

ii) A 600 bp fragment was amplified from by PCR using the primers P20 and P21 (Table 3.1), and plasmid pC.33K DNA as a template. Similarly, a 373 bp fragment was

amplified by PCR using the primers P22 and P23 (Table 3.1), and plasmid pC.33K DNA as a template. In a third PCR reaction, both amplified fragments were annealed and external primers P20 and P24 (Table 3.1), were used to PCR across to give a 804 bp amplicon. This PCR product was digested with *Sac*II-*Eco*RI and ligated to 5.4 kb *Sac*II-*Eco*RI digested pC.33K creating plasmid pC.33Kd6a (200-211 amino acid deletion).

iii) A 600 bp fragment was amplified from by PCR using the primers P20 and P25 (Table 3.1), and plasmid pC.33K DNA as a template. Similarly, a 300 bp fragment was amplified from by PCR using the primers P22 and P26 (Table 3.1), and plasmid pC.33K DNA as a template. In a third PCR reaction, both amplified fragments were annealed and external primers P20 and P24 (Table 3.1), were used to PCR across to give a 753 bp amplicon. This PCR product was digested with *Sac*II-*Eco*RI and ligated to *Sac*II-*Eco*RI digested pC.33K creating plasmid pC.33Kd6b (204-231 amino acid deletions).

iv) A 817 bp fragment was amplified from by PCR using the primers P20 and P24 (Table 3.1), and plasmid pEY.33K (230-240d) DNA as a template. This PCR product was digested with *Sac*II-*Eco*RI and ligated to 5.4 kb *Sac*II-*Eco*RI digested pC.33K creating plasmid pC33Kd6c (230-240 amino acid deletions).

3.2.4 Production of antisera

Production and characterization of antibody recognizing both 33K and 22K have been described (Kulshreshtha et al., 2004). To produce protein specific antiserum, two peptides (amino acid ^{141}KLTKTATQSKKSRRSASAARPRPPPLPPKRARAPRRPKGQ RHQAD^{185} and ^{156}ASAARPRPPPLPPKRARAPRRPKGQRHQADDASTEGRDKLREL IF^{200}) representing 33Ks and one peptide (amino acid^{191}CRPEADQNRHSEQKEPPECQ RGAPSPSSSSSQACSGAPPPQRPAPSGRRRK^{241}) representing 22K were synthesized on the Pioneer Peptide Synthesis system (Perkin Elmer) and conjugated to keyhole limpet hemocyanin (KLH) as a carrier molecule. Rabbits were immunized with conjugated peptide (500μg/rabbit) emulsified with Freunds Complete Adjuvant (FCA) followed by two injections (conjugated peptide, 250 μg/rabbit) in Freund's incomplete adjuvant (FIA) at four weeks apart. Serum was collected twelve days after the third injection to test for protein specific antibodies.

3.2.5 Western blot analysis

Monolayers of MDBK or 293T cells were infected with wild-type BAdV-3 (MOI of 5) or transfected with individual plasmid DNA (2-5μg/10^6 cells). At indicated times post infection, the cells were collected and analyzed by Western blot as described (Kulshreshtha et al., 2004) using protein-specific antisera.

3.2.6 Immunofluorescence microscopy

The HeLa cells were seeded in four-well Lab-Tek chamber slides. After eighteen hours, the cells were transfected with 1μg individual plasmid DNA using lipofectamine as per manufacturer's instructions (Invitrogen). Forty eight hours post-transfection, the cells were fixed in 4% paraformaldehyde for 15 minutes, washed four times with PBS and permeabilized with ice cold acetone for 5 minutes. The cells were then rinsed with PBS and incubated with 4'-6-Diamidino-2-phenylindole (DAPI) for 5 min at room temperature. Finally, the cells were washed three times with PBS, mounted in DABCO mounting media (Fluka) and analyzed by confocal microscopy.

3.2.7 Transcriptional Activation Assay

Monolayers (80% confluency) of Hela cells in one well of 12 well tissue culture plates (Costar) were co-transfected with 0.5 μg per/well of pGL-MLP (luciferase under the control of MLP) or pGL-E1A (luciferase under the control of E1A promoter) (Zakhartchoukand Tikoo,, unpublished data) with appropriate plasmid. Each transfection was performed in triplicate using Lipofectin transfection reagent (Invitrogen) according to manufacturer's directions. 48 hours post-transfection, luciferase expression was measured using the Enhanced Luciferase Assay kit (BD Pharmingen™) according to manufacturer's directions. Briefly, following cell lysis, cellular debris was pelleted and 100 μl of supernatant was taken to luminometer tubes. 100 μl of reaction buffer A was added to supernatants and immediately before readings, 100 μl of reaction buffer B was added and relative light units were read using the model TD 20/20 luminometer. Results were then tabulated and graphed.

3.3. Results

3.3.1 Determination of 33K and 22K ORFs

A recent report suggested that both spliced (33K) and unspliced (22K) forms of 33K mRNAs are present in HAdV-5 infected cells (Ali et al., 2007). To determine if both forms of 33K mRNA is present in BAdV-3 infected cells, we performed RT-PCR analysis of mRNA isolated from BAdV-3 infected cells using primers specific for both 33K spliced and 22K unspliced ORFs (based on Gene bank accession # AC 000002 sequence). As seen in fig. 3.1A, RT-PCR generated cDNAs of 930 bps using specific primers. The cDNA were cloned and sequenced using specific primers. Analysis of DNA sequence identified two types of cDNA clones. The DNA sequence of one type of cDNAs was similar to the genomic DNA sequence of predicted 22K. In contrast, DNA sequence of second type of cDNAs generated showed an internal splicing of 161 bp (Fig.3.1B). Analysis of amino acid sequence of proteins encoded by unspliced (22K) and spliced (33K) cDNAs predicted that both proteins have different C-termini but share a N-terminus 138 amino acids.

3.3.2 Characterization of BAdV-3 33K and 22K proteins

In order to characterize the 33K and 22K proteins, protein specific antibodies were generated by immunizing the rabbits with 500ug of peptides representing unique regions of predicted 33K (anti-33Kp serum) protein and 22K (anti-22Kp serum) protein. Serum collected after the final boost was analyzed for its specificity by Western blot using plasmid DNA (Fig. 3.2A) transfected 293T cells and BAdV-3 infected MDBK cells. Anti-33Kp serum detected five major proteins of 42 kDa, 39kDa 37kDa, 21 kDa and 19kDa, and three minor proteins of 35kDa, 25kDa and 23kDa in BAdV-3 infected MDBK cells at 48 hr post infection (Fig. 3.2B). The 21 kDa band appears to be a doublet. Similar protein bands (different intensities) are also detected in 293T cells transfected with plasmid expressing 33K protein (Fig. 3.2B). Anti-33Kp serum detected four major proteins of 42 kDa 25 kDa, 21kDa and 19kDa in 293T cells transfected with plasmid pC.33Kg2 DNA (Fig. 3.2B). The 21 kDa band appears to be a doublet. However, only three proteins of 25, 21kDa and 19kDa could be detected in cells transfected with plasmid pC.33Kg3 DNA (Fig. 3.2B). Similarly, three proteins of 39kDa, 37 kDa and 21 kDa could be detected in cells transfected with plasmid pC.33Kd6b DNA (Fig.3.2B)

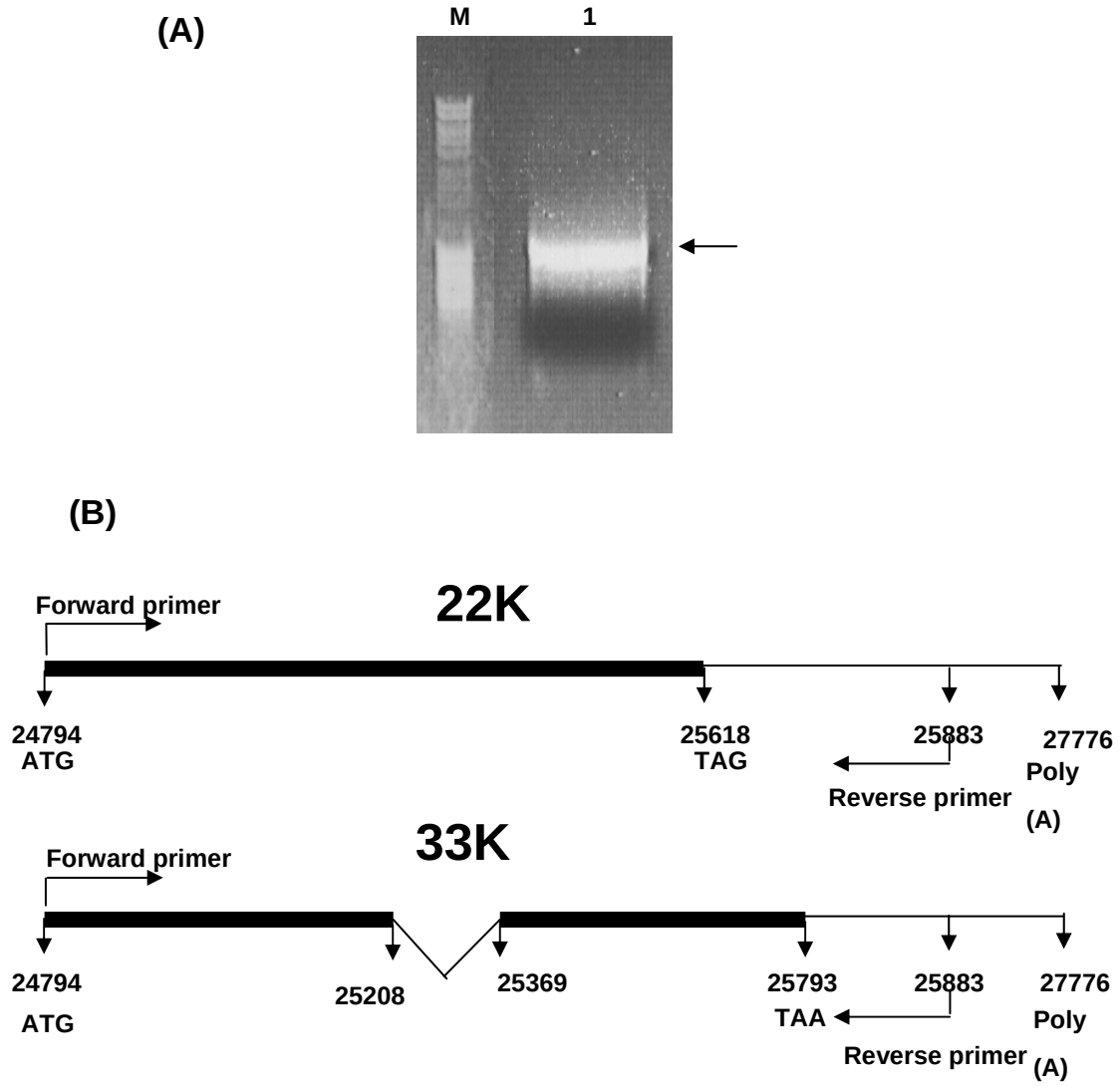


Fig. 3.1. 33K and 22K genes. (A) RT-PCR analysis. Total RNA isolated from MDBK cells at 48 hrs post infection was reverse transcribed using oligo (dT) as a primer. The 33K/22K specific cDNAs (lane 1) were amplified using primer 5'- GGAATTC TGAT GAAA CCCCGCAGCATGTC-3' and primer 5'-GTAATCTTGACTGGCACC TG-3'. Molecular weight marker DNA (M); **(B)** Schematic representation of 33K and 22K genes. The translation start codon, stop codon and poly A site are depicted. The nucleotide numbers represent the genome sequence (GenBank accession # AC 000002). The location of the primers is depicted by arrows.

(A)

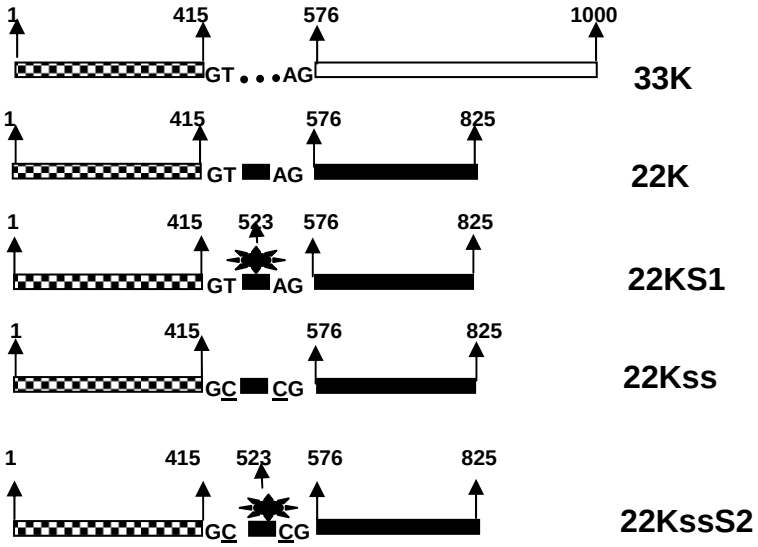


Fig. 3.2. Analysis of BAdV-3 33K and 22K proteins. (A). Schematic representation of BAdV-3 33K and 22K. The coding sequences shared by 33K and 22K (▩) or specific for 33K (▭) and 22K (▬) are depicted. The wild-type (GT…AG) and mutated (G<u>C</u>…<u>C</u>G) splice acceptor / donor sites are depicted. The nucleotide numbers represent the start and stop codons of the respective genes. The mutated nucleotide residues are underlined. The star represents the stop codon. The name of the encoded protein is depicted on the right of the panel.

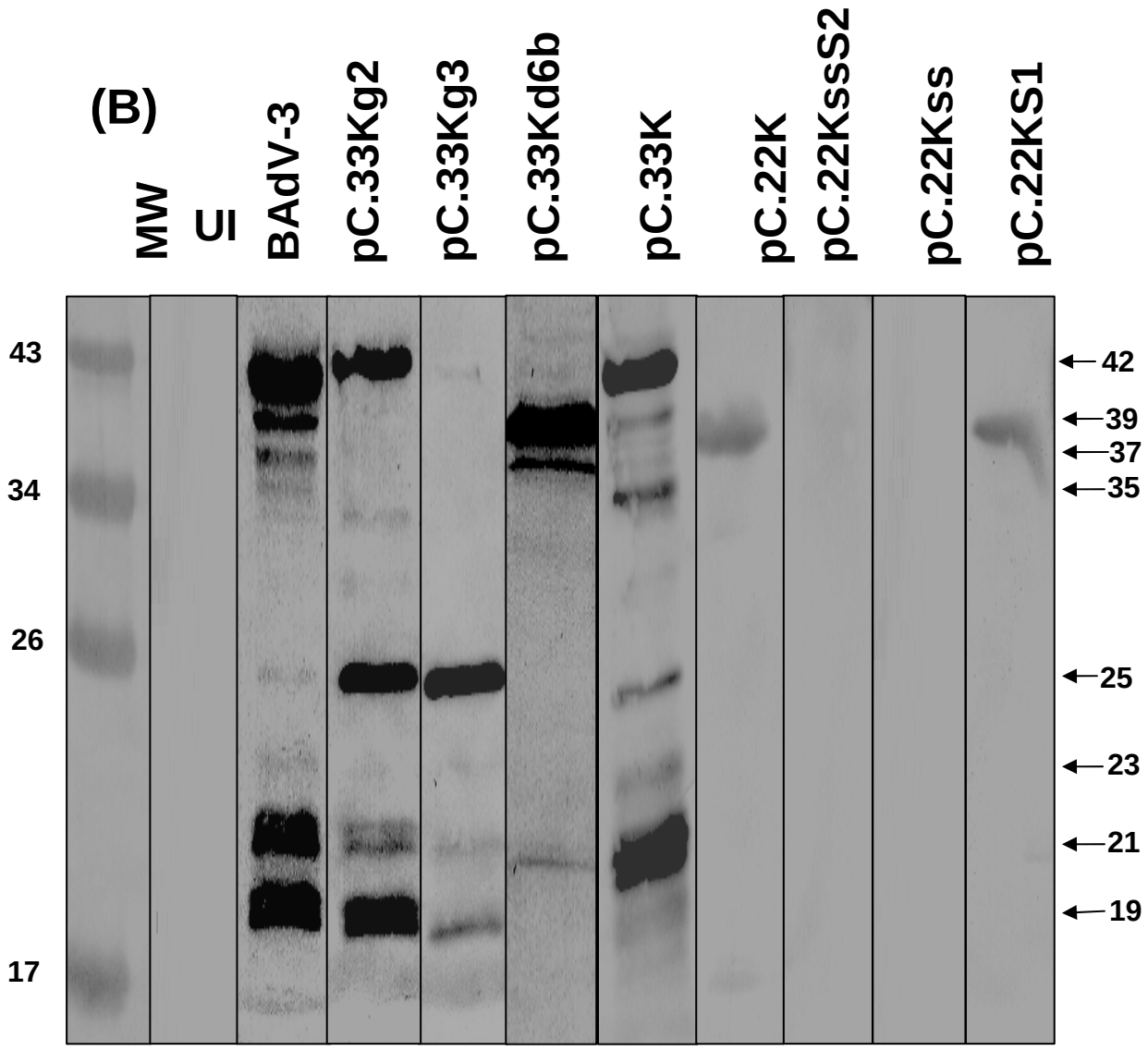


Fig. 3.2 (B). Western blot Analysis**.** Protein lysates of BAdV-3 infected MDBK cells or plasmid DNA transfected 293T cells were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-33Kp serum. The position of the molecular weight markers (M) in kDa is shown to the left of the panel. Arrows on the right of each panel indicate the position of the identified protein in kDa.

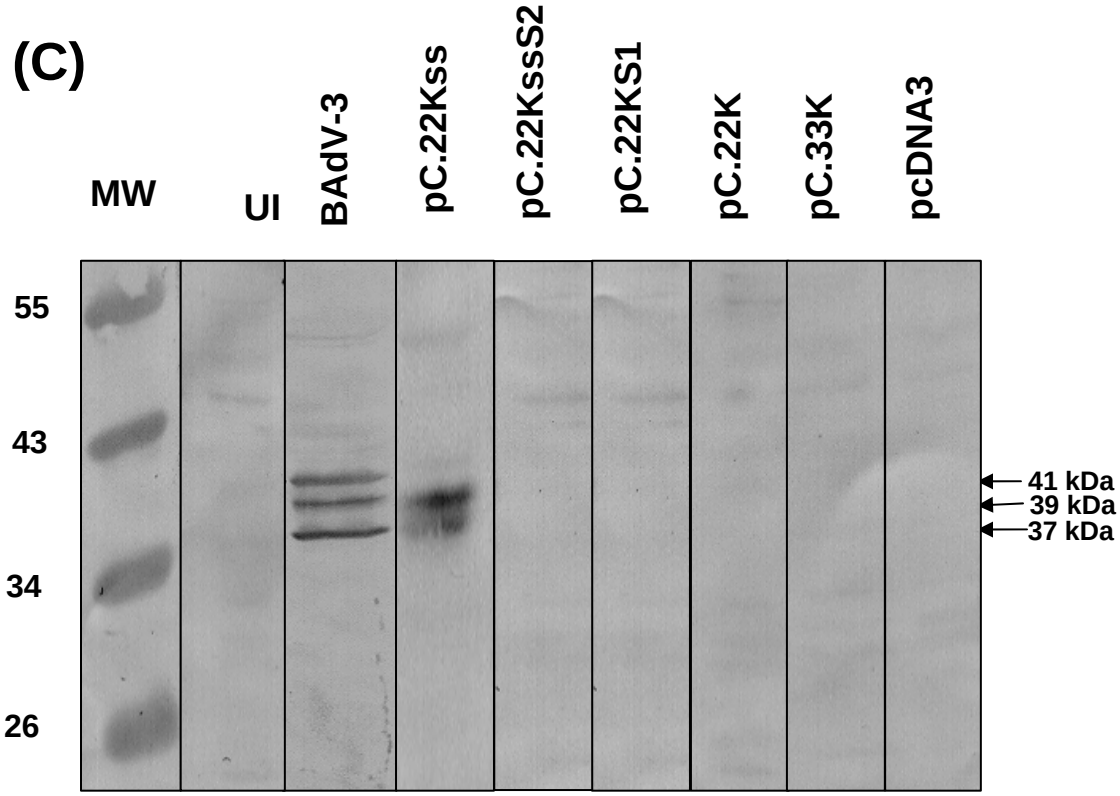


Fig. 3.2 (C). Western blot analysis. Protein lysates of BAdV-3 infected MDBK cells or plasmid DNA transfected 293T cells were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-22Kp serum. The position of the molecular weight markers (M) in kDa is shown to the left of the panel. Arrows on the right of each panel indicate the position of the identified protein in kDa.

Surprisingly, anti-33Kp serum detected a major protein of 39 kDa in cells transfected with plasmid pC.22K DNA (expressing 22K protein) or plasmid pC.22KS1 DNA. No such protein could be detected in cells transfected with plasmid pC.22Kss DNA or plasmid pC22KssS1 DNA (Fig. 3.2B).

Anti-22Kp serum detected three major proteins of 41 kDa, 39kDa and 37 kDa in BAdV-3 infected cells at 48 hrs post infection (Fig.3.2C). Anti-22Kp serum detected proteins of 39 kDa and 37 kDa in cells transfected with plasmid pC.22Kss DNA (Fig. 3.2C). As expected, anti-22Kp serum did not detect any protein in cells transfected with plasmid pC.22KssS2 DNA (Fig. 3.2C). No such protein(s) could also be detected in mock infected cells (Fig. 3.2C), or cells transfected with plasmid pC.33K DNA (Fig. 3.2C), plasmid pC.22K DNA (Fig. 3.2C), plasmid pC.22KS1 DNA (Fig. 3.2C) and plasmid pCDNA DNA (Fig. 3.2C).

3.3.3 Nuclear localization of 33K

Earlier, using anti-33K serum (which recognizes both 33K and 22K) we demonstrated that 33K / 22K proteins localize to the nucleus of BAdV-3 infected cells (Kulshreshtha et al., 2004). To determine if 33K protein is transported to the nucleus of the cells, MDBK cells were infected with wild-type BAdV-3 and examined by confocal microscopy using anti-33Kp serum. As seen in Fig.3.3, 33K was predominantly localized in the nucleus of the infected cells. To determine if 33K protein is transported to the nucleus in the absence of other viral proteins, we analysed plasmid pEY.33K DNA transfected HeLa cells by con-focal microscopy. Like BAdV-3 infected cells (Fig. 3.3), EY.33K was predominantly localized in the nucleus of the transfected cells. Similarly, EY.33Kg2 protein initiated at 2nd methionine residue (amino acid 6) or EY.33Kg3 protein initiated at third methionine residue (amino acid 87) predominantly localized to the nucleus of transfected cells.

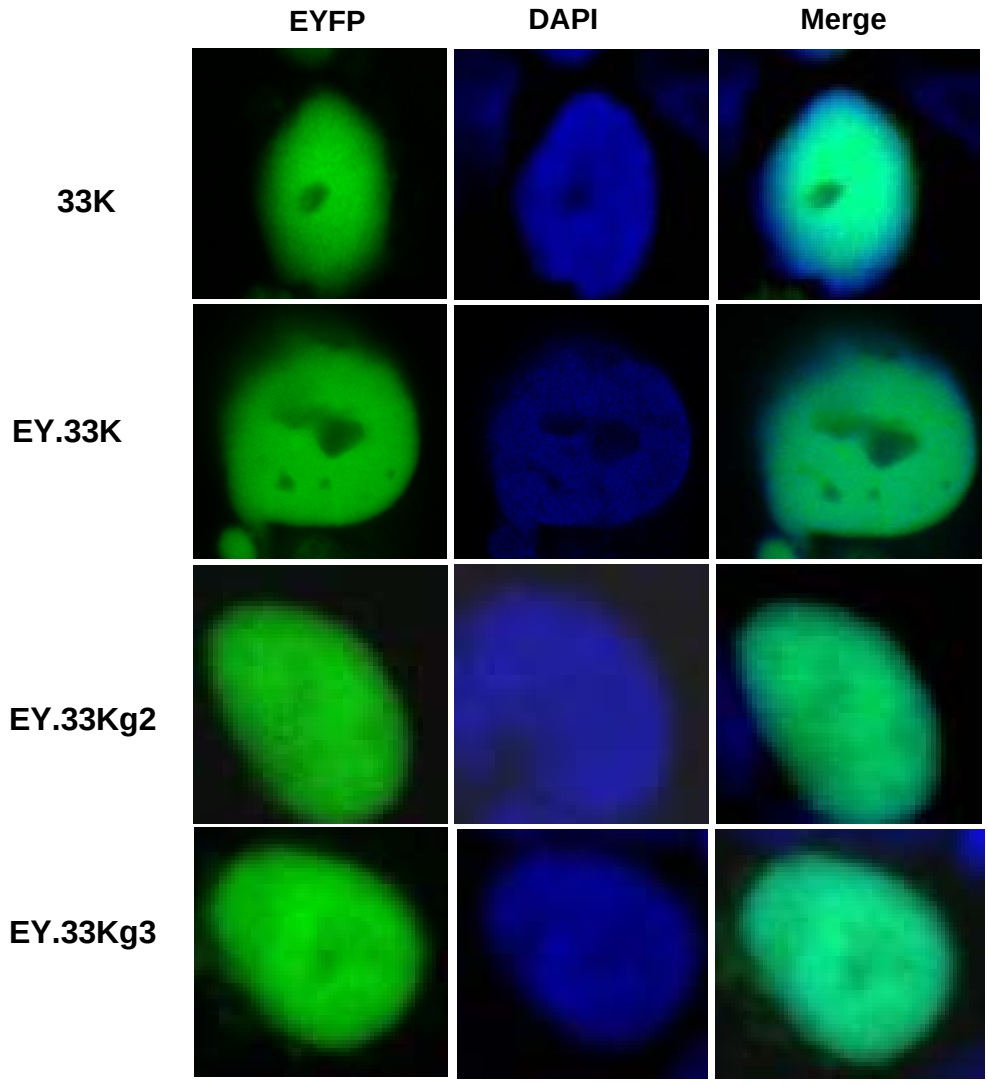


Fig. 3.3. Indirect immunofluorescence. Monolayers of MDBK cells were infected with BAdV-3. After 24 h post infection, the cells were fixed with 100% methanol and analyzed by indirect fluorescence using anti-33Kp serum. Similarly, monolayers of HeLa cells were transfected with individual plasmid DNAs expressing 33K proteins initiating from different ATGs. After 48h, the transfected cells were stained with DAPI and visualized by confocal microscope.

To determine the domain(s) responsible for nuclear localization of 33K protein, a panel of plasmids encoding mutant proteins were constructed (Fig. 3.4). These deletions were confirmed by restriction enzyme analysis and sequencing of mutant plasmid DNA. HeLa cells were transfected with individual mutant plasmid DNAs and analyzed at 48 hrs post-transfection by confocal microscopy. As seen in Fig. 3.5, the deletion of amino acids 2-40 (EY.33Kd1), 41-80 (EY33Kd2), 81-120 (EY.33Kd3) and 120-160 (EY.33Kd4) did not affects nuclear localization (Fig. 3.5). Similarly, mutant 33K proteins lacking amino acid 161-200 (EY33Kd5) and 241-275 (EY33Kd7) also localized to the nucleus (Fig. 3.5). However, compared to 33K, mutant EY.33Kd5 and EY33Kd7 showed granular distribution within the nucleus. In contrast, mutant EY.33Kd6 lacking amino acid 201-240 localized predominantly in the cytoplasm (Fig. 3.5). Taken together these results indicate that amino acid 201-240 contain NLS for nuclear localization of BAdV-3 33K protein (Fig 3.5).

To further localize the NLS, we constructed plasmids containing smaller deletions in this region of 33K (Fig.3.6A) and analyzed the localization of mutant proteins in transfected cells using confocal microscopy. As seen in Fig. 3.6B, none of these deletions localized predominantly in cytoplasm. These results suggest that amino acid 201-240 of 33K may contain multiple NLSs.

Analysis of the amino acid sequence of BAdV-3 33K protein containing putative NLS did not reveal stretches of basic amino acid residues that resemble the classical NLS. To determine if amino acids 201-240 contain NLS, we fused this domain to chimeric GFP/β-gal protein (Fig. 3.6A; Wu et al., 2004). As expected (Wu et al., 2004), the chimeric GFP/β-gal protein was predominantly located in the cytoplasm (Fig. 3.6B).

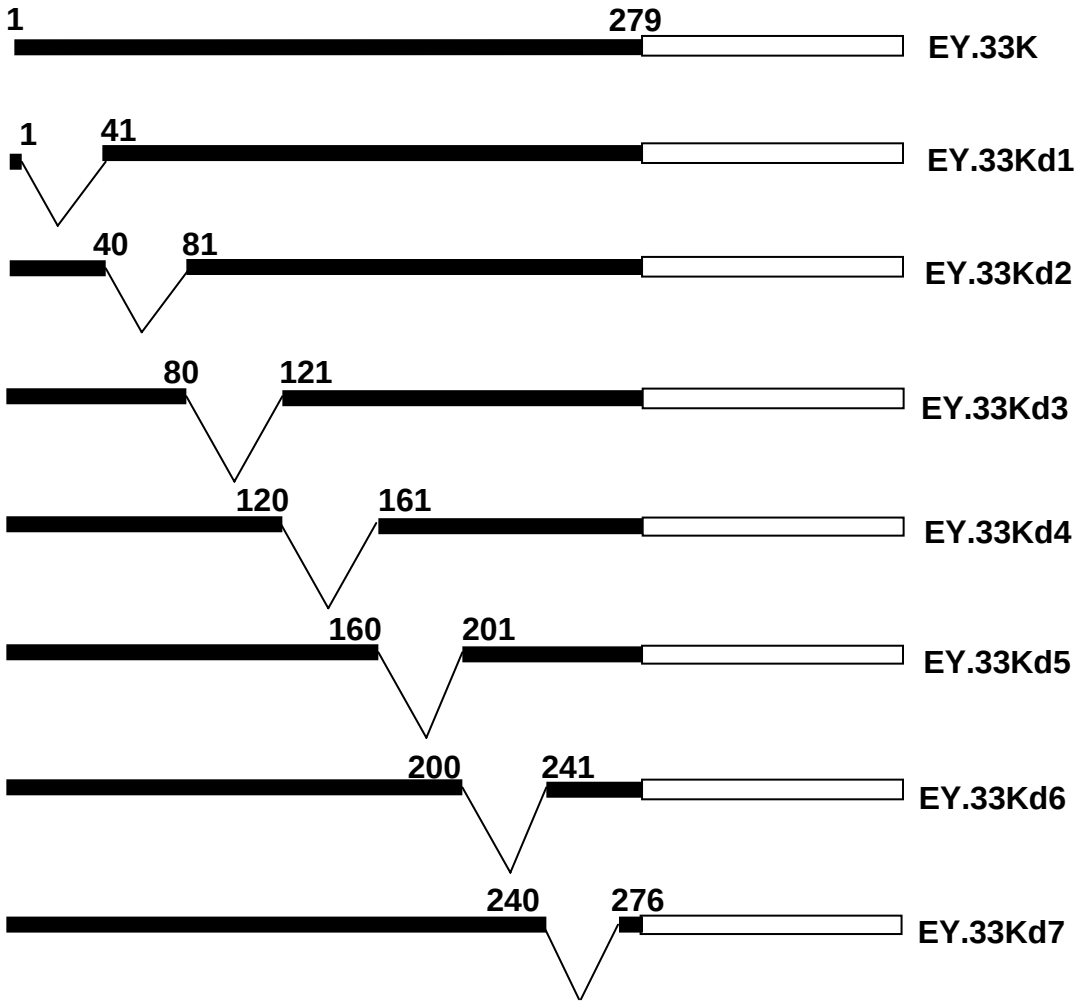


Fig. 3.4. Schematic representation of BAdV-3 33K-EYFP fusion proteins. The number above the box denotes the amino acid number for 33K protein. Thick box represent BAdV-3 DNA; hollow box represents EYFP DNA. Dotted lines represent deleted regions. The name of the individual mutant protein is given on the right.

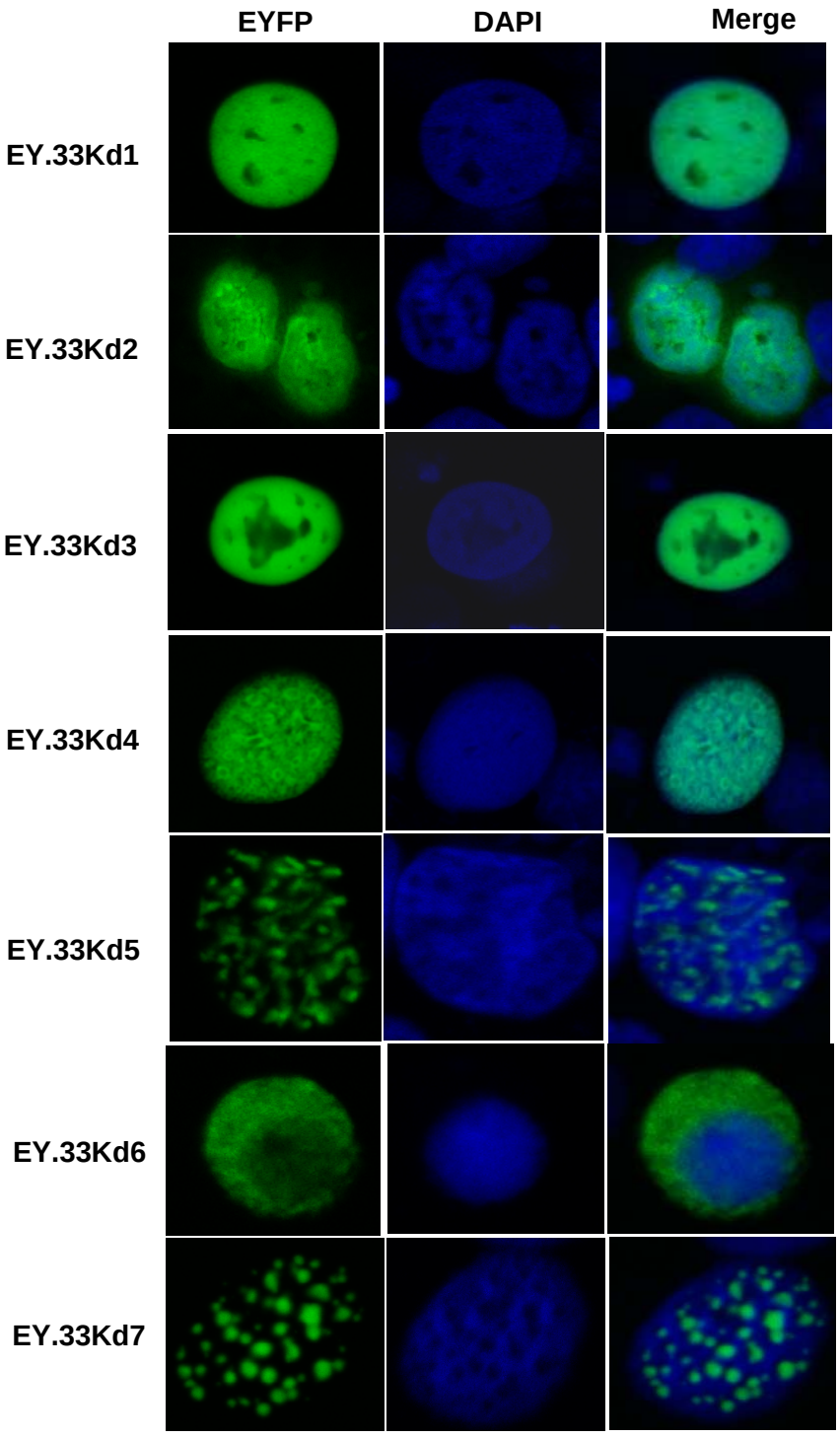


Fig. 3.5. Subcellular localization of 33K mutant proteins. Monolayers of HeLa cells were transfected with individual plasmid DNA expressing 33K-EYFP fusion proteins. At 48hrs post transfection, the cells were stained with DAPI and visualized by confocal microscope.

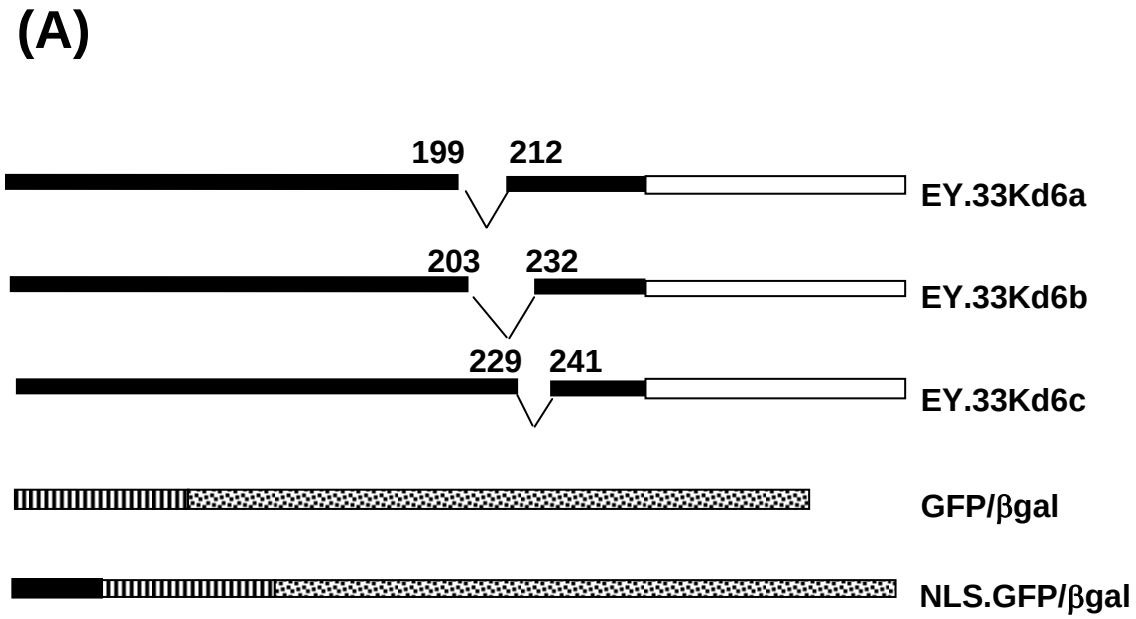


Fig. 3.6. Nuclear localization domain of 33K. (A) Schematic representation of BAdV-3 33K. The coding sequence of 33K (▬), EYFP (▭), GFP (▥) and βgal (▩) is depicted. The thin lines represent deleted sequences. The name of the expressed protein is shown of the right of the panel.

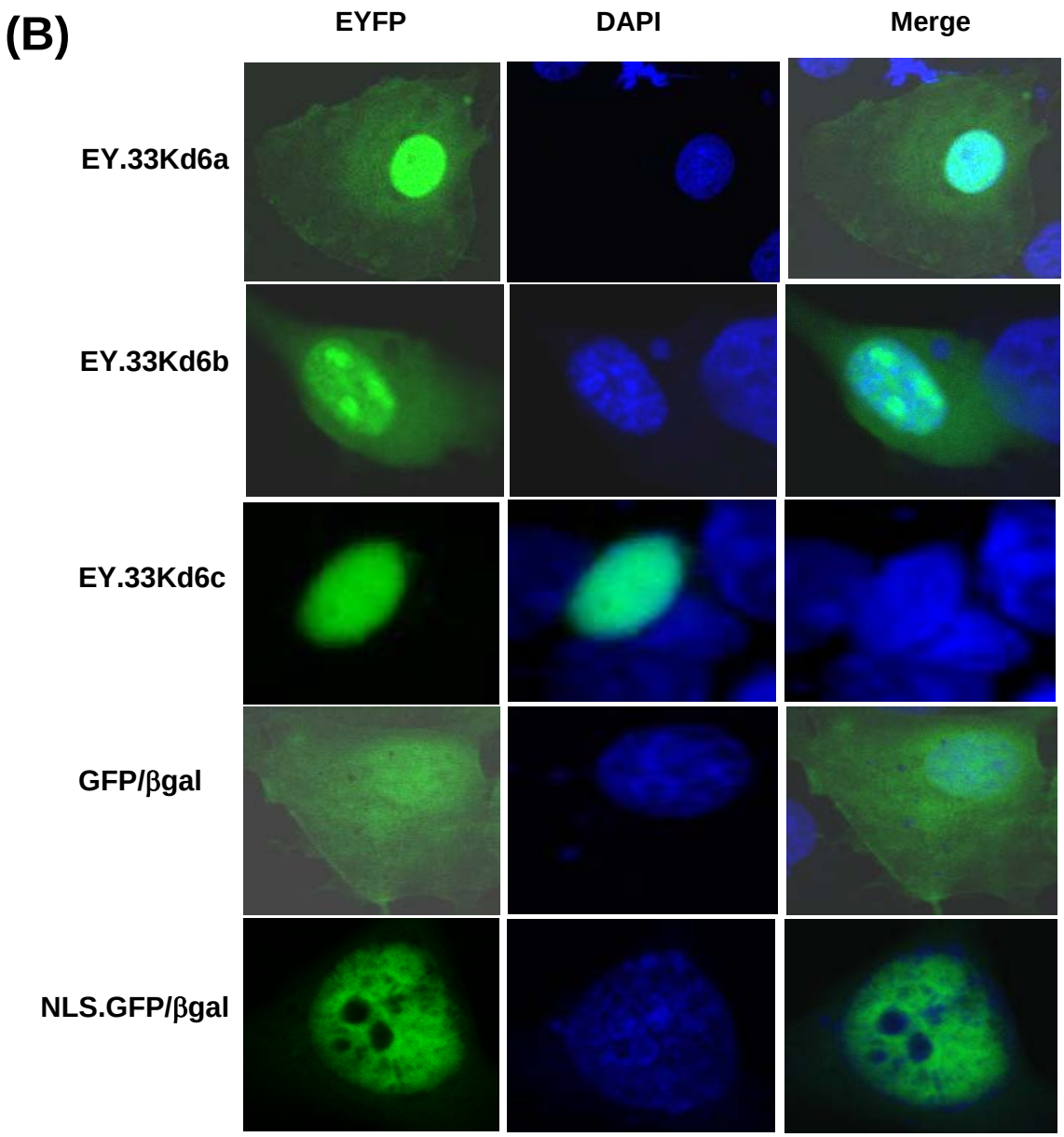


Fig. 3.6. Nuclear localization domain of 33K. (B) Indirect immunofluorescence. Monolayers of HeLa cells were transfected with individual plasmid DNAs. After 48hrs , the transfected cells were stained with DAPI and visualized by confocal microscope.

However, the chimeric GFP/β-gal protein fused to 33K NLS domain was predominantly localized to the nucleus of the cells (Fig. 3.6B). These results confirm that amino acid 201-240 contain essential element(s) of the NLS of BAdV-3 33K protein.

3.3.4 Major Late Promoter Transcription Activation

To determine if 33K can act as a transactivator, plasmid pMLP.Luc , containing a luciferase reporter gene under the control of AdV major late promoter (MLP) was co-transfected into HeLa cells together with individual plasmid pC.33K or pC.IVa2 (Fig. DNAs (Fig. 3.7A). As seen in Fig. 3.7B, luciferase expression increased 6-fold when pC.33K or pC.IVa2 was co-transfected with the pMLP.Luc. However, there was no increase in luciferase expression when plasmid pC.33K DNA, plasmid pC.IVa2 DNA or plasmid pCDNA3 DNA (Fig. 3.7B) was individually co-transfected with the plasmid pE1A.Luc DNA (Fig. 3.6B). To determine the domain involved in the activation of MLP, selected 33K mutant proteins (Fig. 3.8A) were tested for the transactivation of MLP. As seen in fig.3.8B, deletion of amino acid 161 to 200 of 33K (pC.33Kd5) did not effect the transactivation of MLP. However, deletion of a amino acid 201 to 240 of 33K (pC.33Kd6) abolished the transactivation of MLP (Fig. 3.7B). To further identify the boundaries of the domain, involved in transactivation, we constructed plasmids containing smaller deletions between amino acid 201 to 240 of 33K (Fig.3.8A,B) and tested them in transactivation assay. As seen in fig. 3.8C deletion of amino acid 200-211 (pC.33Kd6a) or amino acid 230-240 of 33K (pC.33Kd6c) did not abolish the transactivation of MLP. However, deletion of amino acid 204-231 (pC.33Kd6b)

(A)

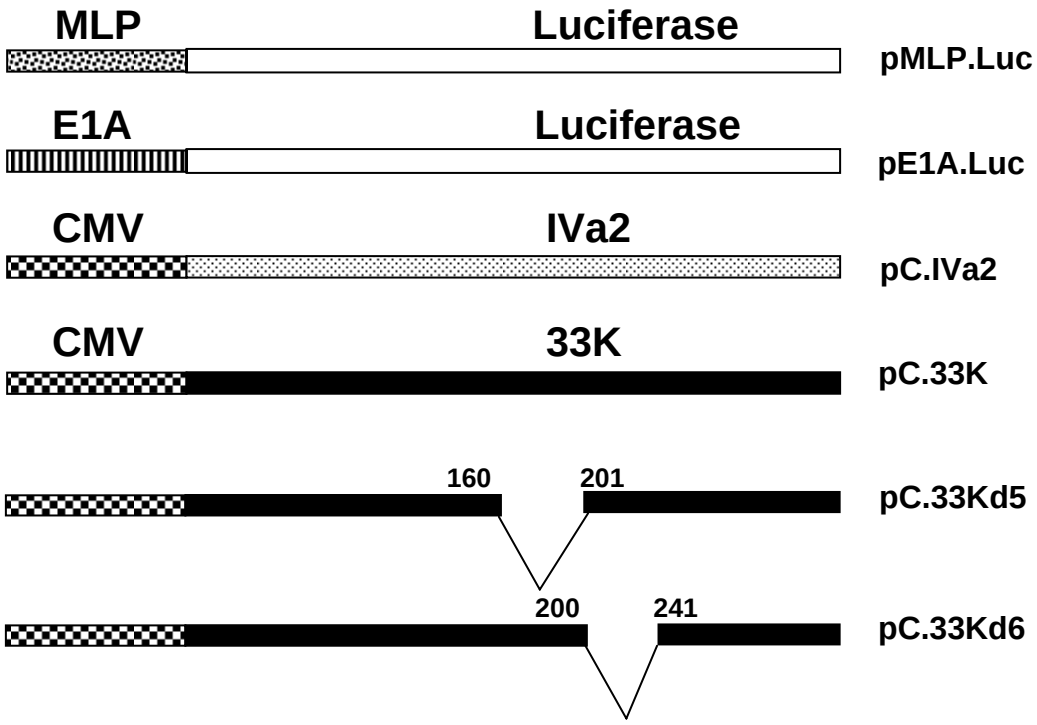


Fig. 3.7. Transactivation of MLP by 33K. **(A)** Schematic representation of plasmids DNAs. The thin lines represent deleted sequences. Numbers above the bars denote residue numbers of BAdV-3 33K. The name given to each plasmid is shown on the right.

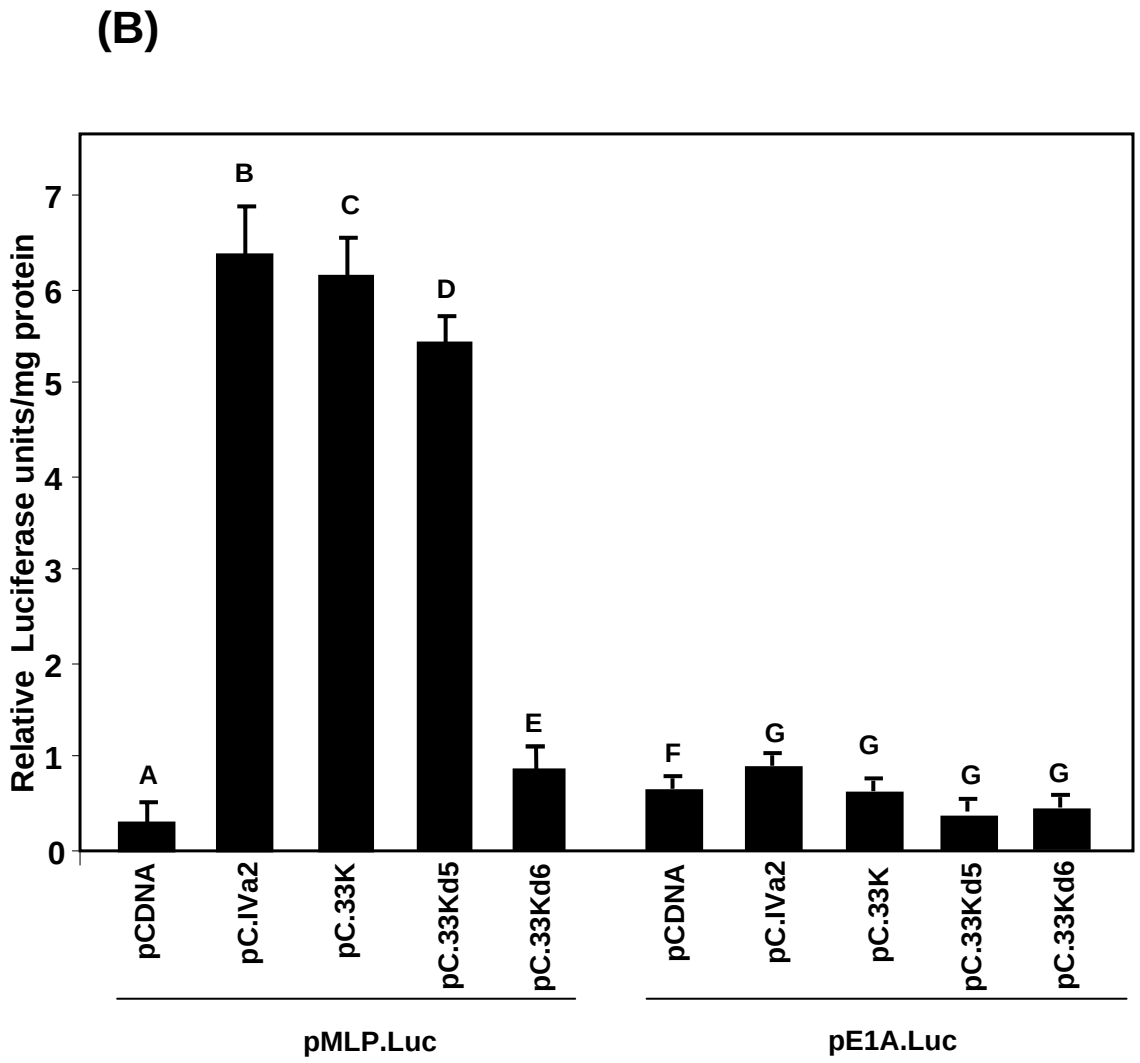


Fig. 3.7. Transactivation of MLP by 33K. (B) Luciferase assay. Monolayers of HeLa cells were transfected with pMLP.Luc or pE1A.Luc plasmid alone or together with indicated individual plasmid DNAs. The cells were collected 48 hrs post transfection and analysed for luciferase activity. Values are expressed as relative light units (RLU). Relative luciferase units (means from three independent experiments) are represented with corresponding standard deviations. Presence of 33K activated the MLP resulting in luciferase expression that was significantly higher than pGL-MLP alone. The statistical differences ($P < 0.05$) are indicated by letters at the top of each bar. B is different than A; C, D are different than A and E; no difference between Cand D, A and E; No difference between F and G.

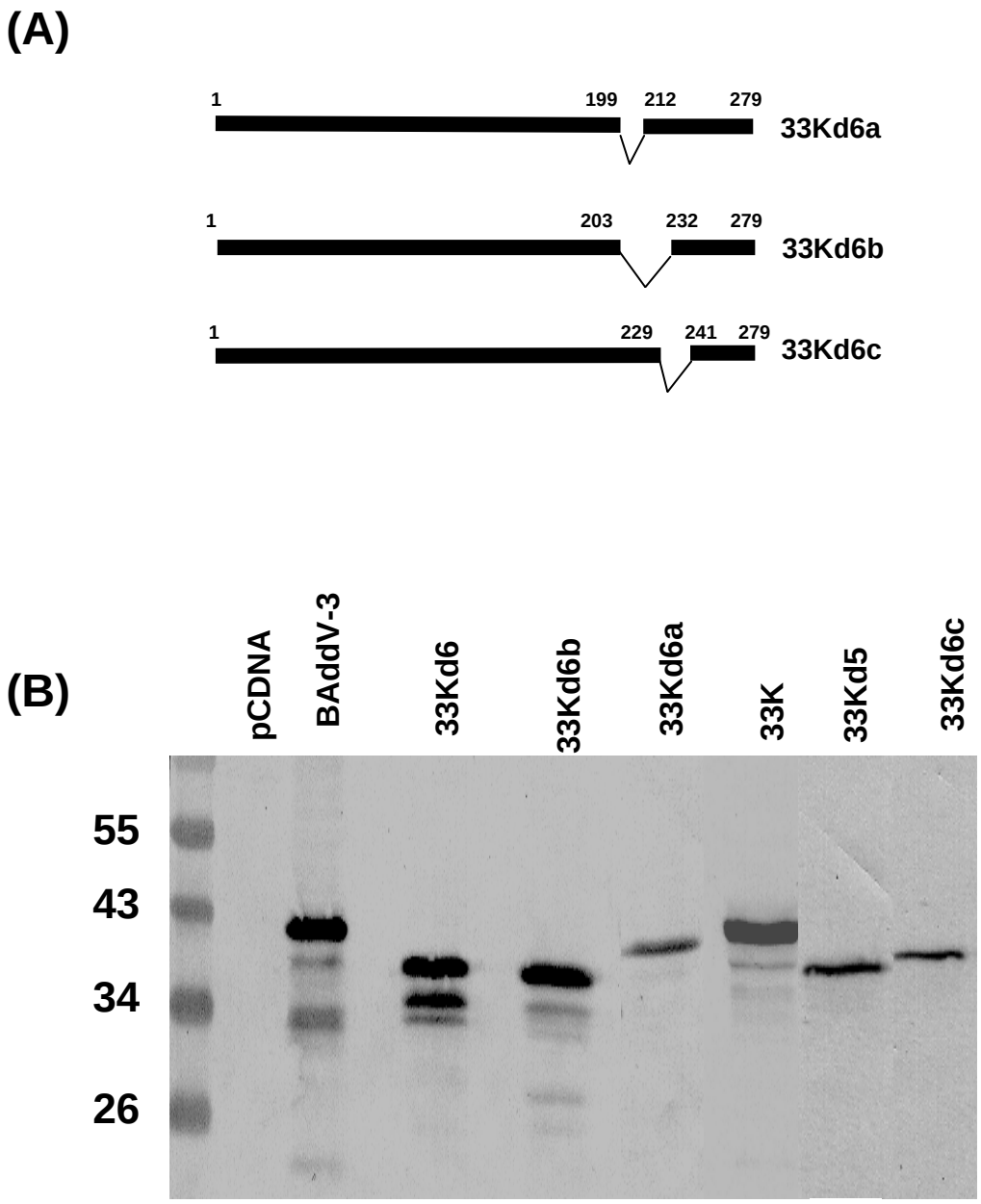


Fig. 3.8. Identification of transactivation domain of 33K. (A). Schematic representation of plasmid DNAs. The thick bars represent coding sequences for BAdV-3 33K. The thin lines represent deleted sequences. Numbers above the bars denote amino acid numbers for BAdV-3 33K. The name given to each protein is shown on the right. **(B)** Western blot. Proteins from the lysates of BAdV-3 infected MDBK or plasmid DNA transfected 293T cells were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-33Kp serum The position of the molecular weight markers (M) in kDa is shown to the left of the panel.

(C)

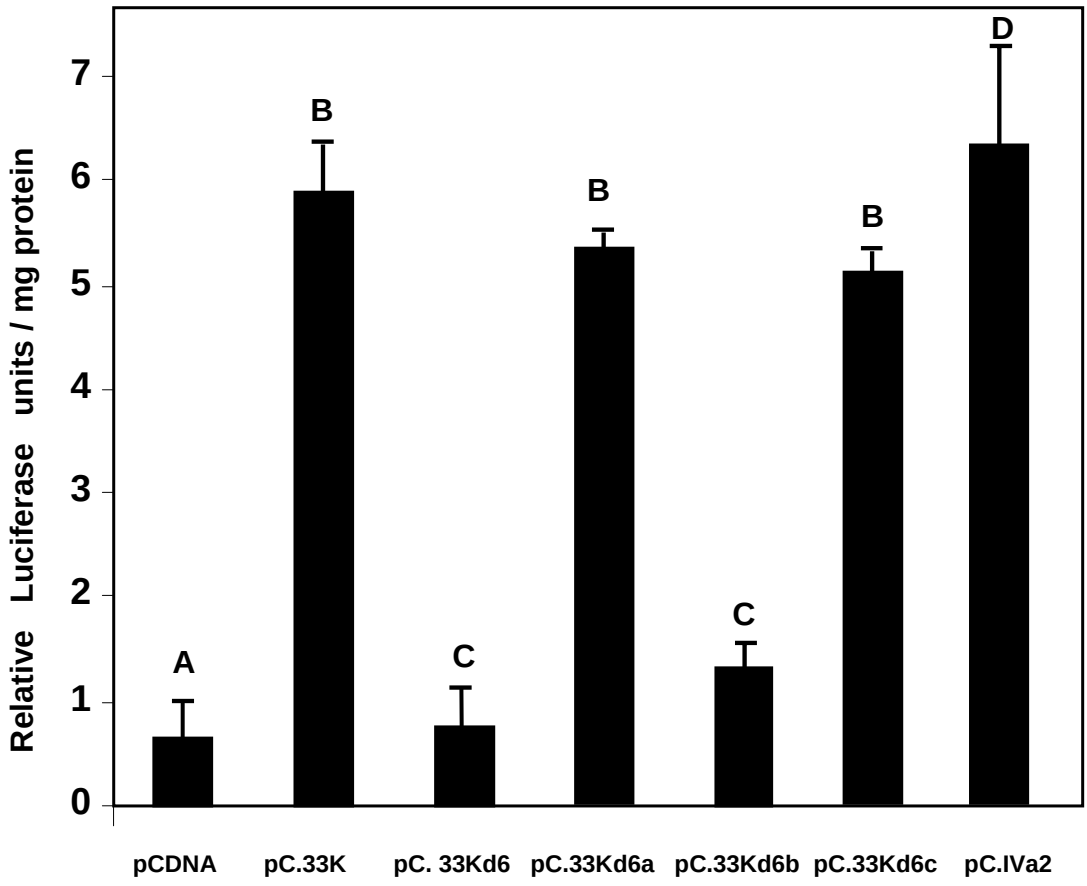


Fig. 3.8. Identification of transactivation domain of 33K. (C) Luciferase assay. Monolayers of Hela cells were transfected with pMLP.Luc together with indicated individual plasmid DNAs. The cells were collected 48 h post transfection and analyzed for luciferase activity. Values are expressed as relative light units (RLU). Relative luciferase units (means from three independent experiments) are represented with corresponding standard deviations. The statistical differences ($P < 0.05$). are indicated by letters at the top of each bar. B is different than A or C; D is different from A)

significantly reduced the transactivation of MLP. These results suggest that amino acid 204-231 of 33K are involved in transactivation of MLP.

3.4 Discussion

The L6 region of BAdV-3 encodes non-structural (33K and 100K) and structural (pVIII) proteins (Reddy et. al, 1998). Earlier, we reported that BAdV-3 33K detected as three proteins of 42, 38 and 33kDa in infected cells appear to be required for capsid assembly and efficient DNA capsid interaction (Kulshrestha et al., 2004). However, recent report suggest that 33K and 22K proteins are produced from spliced and unspliced forms of L4 transcripts of HAdV-2, respectively (Ostapchuk et al., 2006). Since little is know about the existence of different forms of 33K in BAdV-3 infected cell, we sought to analyze this in detail. Here, we report further characterization, cellular distribution and putative functions of 33K protein produced from spliced L6 mRNA of BAdV-3.

Analysis of our earlier data (Kulshrestha et al., 2004) suggested that antisera, generated against C-terminus 197 amino acids of putative 22K protein could recognize both 33K and 22K proteins in BAdV-3 infected cells. Thus, reduced formation of mature virions in mutant BAdV-3 infected cells (Kulshrestha et al., 2004) could be due to inactivation of both 33K and 22K proteins. The present report confirm that both spliced and unspliced mRNAs encoding 33K and 22K proteins, respectively also could be detected in BAdV-3 infected cells. The spliced and unspliced mRNA are predicted to encode proteins of 279 and 274 amino acids respectively. Similar spliced and unspliced mRNAs encoding 33K and 22K respectively, have been detected in other adenoviruses (Ostapchuk et al., 2006; Ali et al., 2007).

Although 33K mRNA is predicted to encode a protein of 279 amino acids, 33K specific antisera detected five major proteins of 42 kDa, 39 kDa, 37 kDa, 21 kDa (appears doublet) and 19 kDa, and three minor proteins of 35 kDa, 25 kDa and 23 kDa. It is possible that different forms of 33K are generated by different mechanisms including translation from different ATG codons and by alternate splicing. Analysis of our data suggests that 25 kDa protein appears to be translated from third ATG (amino acid 87). Similarly 39 kDa protein appears to be generated by alternative splicing. Although we have not detected such mRNA in BAdV-3 infected cells, such rare mRNA has been

detected in HAdV-5 infected cells (Tormanen et al., 2006).

Anti-22Kp serum detected three proteins of 41 kDa, 39 kDa and 37 kDa in BAdV-3 infected cells. Surprisingly, anti-22Kp serum did not detect such proteins in cells transfected with 22K expression plasmid. It is possible that 22K expressed in transfected cells is translated from a spliced mRNA. Several observations support this prediction. First, anti-33Kp detects a protein of 39 kDa in cells transfected with 22K expression plasmid. Secondly, similar protein of 39 kDa is detected in cells transfected with 22K expression plasmid containing insertion of a stop codon in the region spliced out in 33K. Third, anti-22Kp serum detects proteins of 39 kDa and 37 kDa in cells transfected with 22K expression plasmid containing mutated splice accepter/ donor sites but not in cells transfected with 22K expression plasmid. Fourth, as expected anti-22Kp serum did not detect any protein in cells transfected with 22K expression plasmid containing mutated splice acceptor /donor sites and stop codon in the region spliced out in 33K. Earlier, conflicting observations have been reported regarding 22K of HAdV-5 (Ostapchuk et al., 2006). It has been suggested that different molecular weight 22K could be detected due to anomalous migration of proteins in SDS-PAGE (Jansen-Durr et al., 1989; Ostapchuk et al., 2006). Since 22K specific antisera has not been used to determine the molecular weight(s) of 22K protein(s) in HAdV-5 infected cells (Ostapchuk et al., 2006), we believe that like BAdV-3, different forms of 22K are expressed in the HAdV-5 infected cells.

The 33K proteins are detected predominantly in the nucleus of BAdV-3 infected cells. Although proteins less than 40 kDa in size can diffuse passively into the nucleus (Pante and Aebi, 1996), it is unlikely that BAdV-3 33K protein enters nucleus by simple diffusion mechanisms. Support for this comes from the fact that EYFP (a cytoplasmic protein) fused to 33K is predominantly localized to the nucleus of infected cells. Secondly, nuclear transport by diffusion mechanism is expected to result in equal distribution of the protein throughout the cell rather than accumulating in the nucleus.

Analysis of mutant BAdV-3 33K proteins demonstrated that amino acid 201-240 contain potential NLS, which was sufficient to transport predominantly cytoplasmic GFP/β-galactosidase fusion protein. Interestingly, this protein domain contains a pseudo RS domain (three RS motifs and one SR motif; Fig. 3.9). The RS domain of proteins has been shown to be involved in splicing (Caceres et al., 1994; Cazalla et al. 2002;

Hamelberg et al, 2007). Interestingly, observed RS motifs are conserved in HAdV-5 33K and are suggested to be involved in activating IIIa splicing (Tormanen et. al, 2006**)**.

The RS domain has been demonstrated to act as a NLS and is solely responsible for nuclear localization of SR proteins (Caceres et al., 1994; Cazalla et al., 2002; Hamelberg et al, 2007). The nuclear transport is mediated by interaction with SR protein specific nuclear import receptor (Lai et al., 2001). The phosphorylation of RS domain is required for interaction of SR proteins with SR protein specific nuclear import receptor(s) (Lai et al., 2000)

Several evidences support the observation that the RS domain (Fig. 3.9) is not solely responsible for the nuclear localization of BAdV-3 33K protein. First, RS domain deleted 33K is not located predominantly in the cytoplasm. Secondly, 33K protein does not interact with SR protein nuclear import receptor TRN SR2 (data not shown). Thirdly, RS domain of 33K is not phosphorylated. Our results suggest that amino acid 201-240 contain multiple NLS required for efficient nuclear localization of BAdV-3 33K. Earlier, adenovirus intermediate proteins (pIX and IVa2) have been shown to act as transcriptional activators for MLP, E1A and E4 promoters (Lutz and Kedinger, 1996; Lutz et al., 1997; Parks, 2004; Rosa-Calatrava et al., 2001). Similarly, BAdV-3 33K appears to act as a transcriptional activator for MLP as it shows significant increase in the expression of luciferase protein when expressed in cells transfected with pMLP.Luc. In contrast, there was no increase in the expression of luciferase protein when BAdV-3 33K was expressed in cells transfected with pE1A.Luc. Our analysis suggests that amino acid 201 to 240 are involved in the transactivation of MLP. Viral transcription regulators can act a) by recognizing specific DNA sequences (Lutz et al, 1997), by interacting with cellular DNA binding proteins, and \ or c) by acting as cellular co-factors (Lutz and Kedinger, 1996; Perez-Romero et al., 2005). It is possible that 33K transactivates MLP by recognizing sequences unique to MLP. Alternatively, it is possible that 33K (provides transactivation function) binds to other cellular protein (provides specific DNA binding function) with a specific DNA binding activity. Further experiments are needed to prove these speculations.

Fig. 3.9. Amino acid homology of BAdV-3 33K like proteins. Alignment of deduced amino acid sequences of BAdV-3 33K homologs with those of CAdV-2 (GeneBank Accession # AC_000020), HAdV-2 (GeneBank Accession # AC_000007), HAdV-5 (GeneBank Accession # AC_000008), PAdV-3 (GeneBank Accession # AC_000009), OAdV-7 (GeneBank Accession # NC_004037), and HAdV-35 (GeneBank Accession # AC_000019). The potential RS domain is overlined. Conserved amino acid regions are shaded. Leucine residues of potential leucine zipper are marked by filled circle.

The leucine zipper motif has been shown to be essential for a) protein dimerization, required for DNA binding (Landschulz et al., 1988), b) transactivation, and repression of viral proteins (Goodwin et al., 2000), and c) protein-protein interaction and transactivation (Geisberg et al., 1994; Sanchez et al., 2000). Analysis of 33K proteins of different animal and human adenoviruses revealed a highly conserved domain located between amino acids 195 to 255 of BAdV-3 (Fig. 3.9). Moreover, this conserved domain contains a leucine zipper motif (amino acids 217-240; Fig.3.9), which may help in dimerization of 33K required for DNA binding. Analysis of mutant BAdV-3 33K proteins demonstrated that deletion of amino acids 213-232, which disrupts the leucine zipper motif are critical for transcriptional activity of 33K. Further experiments are needed to prove the importance of specific residues of leucine zipper motif in DNA binding and transcriptional activation of MLP.

4.0 INTERACTION OF BOVINE ADENOVIRUS-3 33K PROTEIN WITH OTHER VIRAL PROTEINS

4.1 Introduction

Adenoviruses contain a non-enveloped, double-stranded DNA genome, which is organized into complex transcriptional units comprising of early, intermediate and late regions in members of genus *Mastadenovirus* (Russell, 2000). Expression of the early and intermediate transcription units precedes the onset of viral DNA replication. However, expression of the late transcription unit is dependent on the initiation of viral DNA replication and depends on the expression of the major late transcription unit (MLTU), which is controlled by the major late promoter (MLP) (Thomas and Mathews, 1980).

We have been carrying out molecular characterization of bovine adenovirus (BAdV) 3 (Lehmkuhl et al., 1975) with the aim of developing it as a vaccine delivery vehicle for animals and humans (Rasmussen et al., 1999; Tikoo, 2001; Kulshreshtha et al., 2004; Xing and Tikoo, 2007). Like other members of *Mastadenovirus*, the BAdV-3 genome is organized into early, intermediate, and late regions (Reddy et al., 1998). The late region of BAdV-3 genome is organized into seven regions L1–L7 (Reddy et al., 1998). The L6 region of late transcription unit of BAdV-3 encodes 33K and 22K protein (Reddy et al., 1998; Kulshreshtha et. al, 2004). The L6 33K protein is a product of a spliced transcript, while 22K protein is translated from the unspliced form of this transcript (Reddy et. al, 1998; Kulshrestha and Tikoo, unpublished data). The 33K and 22K proteins share the N-terminus region of 138 amino acids (Chapter 3.0).

Adenovirus 33K protein acts as an alternative RNA splicing factor (Tormanen et al., 2006) and a transcriptional activator (Ali et al., 2007), and plays a role in viral capsid assembly as well as efficient capsid DNA interaction (Fessler and Young, 1999; Finnen et al., 2001; Kulshreshtha et al., 2004). Recently, adenovirus 22K protein has been shown to be involved in the packaging of adenovirus genome (Ostapchuk et al., 2006). The conservation of 33K protein in *Mastadenoviruses* together with its multiple functions in the adenovirus life cycle suggests that 33K might be involved in the interaction with

other viral or cellular proteins. In this report, we demonstrate that 33K interacts with 100K and protein V (pV) during the course of BAdV-3 infection.

4.2 Materials and methods

4.2.1 Cell lines and virus

Madin-Darby bovine kidney (MDBK) cells were cultured in minimal essential medium (MEM) with 10% fetal bovine serum (FBS). The wild-type BAdV-3 was cultivated in MDBK cells as described previously (Reddy et al., 1999).

4.2.2 Antibodies

Production and characterization of antibodies specific to BAdV-3 33K protein have been described earlier (Kulshreshtha et al., 2004). Details concerning the production of antibodies specific to 100K and pV will be described elsewhere. Briefly, anti-100K raised against synthetic peptides recognizes a protein of 130 kDa in BAdV-3 infected cells, while anti-pV serum raised against synthetic peptides recognizes a protein of 56 kDa in BAdV-3 infected cells.

4.2.3 Plasmid construction

The plasmid vectors were constructed as per standard procedures using the DNA modifying enzymes and restriction enzymes (Sambrook and Russell, 2000).

A) Construction of yeast expression plasmids. Individual BAdV-3 genes acting as bait or prey were PCR amplified using specific primer sets (Table 4.1) and plasmid pFBAV302 (Zakharcthouk et al., 1998) DNA as a template. The appropriate PCR amplified DNA fragments were digested with restriction enzymes (Table 4.1) and ligated to restriction enzyme digested plasmids pGBKT7 or pGADT7 (Clontech: Table 4.1).

i*) Construction of p33Kd1.* A 213 bp fragment was amplified by PCR using primers P1and P2 (Table 4.2), and plasmid pGBK-33K DNA as a template. Similarly, a 1000 bp fragment was isolated by PCR using primers P3 and P4 (Table 4.2), and plasmid pGBK-33K DNA as a template. In a third PCR reaction, both amplified fragments were annealed and external primers P1 and P4 (Table 4.2) were used to PCR across to give a final 1.2 kb amplicon. This PCR product was digested with *Nde*I- *Eco*RI and ligated to *Nde*I- *Eco*RI

digested pGBKT7 creating plasmid p33Kd1.

ii) *Construction of p33Kd2.* A 7.9 kb SacII-AatII fragment of plasmid pGBKT7-33K was isolated and ligated to oligos O1 and O2 (Table 4.2) to create plasmid p33Kd2.

iii) *Construction of p33Kd3.* A 235 bp fragment was amplified by PCR using primers P5 and P6 (Table 4.2), and plasmid pGBK-33K DNA as a template. Similarly, a 500 bp fragment was isolated by PCR using primers P7 and P4 (Table 4.2), and plasmid pGBK-33K DNA as a template. In a third PCR reaction, both amplified fragments were annealed and external primers P5 and P4 (Table 4.2) were used to PCR across to give a final 735 bp amplicon. This PCR product was digested with *Nde*I- *Eco*RI and ligated to *Nde*I-*Eco*RI digested pGBKT7 creating plasmid p33Kd3.

iv) Construction of p33Kd4. A 360 bp fragment was amplified by PCR using primers P5 and P8 (Table 4.2), and plasmid pGBK-33K DNA as a template. Similarly, a 375 bp fragment was isolated by PCR using primers P9 and P4 (Table 4.2), and plasmid pGBK-33K DNA as a template. In a third PCR reaction, both amplified fragments were annealed and external primers P5 and P4 (Table 4.2) were used to PCR across to give a final 735 bp amplicon. This PCR product was digested with *Nde*I- *Eco*RI and ligated to *Nde*I-*Eco*RI digested pGBKT7 creating plasmid p33Kd4.

v) Construction of p33Kd5. A 490 bp fragment was amplified by PCR using primers P5 and P10 (Table 4.2), and plasmid pGBK-33K DNA as a template. Similarly, a 850 bp fragment was isolated by PCR using primers P11 and P12 (Table 4.2), and plasmid pGBK-33K DNA as a template. In a third PCR reaction, both amplified fragments were annealed and external primers P5 and P4 (Table 4.2) were used to PCR across to give a final 735 bp amplicon. This PCR product was digested with *Nde*I- *Eco*RI and ligated to *Nde*I- *Eco*RI digested pGBKT7 creating plasmid p33Kd5.

vi) Construction of p33Kd6. A 610 bp fragment was amplified by PCR using primers P5 and P13 (Table 4.2), and plasmid pGBK-33K DNA as a template. Similarly, a 730 bp fragment was isolated by PCR using primers P14 and P12 (Table 4.2), and plasmid pGBK-33K DNA as a template. In a third PCR reaction, both amplified fragments were annealed and external primers P5 and P4 (Table 4.2) were used to PCR across to give a final 735 bp amplicon. This PCR product was digested with *Nde*I- *Eco*RI and ligated to *Nde*I- *Eco*RI digested pGBKT7 creating plasmid p33Kd6.

Table 4.1: List of primers used in the construction of yeast plasmids

Gene	Primer sequence	PCR Fragment	Ligated to plasmid
33K	5'GGAATTCCATATGATGAAACCCCGCAGCATGTCG		
	5'-GGAATTCTTAGGCGGGTCCGGATTCG	0.840kb$^{\Psi}$	pGBKT7$^{\Psi}$
100K	5'-GGAATTCCATATGATGGCAGAGAAAGGCAGTG		
	5'-GGAA TTCCTACTCTTCTTGCCCTGG	2.500kb$^{\Psi}$	pGADT7$^{\Psi}$
52K	5'-GGAATTCCATATGATGATGCATCCCGCTTTACG		
	5'-GGAATTCTCAGAATCGCCAGTGGTTAG	1.000kb$^{\Psi}$	pGADT7$^{\Psi}$
pIX	5'-GGAATTCCATATGAACATGGCCGAGGAAGG		
	5'-GGAATTCTTAAACAAAGGGGTTAACTTGG	0.375kb$^{\Psi}$	pGADT7$^{\Psi}$
IVa2	5'-GGAATTCCATATGATGCTGGATGGAGATGTAC		
	5'-GGAATTCTCAATAAAATTCTTTATTTTTCCTG	1.100kb$^{\Psi}$	pGADT7$^{\Psi}$
pIII	5'-GGAATTCCATATGATGCTCCAGCCCGAACTGC		
	5'-GGAATTCTTAAAACGTGCGGCTAGATAGC	1.400kb$^{\Psi}$	pGAD
DBP	5'-CGGGGGTACCCATATGATGAATCGCAGCGGTGA		
	5'- CGCGGATCCTTAAAACAAAGAGTCATCTGC	1.300kb$^{\Psi}$	pGADT7$^{\Psi}$
pV	5'-GGAATTCATGGCCTCCTCTCGGTTGATTAAA		
	5'-CGGGATCCCTAGGGTGATAGCGCACGCC	1.200kb$^{\Omega}$	pGADT7$^{\Omega}$
pVI	5'- CCGGAATTCATGGACGAATACAATTACGCG		
	5'- CGCGGATCCGCGGCCGCTCAATAGCACCGCCG GCG	0.791kb$^{\Omega}$	pGADT7$^{\Omega}$
pVII	5'- CCGGAATTCATGCGTTTTAATCTGGGCAG		
	5'- CGCGGATCCGCGGCCGCTCAGAGGCCCACG ATG TCATTC	0.520kb$^{\Omega}$	pGADT7$^{\Omega}$
pTP	5'- CCGGAATTCATGTTTTTTGCAGAGCGCG		
	5'- CGCGGATCCGCGGCCGCTTAAAGGGGACGTC GAGG	1.900kb$^{\Omega}$	pGADT7$^{\Omega}$
pX	5'- CCGGAATTCATGAGTCCCCGCGGAAATC		
	5'- CGCGGATCCCTCGAGCTATTTGTTGTGGGCC GCC	0.245bp$^{\Omega}$	pGADT7$^{\Omega}$

Restriction enzyme sites are underlined; $^{\Psi}$digested with *Nde*I-*Bam*HI;
$^{\Omega}$digested with *Eco*RI-*Bam*HI;

Table 4.2: List of primers used in the construction of 33K deletions

Primer	DNA Sequence
P1	5'-GATGCCGTCACAGATAGATAGATTG-3'
P2	5'-CATCATATGCAGGTCCTCCTC-3'
P3	5'-GCTTCTCTGAATCCCACCGC-3'
P4	5'-GGAATTCTTAGGCGGGTCCGGATTCG-3'
P5	5'-GGAATTCCATATCATGAAACCCCGCAGCATGTCG-3'
P6	5'-CCTACTCTTGTCCGTGAAGTCGCTGAAA-3'
P7	5'-TTCACGGACAAGAGTAGGTGGGACCAGC-3'
P8	5'-AGGGCGAGGCTTGCCCTGGCCCTTCTTG-3'
P9	5'-CAGGGCAAGCCTCGCCCTCCTCCTCTT-3'
P10	5'-GAGAGTGGGCGCGCTGGCACTCCGC-3'
P11	5'-GCCAGCGCGCCCACTCTCTATGCCATAT-3'
P12	5'-GAGTGAGCTGATACCGCTC-3'
P13	5'-GGTTCGCTGAAAGATCAGCTCCCGAAGCTT-3'
P14	5'-CTGATCTTTCAGCGAACCCTAGCAGACT-3'
P17	5'-GGCGGGTCCGAGCTGTTCCTCCCTTGTTGT-3'
P18	5'-GAACAGCTCGGACCCGCCTAAGAATTCC-3'
P19	5'-GGAATTCTTAGGCGGGTCC-3'
O1	5'-GGCTCAGGCTTCGGAGGGCCAACAGCTGCCGC CACAGACAGCGGGGCTGCAGCCGAGCAAGAGG-3'
O2	5'-CTACTACCTTCCTCACAGCCCTCTTGCTCGGCTG CAGCCCCGCTGTCTGTGGCGGCAGCTGTTGGCC CTCCGAAGCCTGAGCCGC-3'

vii) Construction of p33Kd7. A 730 bp fragment was amplified by PCR using primers P5 and P17 (Table 4.2), and plasmid pGBK-33K DNA as a template. Similarly, a 620 bp fragment was isolated by PCR using primers P12 and P18 (Table 4.2), and plasmid pGBK-33K DNA as a template. In a third PCR reaction, both amplified fragments were annealed and external primers P5 and P19 (Table 4.2) were used to PCR across to give a final 735 bp amplicon. This PCR product was digested with *Nde*I- *Eco*RI and ligated to *Nde*I- *Eco*RI digested pGBKT7 creating plasmid p33Kd7.

B) Construction of plasmid pGADT7 plasmids. A 375 bp fragment (pIX ORF), 1.1 kb fragment (IVa2 ORF) and 1kb fragment (52K ORF) and were isolated by PCR amplification [using specific primers (Table 4.1) and plasmid pFBAV302 DNA as a template], digested with *Nde*I-*Eco*RI and individually ligated to 7.9 kb *Nde*I-*Eco*RI digested plasmid pGADT7 creating plasmids pGADT7-pIX, pGADT7-pIVa2 and pGADT7-52K, respectively. Similarly, a 1.2kb fragment containing pV was isolated by PCR amplification [using specific primers (Table 4.1) and plasmid pFBAV302 DNA as a template] digested with *Eco*RI-*Bam*HI and ligated to *Eco*RI-*Bam*HI digested plasmid pGADT7 creating plasmid pGADT7-pV.

C) Construction of plasmids pGEX plasmids. A 830 bp *Nde*I-*Eco*RI fragment containing spliced 33K gene excised from plasmid pGBKT7-33K was blunt end repaired with T4 DNA polymerase and ligated to *Sma*I digested plasmid pGEX-5X-1 to create plasmid pGEX-33K. A 1.2 kb *Eco*RI-*Bam*HI fragment containing pV gene, excised from plasmid pGADT7-pV, was blunt end repaired with T4 DNA polymerase and ligated to *Sma*I digested plasmid pGEX-5X-1 to create plasmid pGEX-pV. Similarly, a 2.5 kb *Nde*I-*Eco*RI fragment containing 100K gene excised from plasmid pGBKT7-100K was blunt end repaired with T4 DNA polymerase and ligated to *Sma*I digested plasmid pGEX-5X-1 to create plasmid pGEX-100K.

4.2.4 Yeast two hybrid system

The Matchmaker two hybrid systems 3 using *Saccharomyces cerevisiae* (Clontech) was used to detect the interactions between 33K protein and other viral proteins. These interaction studies involved the use of yeast expression plasmids pGBKT7 (bait plasmid) and prey plasmid pGADT7 (Clontech). The bait and prey

constructs were co-transformed in the yeast strain AH109 as per Clontech protocol and grown on the SD/-Leu/-Trp medium (low stringency selection) at 30°C to select the DNA BD and AD plasmid DNAs. These co-transformants were further screened for HIS3 expression by growing transformed yeast on SD/-Leu/-Trp/-His medium (medium stringency selection). Subsequently, the His positive colonies were further screened for ADE2 and MEL1 expression by growing on high stringency selection medium (SD/-Leu/-Trp/-His/-Ade/X-alpha-Gal).

4.2.5 GST pulldown assays

The plasmids pGEX-5X-1, pGEX-33K , pGEX-pV and pGEX-100K were individually transformed in *E. coli* BL21 cells. The GST alone, GST-33K and GST-100K fusion proteins were induced by 0.25mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Gibco-BRL) and immobilized on Glutathione-Sepharose beads (Pharmacia) as described elsewhere (Zhou and Tikoo, 2001). The identities of the proteins were analyzed by Western blot using anti-GST serum.

Plasmid DNA (pGADT7-V, pGADT7-100K, pGBKT7-33K, p33Kd3, p33Kd4 or p33Kd5), were *in vitro* transcribed and translated using TNT T7 quick-coupled transcription / translation kit (Promega) in the presence of 30 μCi of [^{35}S]methionine. For *in vitro* binding, 10μg of GST or GST-33K fusion protein on Glutathione-Sepharose 4B beads was incubated with 5μl of *in vitro* translated pV (pGADT7-V) or 100K (pGADT7-100K) proteins in a final volume of 500μl of binding buffer (30mM Hepes pH 7.4, 50mM KCL, 0.5% Tween 20, 0.5% non fat dry milk, 2mM PMSF). Similarly, 10μg of GST or GST-100K fusion on Glutathione-Sepharose 4B beads was incubated with 5μl of *in vitro* translated protein 33Kd3 (p33Kd3), 33Kd4 (p33Kd4) in a final volume of 500 μl of binding buffer. After incubating at 4°C for 4 hours with rotation, the beads were then washed four times with the binding buffer. The bead bound proteins were separated by SDS-PAGE and analyzed by autoradiography.

4.2.6 *In vitro* translation

Plasmid DNA (pGADT7-pV, pGBKT7-33K, pGBKT7-33Kd2 or pGBKT7-33Kd3) were *in vitro* transcribed and translated using TNT T7 quick coupled

Transcription / Translation kit (Promega). Equal amounts of non labeled proteins were mixed at 4^0C for 4-6 h. Proteins were immunoprecipitated with protein specific serum, separated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. The separated proteibns were probed by Western blot as described (Kulshrestha et al., 2004) using protein specific antisera.

4.2.7 Immunoprecipitation / Western blotting

Madin Darby bovine kidney (MDBK) cells infected with wild-type BAdV-3 at an MOI of 5 were harvested at 36 hrs post-infection. The infected cells lysed with triton lysis buffer (1% Triton X-100, 10 mM HEPES [pH 7.4], 2 mM EDTA, 2 mM sodium orthovanadate, 0.1% β-mercaptoethanol, protease inhibitors[2mM PMSF and 1 μg each of leupeptin and pepstatin per ml]) were incubated overnight at 4^oC with preimmune serum, anti-pV serum or anti-100K serum. The protein A agarose bead were then added and mixtures were incubated for another 4 hrs at 4°C, before washing the beads four times with lysis buffer. After addition of 2X SDS-sample buffer, beads were boiled for two minutes, subjected to SDS-PAGE and transferred to Immobilon-P membrane (Millipore Corporation). Finally, the membrane was probed with anti-33K serum and visualized by treating the membrane with BCIP/NBT solution (Sigma). Similarly, BAdV-3 infected cell lysates were immunoprecipitated with preimmune serum as well as anti-33K serum and probed with anti-pV serum as well as anti-100K serum.

4.3 Results

4. 3.1 Yeast two hybrid system

To identify the interactions of 33K protein with other viral proteins, matchmaker GAL4 yeast two hybrid system was used. In this system, the bait and prey plasmids are co-transformed in the yeast and the resulting interaction between bait and prey proteins, are identified by the transcriptional activation of reporter genes like *ADE2*, *HIS3*, *lacZ*. The RT-PCR amplified 33K was fused in-frame to the GAL4 DNA binding domain (aa 1–147) of pGBKT7 and used as a bait (Table 4.1). The PCR amplified late viral genes were fused in frame to the GAL4 activation domain (aa 768–881) in the prey vector pGADT7 and used as a prey (Table 4.1). These constructs were analyzed by restriction enzymes analysis of DNA and confirmed to be in-frame by DNA sequencing.

Both the pGBKT7- and the pGADT7- derived constructs were co-transformed in the yeast and the interactions were screened though medium and high selection stringencies (Singh et al. 2005). Interaction between two proteins led to the activation of genes, which allowed the growth on media lacking histidine (His) and adenine (Ade) (Singh et al., 2005) The higher stringency selection revealed the blue colonies upon co-transforming pGBKT7-33K along with pGADT7- pV or pGADT7-100K (Fig 4.1). No blue colonies were observed on co-transforming pGBKT7-33K and pGADT7 plasmid in the yeast. These findings suggest that 33K protein may be specifically interacting with pV and 100K protein**.**

To identify the domains of 33K protein involved in these interactions, we constructed plasmids containing deletions in 33K ORF (Fig 4.2A) cloned individually in pGBKT7 plasmid. These deletions were analyzed by restriction enzyme analysis of DNA, DNA sequencing and by Western blot for expression of mutant 33Kd1 to 33Kd7 proteins (Fig. 4.2B). These pGBKT7 plasmids containing 33K deletions were used as bait with plasmid pGADT7-pV or pGADT7-100K as a prey in yeast two hybrid assays. Blue colonies were only observed when mutant 33K interacted with the prey. As seen in Fig. 4.3, all mutant 33K proteins except 33Kd3 (amino acid 81-120 deleted) interacted with pV. These results suggest that the deletion of amino acid 81-120 of 33K disrupted the interaction of 33K with pV. Similarly, all mutant 33K proteins except 33Kd4 (aa 121-160 deleted) and 33Kd5 (aa160-200 deleted) interacted with 100K (Fig 4.3). These results suggested that the deletion of amino acids 121-200 of 33K disrupted the interaction of 33K protein with 100K protein.

4.3.2 33K protein interacts with protein V and 100K protein *in vitro*

To confirm the results of yeast two hybrid analyses, we tested the interaction of proteins *in vitro* using GST pull down assay. The part of ORF 33K (amino acids----- 279) , ORF 100K (amino acids ---- 850) or ORF pV (amino acids ---- 410) was fused in frame to GST in the pGEX-5X-1 plasmid creating the plasmids pGEX-33K, pGEX-100K and pGEX-V respectively. The GST alone, GST-33K, GST-100K or GST-V proteins were induced by IPTG and the expression of these proteins was confirmed by Western blot using anti-GST, anti-33K, anti-100K serum (Fig. 4.4) or anti- pV serum. Compared to

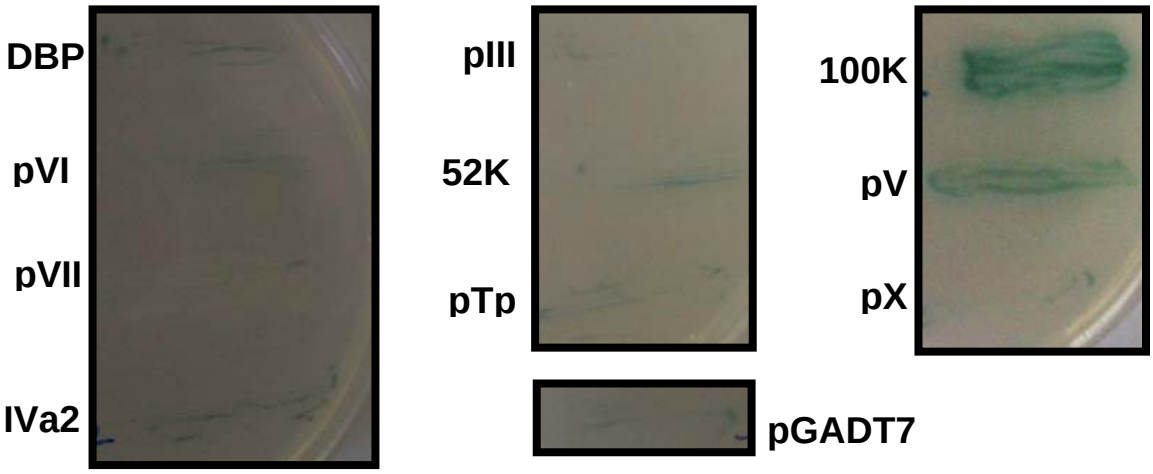
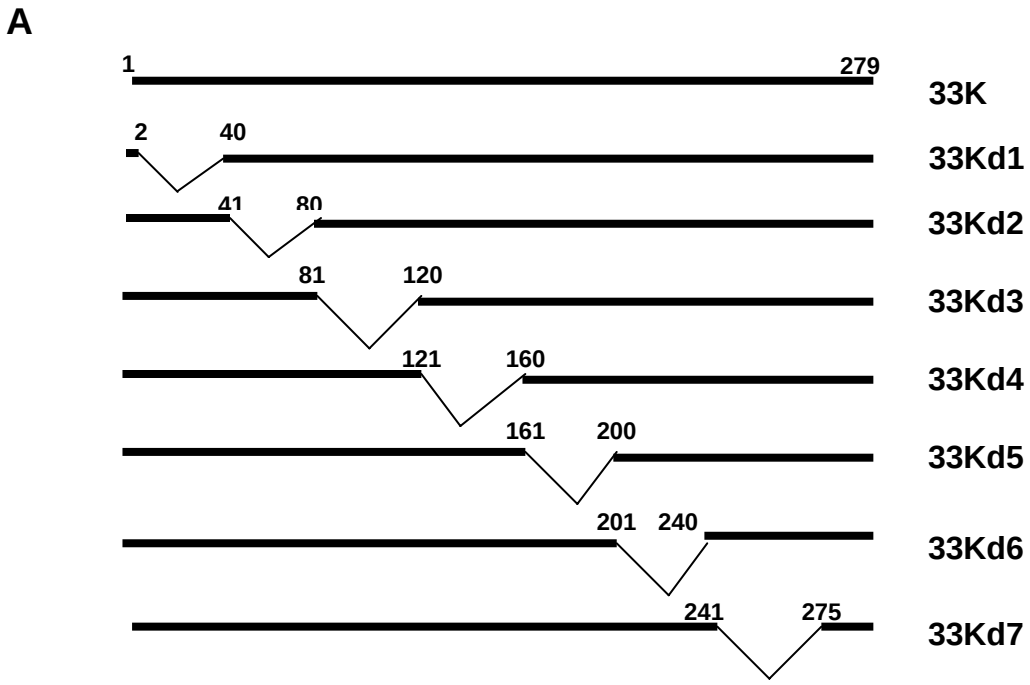


Fig. 4.1. **Yeast two-hybrid analysis**. Plasmid pGADT7 (prey) expressing DBP, pVI, pVII, IVa2, penton, 52K, pTP, 100K, pV or pX, and plasmid pGBKT7 (bait) expressing 33K were co-transformed in yeast strain AH109. The co-transformants were streaked on SD/Leu-/Trp-/His-/Ade-/ X-α-gal-containing plates and incubated at 30°C for 5-7 days. pGADT7 and AD_'T' antigen were used as negative controls.



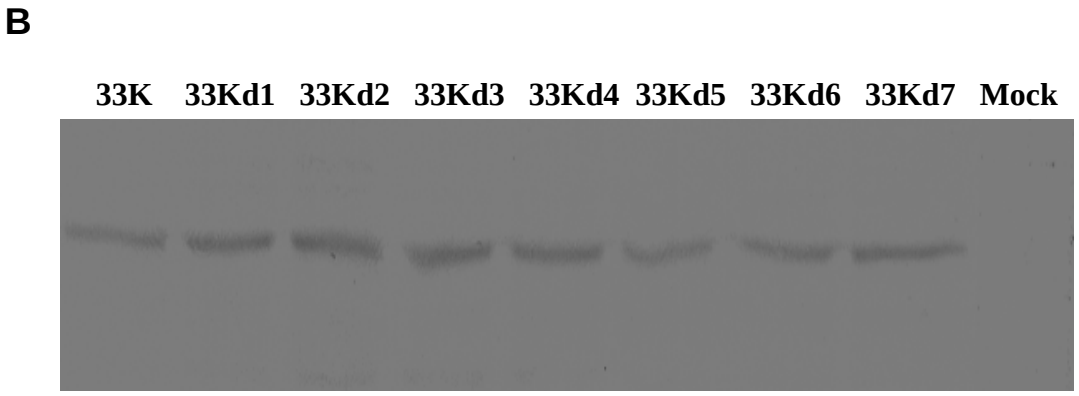


Fig. 4. 2. Analysis of mutant 33K. **A)** Schematic representation of BAdV-3 33K and mutant 33K proteins. **B)** Western blot. Proteins from lysates of AH109 yeast cells transformed with plasmid pGBKT7 (mock), plasmid pGBKT7 expressing 33K or mutant 33K proteins were separated by 10% SDS-PAGE under reducing conditions and transferred to nitrocellulose. The separated proteins were probed in Western blot by anti-33K serum.

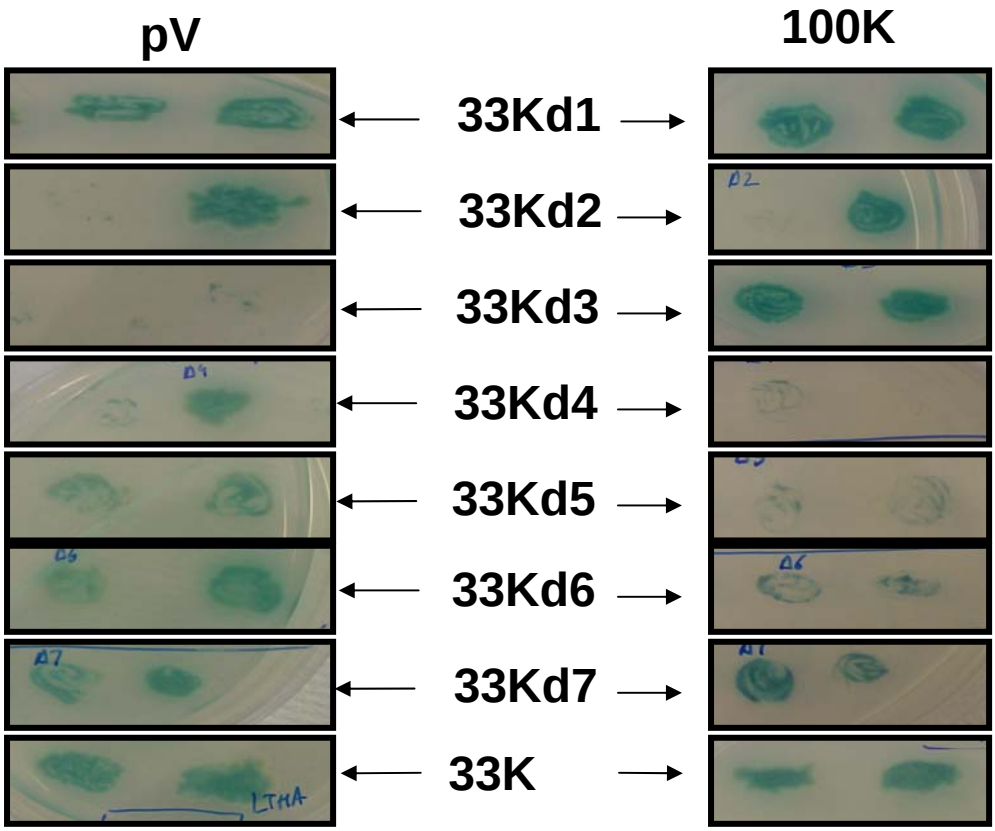


Fig. 4.3. Yeast two hybrid analysis using mutant 33K proteins. Plasmids pGBKT7 expressing 33K, 33Kd1, 33Kd2, 33Kd3, 33Kd4, 33Kd5, 33Kd6 or 33Kd7 (acting as bait) were cotransformed with plasmid pGADT7-100K (prey) or pGADT7-pV (prey) in AH109 yeast. The cotransformants were streaked on SD/Leu-/Trp-/His-/Ade-/ X-α-gal-containing plates and incubated at 30°C for 5-7 days.

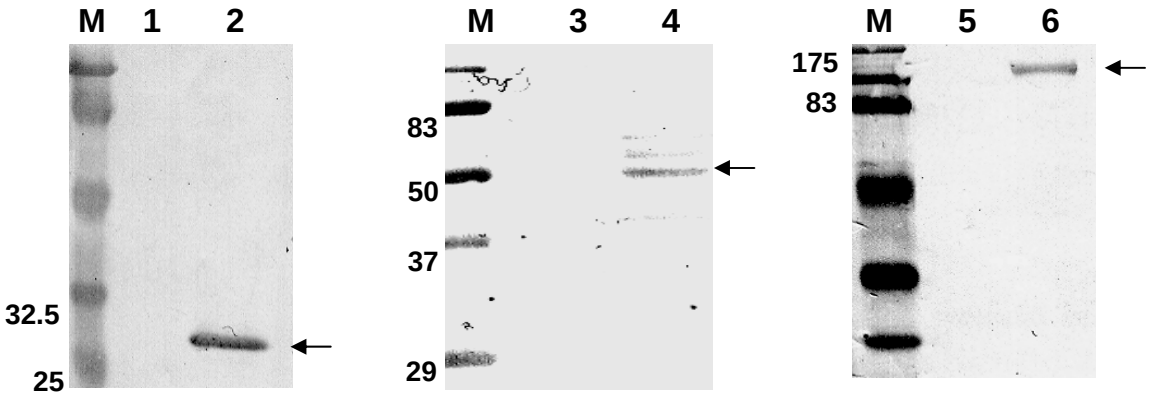


Fig. 4.4. **Immunoblot analysis of Glutathione-Sepharose-immobilized proteins**. Purified GST (lane 2), GST-33K (lane 4) and GST-100K (Lane 6) proteins were separated by 10% SDS–PAGE, transferred to Immobilon-P membrane and analyzed by immunoblotting using anti-GST serum (lane 1,2), anti-33K serum (lanes 3,4) and anti-100K serum (lanes 5,6). Uninfected MDBK cell lysates (lanes 1,3,5) were used as negative controls. The position of the molecular weight markers (M) in kDa is shown to the left of the each panel. Arrows on the right of each panel indicate the position of the identified protein.

other fusion proteins, multiple bands of GST-V fusion protein were detected (data not shown). These results suggested that GST-pV fusion protein is not stable. The induced GST-100K and GST-33K fusion proteins were bound on glutathione sepharose beads as per described (Zhou and Tikoo, 2001).

The plasmids pGADT7-pV, pGADT7-100K, pGBKT7-33K, p33Kd3, p33Kd4 or p33Kd5 containing T7 promoter upstream of protein coding regions were individually transcribed and translated *in- vitro* in the presence of 50 µl of [^{35}S] methionine, using TNT T7-coupled reticulocyte lysate system as described by the manufacturer (Promega).

The GST-fusion proteins bound to glutathione sepharose beads were individually incubated with *in-vitro* translated [^{35}S]-labeled proteins for 6 h at 4^{o}C. After extensive washing with binding buffer, the bead bound products were separated on 10% SDS-PAGE and visualized by autoradiography. As seen in Fig. 4.5A, *in vitro* translated pV interacted with GST-33K protein (Lane 2). Similarly, *in vitro* translated 100K protein interacted with GST-33K protein (Fig. 4.5B, lane 2). No interaction was detected between *in vitro* synthesized pV (Fig. 4.5A) or 100K (Fig. 4.5B) with GST protein alone (Lane 1) demonstrating the specificity of the assay. These results confirm the yeast two hybrid observations and suggest that 33K/pV and 33K/100K interactions are real.

To confirm the domain of 33K protein involved in the interaction with 100K protein, GST pull down assay was carried out as described above. As seen in Fig. 4.6, GST-100K fusion protein interacts with 33K, 33Kd3 and 33Kd4 proteins. No such interaction was observed between GST-100K and mutant 33Kd5 protein (Fig 4.6). These findings confirm the *in vivo* yeast screening analysis and suggest that 161-200 amino acid domain of 33K protein may be involved in the interaction with 100K protein.

Since GST-pV fusion protein was unstable, we carried out an *in vitro* co-immunoprecipitation assay to determine the domain of 33K interacting with pV. The pV, 33K and 33K mutant proteins were translated *in vitro* as described above. For co-imunoprecipitation assay, *in vitro* translated pV was incubated with *in vitro* translated 33K , p33Kd3 or p33Kd4 for 6 h at 4^{o}C before immunoprecipitation with anti-pV serum. The co-immunoprecipitated proteins were separated on 10% SDS-PAGE under

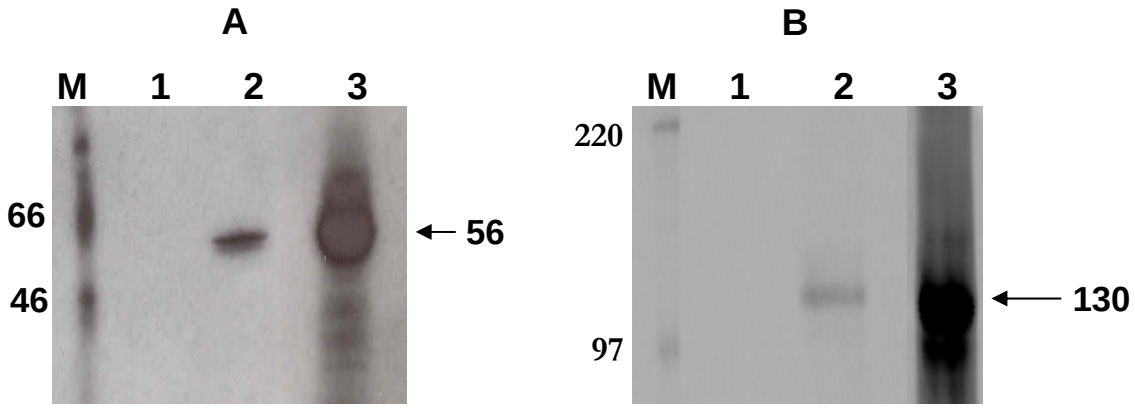


Fig. 4. 5. GST pulldown assay. **(A)** Purified GST alone (lane 1) or GST-33K (lane 2) proteins immobilized on Glutathione-Sepharose 4B beads, incubated with *in vitro* translated [^{35}S] labeled pV, were separated by 10% SDS–PAGE and detected by autoradiography. Lane 3 indicates *in vitro* translated [^{35}S] methionine labeled pV. **(B)** Purified GST alone (lane 1) or GST-33K (lane 2) proteins immobilized on Glutathione-Sepharose 4B beads, incubated with *in vitro* translated [^{35}S] methionine labeled 100K protein, were separated by 10% SDS–PAGE and detected by autoradiography. Lane 3 indicates *in vitro* translated [^{35}S] methionine labeled 100K Protein. The position of the molecular weight markers (M) in kDa is shown to the left of the panel. Arrows on the right of each panel indicate the position of the identified protein in kDa.

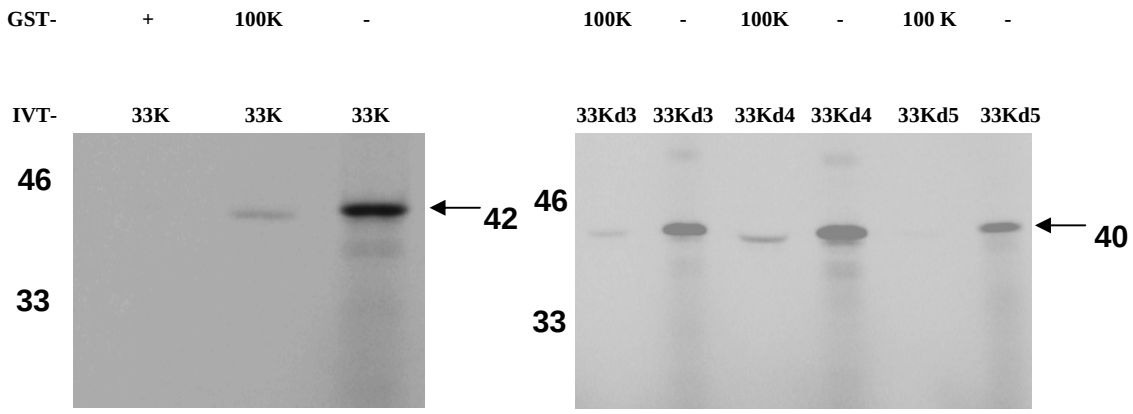


Fig. 4. 6. **Binding of labeled proteins to GST-fusion proteins**. **(A)** Purified GST or GST-100K proteins were immobilized on glutathione-Sepharose 4B beads, incubated with *in vitro* translated [^{35}S] methionine labeled 33K, 33Kd5, 33K3d3 or 33Kd4, The bound proteins were separated by 10% SDS–PAGE and detected by autoradiography. The position of the molecular weight markers (M) in kDa is shown to the left of the panel.

reducing conditions. The separated proteins were transferred to nitrocellulose membranes and probed in Western blot using anti-33K serum. As seen in Fig. 4.7, pV interacts with mutant 33Kd4 protein but not with mutant 33Kd3 protein. These results confirmed the earlier observations and suggested that amino acids 81-120 of 33K are involved in the binding to pV.

4.3.3 In vivo interaction of 33K with 100K and pV

To confirm the interaction of 33K with 100K or pV during BAdV-3 infection, the coimmunoprecipitation studies were carried out in BAdV-3 infected cells using protein specific antibodies. Cleared lysates prepared from BAdV-3 infected cells were immunoprecipitated with anti-33K serum or anti-pV serum. The co-immunoprecipitated proteins were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes and probed in Western blots using anti-pV serum or anti-33K serum. As seen in Fig. 4.8A, a 56 kDa protein representing pV was observed when the infected cell lysates were immunoprecipitated with anti-33K serum and probed in Western blot with anti-pV serum (Lane 2). No such protein was observed when infected cell lysates were incubated with preimmune serum and probed with anti-pV serum (Lane 1). Similarly, a protein of 42 kDa representing 33K protein was observed when infected cell lysates were immonprecipitated with anti-pV serum and probed in Western blot with anti-33K serum (Fig. 4.8B, lane 2). No such protein was observed when infected cell lysates were incubated with preimmune serum and probed in Western blot with anti-pV serum (Fig. 4. 8B, Lane 1). These results suggest that 33K and pV interact specifically during BAdV-3 infection.

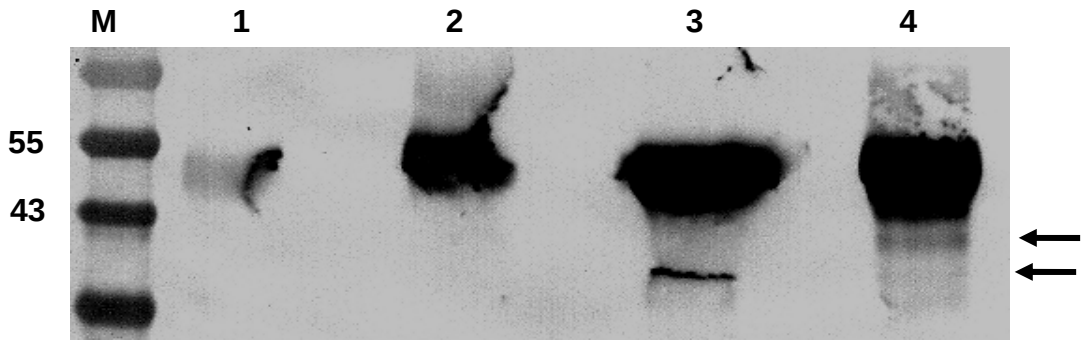


Fig. 4.7. Co-immunoprecipitation of *in vitro* translated proteins. *In vitro* translated pV protein was incubated individually with *in vitro* translated 33Kd3 (lane 2) , 33Kd4 (lane 3) or 33K (lane 4) proteins, The bound proteins were immunoprecipitated with anti-pV serum (lane 2,3,4) or preimmune serum (lane 1) separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. The separated proteins were probed by Western blot using anti-33K serum. The position of the molecular weight markers (M) in kDa is shown to the left of the panel. Arrow on the right of panel indicates the position of the identified protein in kDa.

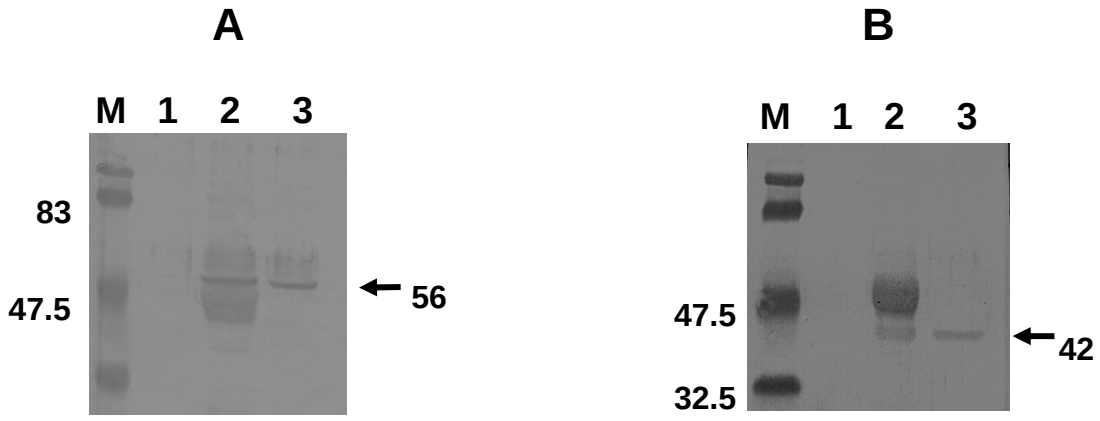


Fig. 4. 8. **Co-immunoprecipitation of 33K and pV proteins in BAdV-3 infected cells**. **(A)** Proteins from the lysates of BAdV-3 infected cells were immunoprecipitated with preimmune serum (lane 1) or anti-33K serum (lane 2), separated by 10% SDS–PAGE, and transferred to Immobilon-P membrane. The separated proteins were probed by Western blot using anti- pV serum. Proteins from the lysates of BAdV-3 infected cell were separated by 10% SDS-PAGE, transferred to Immobilon-P membrane and probed with anti-pV serum (lane 3). **(B)** Proteins from the lysates of BAdV-3 infected cells were immunoprecipitated with preimmune serum (lane 1) or anti -pV serum (lane 2), separated by 10% SDS–PAGE, and transferred to Immobilon-P membrane. Separated proteins were probed by Western blot using anti-33K serum. Proteins from BAdV-3 infected cell lysates were separated by 10% SDS-PAGE, transferred to Immobilon-P membrane and probed with anti-33K serum (Lane 3). The position of the molecular weight markers (M) in kDa is shown to the left of the panel. Arrows on the right of each panel indicate the position of the identified protein in kDa.

Similarly, a protein of 130 kDa representing 100K protein was observed when the infected cell lysates were immunoprecipitated with anti-33K serum and probed in Western blot with anti-100K serum (Fig. 4.9A, Lane 2). No such protein was observed when infected cell lysates were immunoprecipiated with preimmune serum and probed in Western blot with anti- 100K serum (Fig. 4.9A, Lane 1). Similarly, a protein of 42 kDa representing 33K protein was observed when the infected cell lysates were immunoprecipitated with anti-100K serum and probed in Western blot with anti-33K serum (Fig. 4.9B, Lane 2). These results suggest that 33K and 100K interact specifically during BAdV-3 infection.

4.4 Discussion

The 33K protein of adenovirus appears to be a multi-functional protein, which acts as alternative splicing factor (Tormanen et al., 2006) and plays an important role in viral assembly (Fessler and Young, 1999; Finnen et. al, 2001; Kulshreshtha et. al, 2004), stimulation of transcription from the MLP (Ali et. al, 2007) and early to late switch in MLP transcription (Farley et al., 2004). The multifunctional role played by 33K in adenovirus biology suggest that 33K may interact with other viral or cellular proteins. Here, we report for the first time that 33K protein interacts with 100K and pV during BAdV-3 infection.

The yeast two hybrid analyses revealed that 33K protein may be interacting with pV and 100K proteins of BAdV-3. This initial observation is supported by the fact that these interactions between 33K - 100K and 33K - pV were confirmed by GST pull down assay.

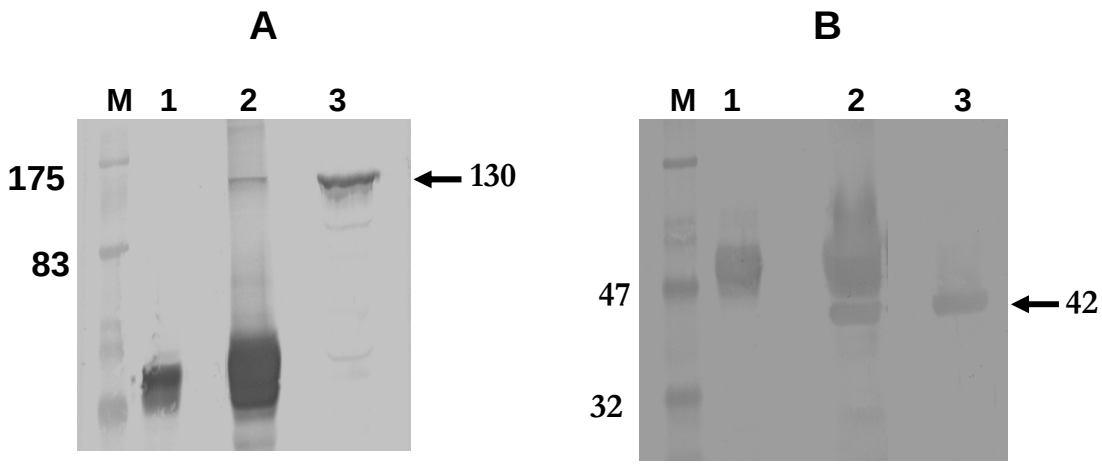


Fig. 4.9. Co-immunoprecipitation of 33K and 100K protein in BAdV-3 infected cells. **A)** Proteins from the lysates of BAdV-3 infected cells were immunoprecipitated with preimmune serum (lane 1) or anti-33K serum (lane 2), separated by 10% SDS–PAGE, and transferred to Immobilon-P membrane. Separated proteins were probed by Western blot using anti-100K serum. Proteins from BAdV-3 infected cell lysates were separated by 10% SDS-PAGE, transferred to Immobilon-P membrane and probed with anti-100K serum (lane 3) **(B)** Proteins from the lysates of BAdV-3 infected cells were immunoprecipitated with preimmune serum (lane 1) or anti-100K serum (lane 2), separated by 10% SDS–PAGE, and transferred to Immobilon-P membrane. Separated proteins were probed by Western blot using anti-33K serum Proteins from BAdV-3 infected cell lysates were separated by 10% SDS-PAGE, transferred to Immobilon-P membrane and probed with anti-33K serum (lane 3). The position of the molecular weight markers (M) in kDa is shown to the left of the panel. Arrows on the right of each panel indicate the position of the identified protein in kDa.

Moreover, these specific interactions between 33K-100K and 33K-pV were detected in BAdV-3 infected cells using co-immunoprecipitation and Western blot assays.

The detection of specific interaction between 33K / 100K proteins and 33K/pV proteins in BAdV-3 infected cells suggested that such interactions have a significant role in the virus replication cycle. However, the exact function of these interactions during the viral infection is currently unclear. The 100K protein associates with hexon monomers, help in the formation of hexon trimers and assist in the transport of hexon trimers to the nucleus of baculovirus infected cells (Hodges et al., 2001). However, expression of hexon and 100K in transfected COS-7 cells did not transport hexon to the nucleus (Hong et al., 2005). It is possible that interaction of 33K with 100K stabilizes the trimerization of hexons and helps to efficiently transport the hexon trimers to the nucleus of BAdV-3 infected cells. Support for this comes from the fact that despite the expression of early and late genes in the cells transfected with mutant BAdV-3 DNA containing insertional mutation in 33K of BAdV-3, the majority of hexon was detected in the cytoplasm of the transfected cells (Kulshrestha et al., 2004). Moreover, the 33K function in baculovirus infected cells may be functionally substituted by a viral or an insect cell protein(s). Alternatively, it is possible that hexon trimerization and efficient nuclear import may be mediated by different proteins including 33K at different stages of the BAdV-3 replication (Hong et al., 2005; Wodrich et al., 2003). Partial localization of hexon in the nucleus of adenovirus pVI transfected cells (Wodrich et al., 2003) or adenovirus 100K expressing insect cells (Hong et al., 2005) support this possibility.

Although adenovirus 33K protein is required for the assembly of virus particles (Finnen et al., 2001; Kulshreshtha et al, 2004), it can interact with adenovirus packaging sequence only in the presence of IVa2 protein (Ali et al., 2007). Since pV is known to specifically bind adenovirus genome (Ugai et al., 2007), the interaction of 33K with pV may help to increase the cooperative binding of 33K protein to the viral DNA packaging sequences. Alternatively, interaction of 33K with pV may help to relieve the repressive effect of pV on major late promoter activity (Ugai et al., 2007). Several lines of evidence support this speculation. First, significant level of 33K specific mRNAs and protein were detected in adenovirus infected cells before the beginning of transcription /translation of other late protein (Beltz and Flint 1979; Larsson et al., 1992). Secondly, 33K protein is

required for the efficient expression of ML transcription unit (Ali et al., 2007; Farley et al., 2004) Thirdly, expression of major late structural proteins was detected significantly earlier in mutant adenovirus containing deletion of pV (Ugai et al., 2007).

Although the boundaries have not been fully defined, our analysis of the deletion mutants suggested that amino acid 81-120 and 161-200 of 33K are involved in the interaction with pV and 100K proteins, respectively. Nevertheless, precise localization of the region and the identification of mutants of 33K that prevent interaction, and assessment of the consequences of these mutations on virus life cycle should provide insights into the functional importance of these novel interactions. Interestingly, while the identified region required for the interaction of 33K with pV protein is shared between 33K and 22K proteins, the identified region required for the interaction of 33K and 100K is not shared by 33K and 22K. It would be of interest to determine the role of each protein in these interactions.

5.0 INTERACTION OF BOVINE ADENOVIRUS-3 33K PROTEIN WITH CELLULAR PROTEINS

5.1 Introduction

Adenoviruses contain a non-enveloped, double-stranded DNA genome (Davison et al., 2000), which is organized into complex transcriptional units comprising of early, intermediate and late regions. Although expression of the early and intermediate transcription units precedes the onset of viral DNA replication, the expression of the late transcription unit is dependent on the initiation of viral DNA replication and expression of the major late transcription unit (MLTU) (Thomas and Mathews, 1980). In human adenovirus (HAdV) -5, the MLTU generates a primary transcript that is processed into more than 20 different cytoplasmic mRNAs. These mRNAs are divided into five (L1–L5) families. Late transcriptional unit encode structural and non-structural proteins involved in the formation of progeny virions.

The L4 region of the late transcription unit of HAdV-5 transcribes a spliced mRNA encoding 33K protein (Oosterom-Dragon and Anderson, 1983; Ostapchuk et al., 2006). The 33K protein is expressed as a 39 kDa phosphoprotein and is predominantly localized in the nuclei of the infected cells (Gambke and Deppert, 1981; Oosterom-Dragon and Anderson, 1983). Adenovirus 33K protein acts as an alternative RNA splicing factor (Tormanen et al., 2006), a transcriptional activator (Ali et al., 2007) and plays a role in viral capsid assembly / efficient capsid DNA interaction (Fessler and Young, 1999; Finnen et al., 2001; Kulshreshtha et al., 2004). Recently, adenovirus 22K protein has been shown to be involved in the packaging of adenovirus genome (Ostapchuk et al., 2006).

Homologs of 33K protein have been identified in other *Mastadenoviruses* including bovine adenovirus (BAdV) -3 (Kulshreshtha et al., 2004; Ali et al., 2007). The L6 region of BAdV-3 transcribes a spliced mRNA encoding 33K protein, which is localized to the nucleus of virus infected cells (Chapter 3). Preliminary study suggested that 33K may be involved in virus assembly (Kulshreshtha et al., 2004). The conservation of 33K protein in *Mastadenoviruses* together with its multiple functions in the adenovirus

life cycle suggests that 33K protein might be involved in the interaction with other viral or cellular proteins. Earlier, we reported that the 33K protein appears to be interacting with protein V and 100K protein (Chapter 4). In this study, we demonstrate that 33K protein of BAdV-3 appears to interact with bovine presenilin-1-associated protein (BoPSAP).

5.2 Materials and methods

5.2.1 Cell lines

Madin-Darby bovine kidney (MDBK) cells were cultured in minimal essential medium (MEM) with 10% fetal bovine serum (FBS). The wild-type BAdV-3 was cultivated in MDBK cells as described previously (Kulshrestha et al., 2004). VIDO R2 (Reddy et al., 1999), a transformed fetal bovine retina cell (FBRC) line expressing the E1 proteins of human adenovirus (HAdV)-5, and 293 cells (Graham et al., 1977) were grown in Eagle's minimum essential medium containing 5-10% fetal bovine serum cells. HeLa cells were cultivated in MEM -medium containing 10% fetal bovine serum.

5.2.2 Yeast and media

Matchmaker GAL4 two-hybrid system 3 and Yeast strain AH109 were obtained from Clontech. The pGADT7 vector containing bovine retina cDNA library was obtained from Wolfgang Baehr laboratory (University of Utah).

5.2.3 Yeast two hybrid analysis

The AH109 yeast strain was inoculated into 10 ml of Yeast peptone dextrose adenine hemisulphate (YPDA) media containing 2% dextrose and grown at 30°C overnight. The 5 ml of overnight culture was transferred to 100 ml of warm YPDA medium and grown at 30°C till the OD_{600} reached 0.5±0.1. The cells were harvested by centrifugation at 1500 rpm for 5 minutes. The pellet was washed with 50 ml of sterile water and resuspended in 1XTE/LiAc buffer. The cells were aliquot into 100ul in each of the 1.5ml of centrifuge tubes. The transformation mixture consisting of 240 µl of polyethylene glycol, 36 µl of 1.0 M LiAc, 50 µl of predenatured salmon sperm DNA (2.0 mg/ml), 5 µg of bovine retina cDNA library (library titer: 2×10^6 cfu/ml) , and 5 µg of

bait (pGB.33K) DNA (Chapter 4) was added to each of these tubes. The tubes were vortexed vigorously and incubated at 30°C for 30 minutes with shaking at 200 rpm and heat-shocked at 42°C for 15 minutes. The cells were pelleted, resuspended in 1X TE and spread on synthetic dropout medium plates containing 2% dextrose but lacking leucine and tryptophan. After six days incubation at 30^0C, the grown up colonies were transferred to the medium stringency media (synthetic dropout media plates lacking tryptophan, leucine and histidine) plates having 2% dextrose and later on transferred to the higher stringency media (synthetic dropout medium plates lacking tryptophan, leucine, histidine and adenine) plates having 2% dextrose and X-α-gal. Blue colonies indicating positive clones were observed after 5-6 days of incubation.

Plasmid DNA was isolated from each of the blue colonies, transformed in DH5α cells and plated on LB-ampicillin selection media. The plasmid DNA from each clone was again isolated, grouped on the basis of restriction enzyme analysis. The representative plasmid DNA selected from each group was sequenced. The DNA sequence obtained was compared with sequences in the GenBank data base to identify the potential cellular genes interacting with 33K protein of BAdV-3.

5.2.4 Isolation of bovine presenilin-1-associated protein and bovine microtubule associated protein ORFs

RNA was extracted from VIDO R2 (Reddy et al., 1999) cells using RNeasy kit (Qiagen). The first strand cDNA was synthesized using superscript II reverse transcriptase and oligo (dT) primers (Invitrogen). The cDNA (containing bovine presenilin-1-associated protein [BoPSAP] ORF) was amplified by PCR using primers BoP1: 5'- GGAATTCCATATG ATGGCGGGAGCCGGAGCTG-3'; BoP2: 5'-GGAAT TCTTACT CCAGGGCAAAG CATGAT -3'. The PCR product was cloned into *Sma*I digested plasmid pGEX-5X-1 (Pharmacia) creating plasmid pGEX.BoPSAP. The plasmid DNA was used to determine the DNA sequence of the amplified PCR product.

Similarly, a cDNA (containing a C-terminal bovine microtubule associated protein [BoMAP] ORF) was amplified by PCR using primers BoM1: 5'-GGA ATTCCATATG ATGATA TCCTTATATGCCTCACTCAC-3'; BoM2: 5'-GGAATTCT TACAGTTCGA TCTTGC ATGCA -3'. The PCR product was cloned into *Sma*I digested

plasmid pGEX-5X-1 creating plasmid pGEX.BoMAP.

5.2.5 Plasmid construction

Construction of plasmids pGEX.33K (Chapter 4.2.3), pC.33K (Chapter 3.2.3), OCT-CFP and OCT-Red (Harder et al., 2004) has been described earlier.

5.2.5.1 Construction of yeast expression plasmids

A 1.1 kb fragment amplified by PCR using primers BoP1 and BoP2 (5.3.2) and plasmid pGEX.BoPSAP as a DNA template was digested with *Nde*I-*Eco*RI and ligated to *Nde*I-*Eco*RI digested plasmid pGADT7 (Clontech) creating plasmid pGA.BoPSAP (containing full length BoPSAP ORF [354 amino acids]). Similarly, a 2.1 kb fragment amplified by PCR using primers BoM1 and BoM2 (5.3.2) and plasmid pGEX.BoMAP as a DNA template. The PCR product was digested with *Eco*RI enzyme and ligated to *Eco*RI digested plasmid pGADT7 (Clontech) creating plasmid pGA.BoMAP (containing full length BoMAP ORF [719 amino acid]). This cloning approach creates inframe HA epitope tag at 5' end of BoPSAP and BoMAP.

5.2.5.2 Construction of plasmid pCF.V.

A 1.2 kb fragment containing BAdV-3 pV ORF (without stop codon) was amplified by PCR using the primers 5'-GGGGTACCATGGCCTCCTC TCGGTTG-3' and 5'-CGGGATCCGGGTGATAGCG CACGCC-3' and plasmid pGA.pV DNA (Chapter 4.2.3). The PCR product was digested with *Kpn*I-*Bam*HI and ligated to *Kpn*I-*Bam*HI digested plasmid OCT-CFP (cyano-fluorescent protein expression vector;-Harder et al., 2004) creating the plasmid pCF.V.

5.2.5.3 Construction of plasmid pCF.BoPSAP

The BoPSAP ORF without stop codon was amplified by PCR using the primers Bo3: 5'-GGGGTACCATGGCGGGAGCCGGAGCT-3' and Bo4: 5'- GAAGAT CTCTCC A GGGCAA AGCATGATC-3' and plasmid pGA.BoPSAP DNA as a template. The PCR product (containing BoPSAP ORF) was digested with *Kpn*I-*Bgl*II and ligated to *Kpn*I-*Bam*HI digested plasmid OCT-CFP (cyano-fluorescent protein expression vector; Harder et al., 2004) creating plasmid pCF.BoPSAP.

5.2.5.4 Construction of plasmid pDsRed.BoPSAP

The BoPSAP ORF without stop codon was amplified by PCR using the primers Bo3: 5'-GGGGTACCATGGCGGGAGCCGGAGCT-3' and Bo4: 5'- GAAGAT CTCTCCAGGGCAA AGCATGATC-3' and plasmid pGA.BoPSAP DNA as a template. The PCR product (containing BoPSAP ORF) was digested with *Kpn*I-*Bgl*II and ligated to *Kpn*I-*Bam*HI digested plasmid OCT-DsRed (DsRed expression vector; Harder et al., 2004) creating plasmid pDsRed.BoPSAP.

5.2.6 GST pulldown assay

The GST alone or GST-33K fusion protein (Chapter 4) were induced by isopropyl-β-D-thiogalactopyranoside (IPTG) (Gibco-BRL) and immobilized on Glutathione-Sepharose beads (Pharmacia) as described elsewhere (Zhou and Tikoo, 2001).

Individual plasmid (pGA.BoPSAP, pGA.BoMAP, pGADT7) DNA was *in vitro* transcribed and translated using TNT T7 quick-coupled Transcription/Translation kit (Promega) in the presence of 30 μCi of [^{35}S] methionine. A 5ul of *in vitro* translated protein was added individually to beads bound 10 ug of GST or GST-33K fusion protein in 500ul of binding buffer (30mM Hepes pH 7.4, 50mM KCL, 0.5% Tween 20, 0.5% nonfat dry milk, 2mM PMSF) and incubated at 4°C for 4 hrs with rotation. The beads were then washed four times with the binding buffer. The bead bound proteins were separated by sodium dodecyl sulphate (SDS) - polyacrylamide gel electrophoresis (PAGE) and analyzed by autoradiography.

5.2.7 Confocal microscopy

HeLa cells plated on Lab-Tek II chamber slide (Nunc) were co-transfected with individual plasmid DNAs. Forty eight hrs post-transfection, the cells were fixed in 4% paraformaldehyde for 15 min at room temperature, washed four times with PBS and permeabilized with ice cold acetone for 5 min. The cells were then rinsed with PBS three times and mounted in DABCO mounting media (Fluka). Finally, the cells were analyzed by confocal microscopy using laser scanning microscope.

5.2.8. Apoptosis assay.

293 cells were transfected with individual plasmid DNA (pCDNA or pC.33K). After 48 hrs, the transfected or non transfected cells were treated with 1μm Stauroprorine. After 4 hrs of staurosporine treatment, the cells were treated with 0.25 % trypsin EDTA for 5 min at 37^0C. The detached cells were collected, washed three times with cold PBS and annexin binding buffer. Cells were later treated with FITC-annexin V and propidium iodide at room temperature for 15 minutes. Following the incubation period, annexin binding buffer was added to the cells and the stained cells were analyzed by flow cytometry. The annexin-V/propidium iodide (PI) assay indicates the percentage of cells undergone early and late apoptosis. The annexin V has a strong affinity to bind to phosp -hatidylserine exposed on the surface of the membrane of the cell during apoptosis.

To identify the cleavage of poly (ADP-ribose) polymerase (PARP), the cells were transfected with individual plasmid DNAs (pCDNA or pC.33K). After 48 hrs of transfection, the transfected or non transfected cells were treated with staurosporine. After 4 hrs of treatment, the cells were lysed and analysed by Western blot analysis as described (Chapter 4) using monoclonal antibody against PARP (BD PharMingen).

5.3. Results

5.3.1 Screening of bovine retina cDNA library

To identify the cellular proteins interacting with 33K protein, the bovine retina cDNA library cloned in plasmid pGADT7 (Clontech) was screened using 33K protein as a bait in yeast two-hybrid system. The screening of cDNA library was made stringent by selecting the interacting proteins by growing cotransformants on SD/Leu-Trp-His- plates (medium stringency) followed by SD/Leu-Trp-His-Ade- plates (high stringency) containing X-α-gal. The Ade provides a strong nutritional selection, HIS3 reduces the incidence of false positive and MEL1 or LacZ encoding β-galactosidase can be assayed on X-α-gal indicator plates. Out of 2×10^6 clones screened, 150 clones showed bluish colonies (Fig. 5.1) suggesting some positive interactions. The plasmid DNAs isolated from these yeast clones was used to transform DH5α cells. Since bovine cDNA library was constructed using Eco*RI* site of plasmid pGADT7 (Zhang et al., 2004), the plasmid

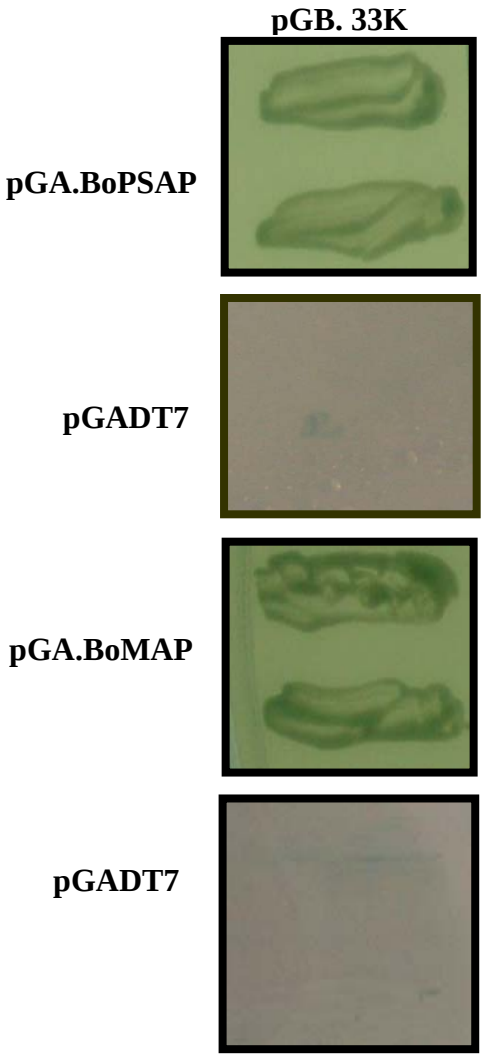


Fig. 5.1. **Yeast cDNA library screening.** Plasmid pGAD7 (prey) containing cDNA library of bovine retina cells and plasmid pGB.33K were co transformed in yeast AH109 cells. Similarly, plasmid pGADT7 and plasmid pGB.33K were co transformed in yeast AH109 cells.The co-transformants were streaked on SD/Leu-/Trp-/His-/Ade-/ X-α-gal-containing plates and incubated at 30°C for 5-7 days.

DNAs isolated from ampicillin resistant DH5α bacteria were digested by *Eco*RI and observed for the release of insert(s). Based on the size of the released DNA inserts plasmid DNAs were grouped into 30 groups. The DNA sequence of an insert DNA from each group was determined. Analysis of the DNA sequences of different inserts showed significant homology to *Homo sapiens* Presenilin-1-associated protein (HuPSAP; GenBank Accession # AF189289) and *Homo sapiens* Microtubule associated protein (HuMAP GenBank Accession # NM 005909) .

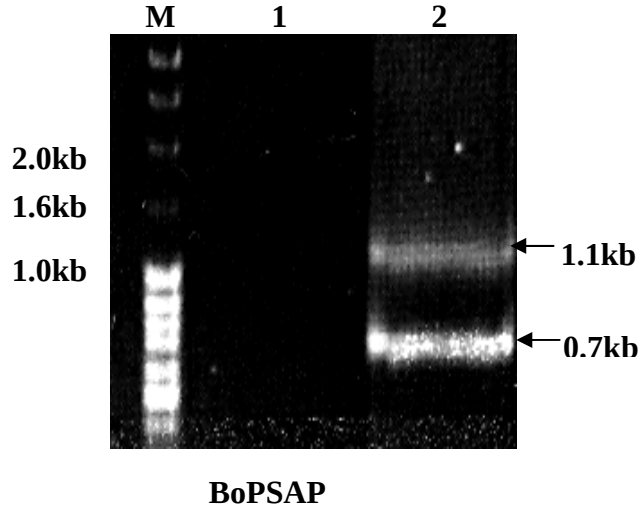
5.3.2 Identification of BoPSAP and BoMAP ORFs

RT PCR analysis of RNA (isolated from VIDO R2 cells) using primers BoP1 / BoP2 amplified two fragments of 1.1kb and 700 bp (Fig. 5.2A). Similarly, RT PCR analysis of RNA (isolated from VIDO R2 cells) using primers BoM1 / BoM2 amplified a fragment of 2.1kb (Fig. 5.2B). The 1.1kb and 2.1kb PCR fragments were individually clones in *Sma*I digested plasmid pGEX-5X-1 to create plasmid pGEX.BoPSAP and plasmid pGEX.BoMAP, respectively.

The DNA sequence of plasmid pGEX.BoPSAP containing 1.1kb PCR fragment, identified an ORF of 1062 nucleotides encoding a protein of 354 amino acids (Fig 5.3A) with a predicted molecular weight of 38 kDa. The protein designated as BoPSAP (Fig.5.3A) contains an N-terminal signal sequence of 19 amino acids and a potential transmembrane anchor of 23 amino acids. The predicted translation product contains a mitochondrial carrier protein sequence (Lamarca et al., 2007) from amino acid 206 to 266. The BoPSAP showed 98% homology with HuPSAP (Xu et al., 2002). Moreover, apoptotic domain identified in HuPSAP (Lamarca et al., 2007) is conserved in BoPSAP (amino acid 65 to 168).

The DNA sequence of plasmid pGEX.BoMAP containing 2.1kb PCR fragment identified an ORF of 2157 nucleotides encoding a protein of 719 amino acids (Fig. 5.3B) with a predicted molecular weight of 79 kDa. The protein designated as BoMAP showed 87% homology with HuMAP (Lien et al., 1994).

(A)



(B)

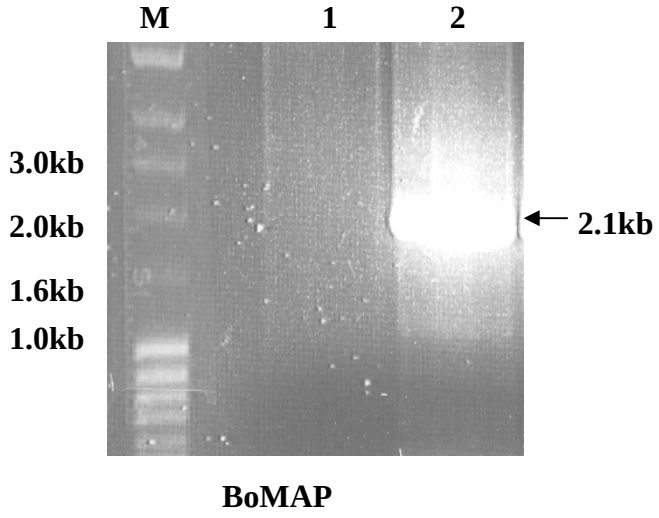


Fig. 5.2. RT-PCR . Total RNA isolated from VIDOR2 cells was reverse transcribed using Oligo (dT) as primer. The BoPSAP specific cDNA **(A)** or BoMAP specific cDNA **(B)** were amplified by PCR using primers BoP1/BoP2 and BoM1/BoM2 respectively. The amplified products were analyzed on a 1 % agarose.

(A)

```
MAGAGAGAGA RGGAAAGVEA RARDPPPAHR AHPRHPRPAA QPSARRMDGA  50
SGGLGSGDNA PTTEALFVAL GAGVTALSHP LLYVKLLIQV GHEPMPPTIG 100
TNVLGRKVLY LPSFFTYAKY IVQVDGKIGL FRGLSPRLMS NALSTVTRGS 150
MKKVFPPDEI EQVSNKDDMK TSLRKVVKVT SYEMMMQCVS RMLAHPLHVI 200
SMRCMVQFVG REAKYSGVLS SIGKIFKEEG LLGFFVGLIP HLLGDVVFLW 250
GCNLLAHFIN AYLVDDSFSQ ALAIRSYTKF VMGIAVSMLT YPFLLVGDLM 300
AVNNCGLQAE LPPYSPVFKS WIHCWKYLSV QGQLFRGSSL LFRRVSSGSC 350
FALE                                                    354
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(B)

```
MISLYASLTS EKVQSLDAEK LSPKSDISPL TPRESSPLYS PSLSDSTSAV  50
KDSTAACHTA SPPPMDAASA EPYGFRASML FDTMPHHLAL NRDLTTPGME 100
EDTGGKTPGD FSYAYEKPET TTRSPDEEGY DYEAYEKTTR TPDVSGYYYE 150
KTESVTKSPC DSSYSYETME KTSKSPEDGG YAYEITEKTT RSPEESGYAY 200
EISEKTIRTP EVSAYSYEKA ERSRRLLDDI SNGYDDSEEG GHTLGDSSYS 250
YETTEKTAGF PESESYSYET STTTTRSPDA SAYCYETPEK ITRAPQVSSY 300
AYETSDQCYT AEKKSPSEAR QDVDLCLVSS CEYKHPKTEL SPSFINPNPL 350
EWFASEEPTE ESEKPLTQSG GAPPPPGGKP QGRQCDETPP TSVSESAPSQ 400
TDSDVPPETE ECPSITADAN IDSEDESETI PTDKTVTYKH MDPPPAPMQD 450
RSPSPRHPDV SMMDPEALAI EQNLGKALKK DLKEKTKTKK PGTKTKSSSP 500
VKKGDGKSKP SAASPKPGAL KESSDKVSKV ASPKKKESVE KATKTTTTPE 550
VKAARGEEKD KETKNAANAS TSKSVKTATA GPGTTKTSKP SAVPPGPPVY 600
LDLCYIPNHS NSKNVDVEFF KRVRSSYYVV SGNDPAAEEP SRAVLDALLE 650
GKAQWGSNMQ VTLIPTHDSE VMREWYQETH EKQQDLNIMV LASSSTVVMQ 700
DESFPACKIE L                                            711
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Fig. 5.3. Deduced amino acid sequence of cellular genes A) Amino acid sequences of Bovine Presenilin-1-associated protein (BoPSAP): Underlined residues indicate apoptotic domain (solid line), mitochondria carrier homology domain (double solid lines) and transmembrane domains (wavy solid line). **B)** Amino acid sequence of Bovine Microtubule Associated protein (BoMAP).

5.3.3 Yeast two hybrid assay

Since the BoPSAP DNA and BoMAP DNA inserts identified by cDNA library screening appeared truncated, we repeated the yeast two hybrid analysis using full length BoPSAP and BoMAP genes. To achieve this, we constructed plasmid pGA.BoPSAP (containing full length BoPSAP ORF [354 amino acids]) and plasmid pGA.BoMAP (containing C-terminal BoMAP ORF [719 amino acid]) amplified by RT-PCR of mRNA isolated from VIDOR2 cells (Reddy et al., 1999). The yeast strain AH109 was cotransformed with bait and prey plasmids and the interactions were screened though different selection stringencies as described earlier (Singh et al., 2005). The higher stringency selection revealed the appearance of blue colonies upon co-transforming plasmid pGB.33K DNA along with plasmid pGA.BoPSAP DNA or plasmid pGA.BoMAP DNA (Fig 5.4). No such blue colonies were observed on co-transforming plasmid pGB.33K DNA and plasmid pGADT7 DNA in the yeast (Fig. 5.4). These findings suggest that 33K protein may be specifically interacting with BoPSAP and BoMAP.

5.3.4 GST-pulldown assay

To confirm the results of yeast two hybrid analyses, we tested the interaction of proteins *in-vitro* using GST pull down assay. The GST alone or GST-33K fusion protein (Chapter 4) were induced by IPTG and the expression of these proteins were confirmed by Western blot using anti-GST and anti-33Kp serum respectively (Fig. 5.5). The induced GST and GST-33K fusion proteins were bound on glutathione sepharose beads as described earlier (Zhou and Tikoo, 2001). Since T7 promoter is located 5' to BoPSAP or BoMAP coding sequence in the plasmid pGA.BoPSAP and plasmid pGA.BoMAP respectively, these plasmids were used to transcribe and translate these proteins *in- vitro* in the presence of 50 μl of [^{35}S] methionine, using TNT T7-coupled reticulocyte lysate system as described by the manufacturer (Promega). The GST or GST-33K proteins bound to glutathione sepharose beads were individually incubated with *in-vitro* translated [^{35}S]-labeled proteins for 6 h at 4^{o}C. After extensive washing with binding buffer, the bead bound products were separated on 10% SDS-PAGE and visualized by autoradiography.

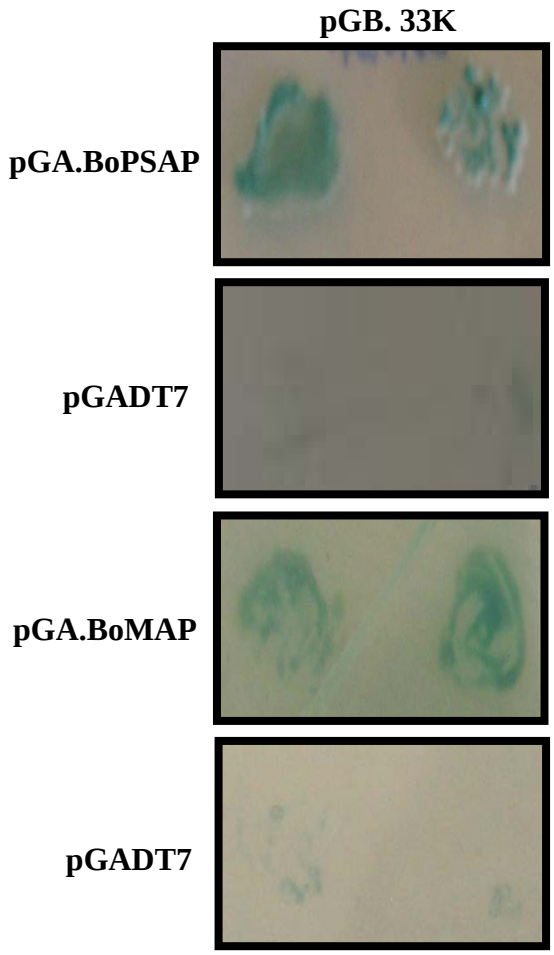


Fig. 5.4 Yeast two-hybrid analysis. Plasmid pGA.BoPSAP DNA or plasmid pGA.BoMAP DNA was individually co-transformed with plasmid pGB.33K DNA in yeast strain AH109. Similarly, plasmid pGADT7 and plasmid pGB.33K were co transformed in yeast AH109 cells.The co-transformants were streaked on SD/Leu-/Trp-/His-/Ade-/ X-α-gal-containing plates and incubated at 30°C for 5-7 days. pGBKT7 was used as negative control.

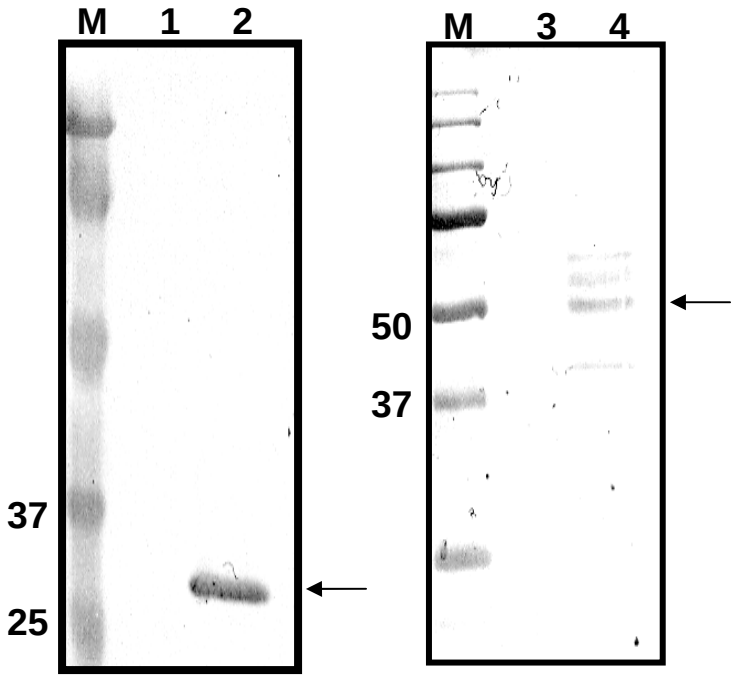
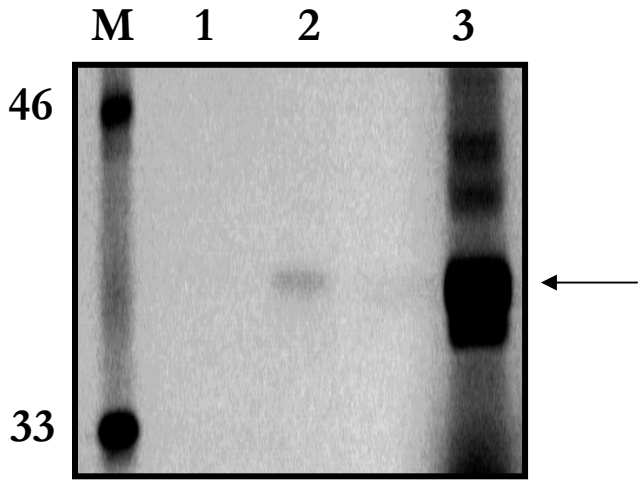


Fig. 5.5. **Immunoblot analysis of Glutathione-Sepharose-immobilized proteins**. Purified GST (lane 2) and GST-33K (lane 4) proteins were separated by 10% SDS–PAGE, transferred to Immobilon-P membrane and analyzed by immunoblotting using anti-GST serum (lanes 1,2) and anti-33Kp serum (lane 3,4) . Uninfected cell lysates (lanes 1,3) were used as negative controls. The position of the molecular weight markers (M) in kDa is shown to the left of the each panel. Arrows on the right of each panel indicate the position of the identified protein.

(A)



(B)

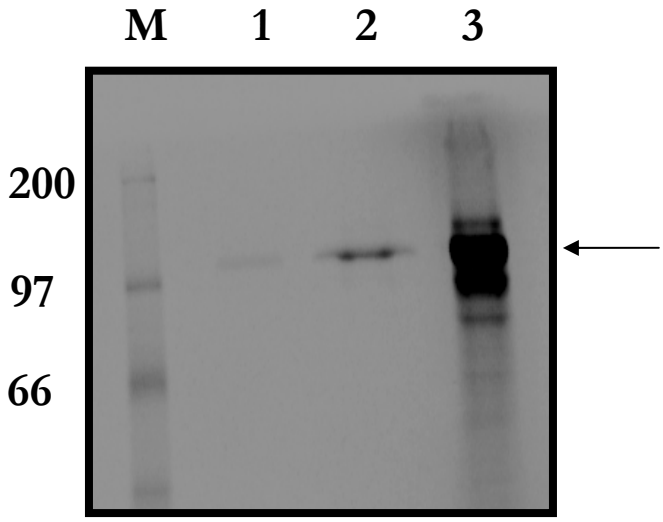


Fig. 5.6. GST pull down assay. (A) Purified GST (lane 1) or GST-33K (lane 2) proteins were immobilized on glutathione-Sepharose 4B beads, incubated with *in vitro* translated [^{35}S] methionine-labeled BoPSAP were sepatated by 10% SDS-PAGE and detected by autoradiography. Lane 3 indicates *in vitro* translated [^{35}S] methionine labeled BoPSAP **(B)** Purified GST (lane 1) or GST-33K (lane 2) proteins were immobilized on glutathione-Sepharose 4B beads, incubated with *in vitro* translated [^{35}S] methionine-labeled BoMAP were sepatated by 10% SDS-PAGE and detected by autoradiography. Lane 3 indicates *in vitro* translated [^{35}S] methionine labeled BoMAP. The position of the molecular weight markers (M) in kDa is shown to the left of the each panel. Arrows on the right of each panel indicate the position of the identified protein.

As seen in Fig 5.6A, *in vitro* translated BoPSAP protein appears to interact with GST-33K protein (lane 2) but not with GST protein (lane 1). No such interaction was detected between i*n vitro* synthesized BoPSAP and GST protein alone (lane 1) demonstrating the specificity of the assay. These results confirm the yeast two hybrid observations, and further suggest that 33K-BoPSAP interaction is real. In contrast BoMAP protein appeared to interact with GST (Fig. 5.6B, lane 1) as well as with GST-33K (Fig. 5.6B, lane 2). These results suggested that in *vitro* translated BoMAP interacted specifically with GST protein. These results suggest that interaction detected using Yeast two hybrid system may not be real.

5.3.5 BoPSAP localizes to mitochondria

Since the human homolog of BoPSAP localizes to mitochondria (Xu et al., 2002), we determined if BoPSAP also localizes to the mitochondria. Hela cells in Lab-Tek II chamber slide (Nunc) were co-transfected with 0.5 μg/well plasmid pCF.BoPSAP DNA and 0.5 μg/well plasmid OCT-DsRed (mitochondrial marker OCT [ornithine carbamyl transferase] pre-sequence fused to DsRed2) DNA (Harder *et al.*, 2004), or plasmid OCT-DsRed DNA 0.5 μg/well and 0.5 μg/well plasmid pCF.V DNA. Forty eight hrs post-transfection, the cells were analyzed by confocal microscopy. As expected, sub cellular distribution of OCT-DsRed was consistent with the localization in mitochondria (Fig. 5.7A). The CF.BoPSAP displayed the fluorescence throughout the cytoplasm of the transfected cells with no diffuse staining (Fig. 5.7A). However the merging of two images showed purple region of colocalization (Fig. 5.7A). In contrast, CF.V displayed predominantly nuclear fluorescence (Fig. 5.7A) and does not appear to colocalize with OCT-DsRed (Fig 5.7A) in the mitochondria. These results suggest that like HuPSAP, BoPSAP protein localizes to the mitochondria.

5.3.6 33K also localizes to mitochondria

The proteins execute their function when they are targeted to the sub-cellular compartments via specific localization signals. Analysis of 33K protein by MioProt program predicted the presence of a mitochondrial localization signal (N-terminus 38 amino acids). To determine if 33K protein also localizes in the mitochondria, Hela cells

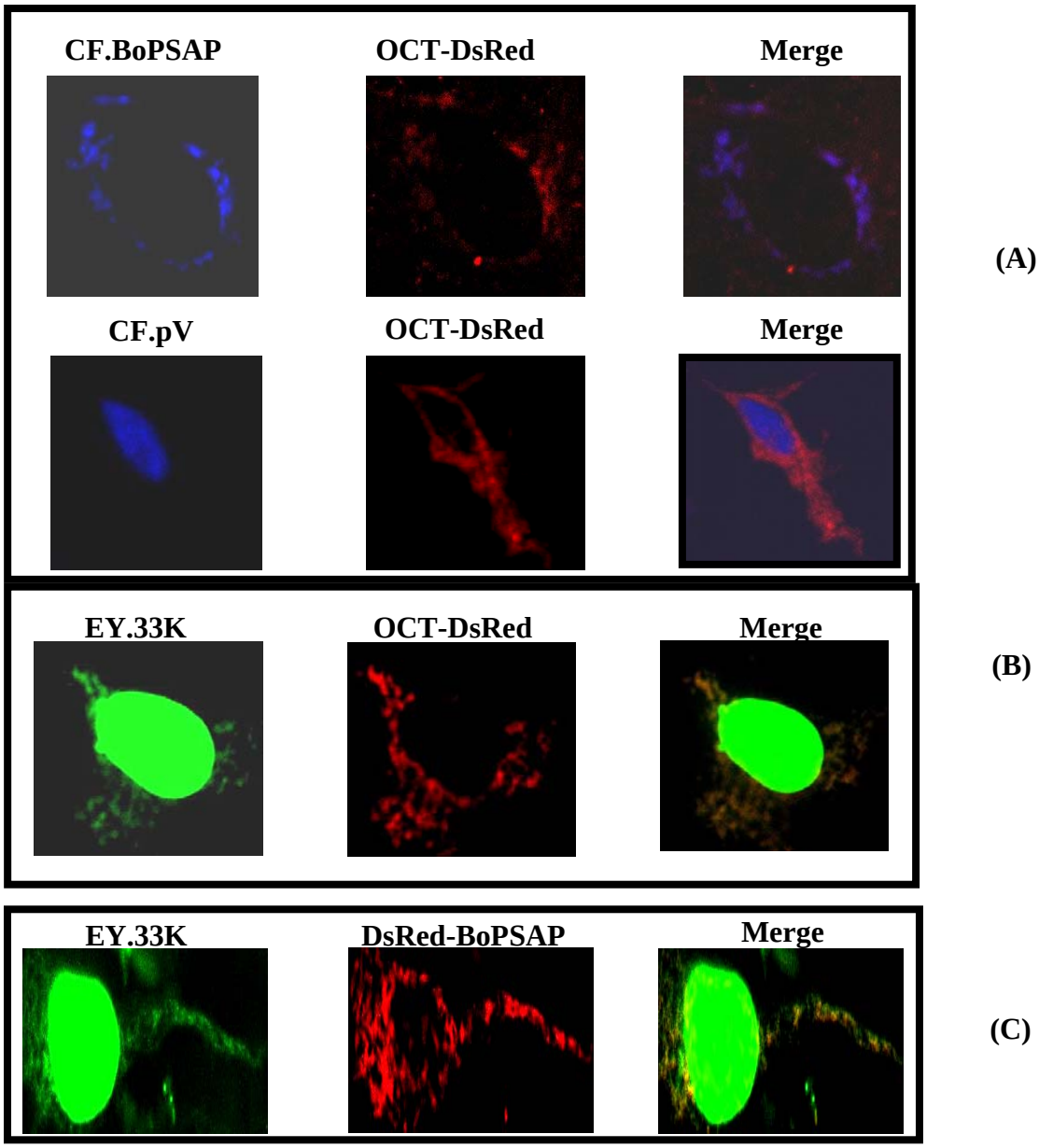


Fig. 5.7. Subcellular localization of proteins. **(A)** BoPSAP protein. Monolayers of HeLa cells in four well Lab-Tek chamber slides were cotransfected individually with plasmid pCF.BoPSAP DNA (0.5μg/well and plasmid OCT-DsRed DNA (0.5 μg/well) or plasmid pCF.V DNA (0.5 g./well) and plasmid OCT-Red DNA (0.5 g/well) . After 48h post-transfection, the cells were visualized by confocal microscope**. (B)** 33K protein. Monolayers of HeLa cells were cotransfected with plasmids pEY.33K DNA (0.5 μg/well) and OCT-DsRed DNA (0.5 μg/well). At 48h post-transfection, the cells were visualized by confocal microscope. **(C)** 33K protein and BoPSAP. Monolayers of HeLa cells were cotransfected individually with plasmids pEY.33K DNA (0.5 μg/well) and pDsRed-BoPSAP DNA (0.5 μg/well). At 48h post-transfection, the cells were visualized by confocal microscope.

were co- transfected with the plasmid OCT–DsRed2 (ornithine carbamyl transferase pre-sequence fused to DsRed2) (Harder et al., 2004) DNA and plasmids pEY.33K DNA. Forty eight hrs post transfection, the cells were fixed with 4% paraformaldehyde and examined by confocal microscopy. As seen in Fig. 5.7B, the EY.33K protein expressed in transfected cells was localized predominantly within the nucleus (Fig. 5.7B). However, a small amount of EY.33K protein was also detected in the mitochondria as it showed co-localization with the mitochondrial localized OCT-DsRed2 protein (Fig.5.7B).

5.3.7. Colocalization of 33K and BoPSAP in mitochondria.

To determine if 33K protein also localizes with BoPSAP in the mitochondria, Hela cells were co-transfected with 0.5 μg plasmid pDsRed-BoPSAP DNA and 0.5 μg/well plasmid pEY.33K DNA. Forty eight hrs post transfection, the cells were fixed with 4% paraformaldehyde and examined by confocal microscopy. As seen in Fig. 5.7C, the EY.33K protein expressed in transfected cells was localized predominantly within the nucleus (Fig. 5.7C). However, a small amount of EY.33K protein was also detected in the mitochondria as it showed co-localization with the mitochondria localized DsRed-BoPSAP protein (Fig. 5.7C).

5.3.8 33K protein induces anti-apoptotic activity

Since the 33K protein is a late protein and a portion of 33K protein localizes to the mitochondria, we determined whether the 33K protein shows anti-apoptotic activity. The annexin V staining precedes the loss of integrity of cell membrane, which accompanies the latest stages of cell death resulting from either apoptosis or necrosis. Hence, the staining with annexin V along with PI (vital dye) helps in identifying early apoptotic cells.

The 293 cells were transfected individually with plasmids pC.33K or pCDNA3 (as a negative control), followed by treatment with stauroporine (a caspase-independent direct activator known to induce apoptosis in cells by blocking the activity of many kinases [Kabir et al., 2002]) for four hrs. After 48 hrs, the transfected cells were collected, washed twice with PBS and stained with annexin V and propidium iodide (PI). The annexin V/PI stained cells were analyzed by flow cytometry. As shown in Fig. 5.8,

compared to plasmid pcDNA3 DNA transfected cells, pC.33K transfected cells showed 60% decrease in annexin V/PI positive cells. These results suggested that the 33K protein may have the potential to counteract the staurosporine induced apoptosis.

The anti-apoptotic activity of 33K protein was further confirmed by cleavage of poly(ADP-ribose) polymerase (PARP). Since the cleavage of the PARP (death substrate) is a hallmark for apoptosis (Dubrez et al., 1996; Bluethner et al., 2007), we analyzed the cleavage of PARP in plasmid DNA transfected cells. The 293 cells were transfected individually with plasmids pC.33K or pCDNA3 (as a negative control), followed by treatment with staurosporine. After 48 hrs, the cells were collected and analyzed by Western blot using anti-PARP serum. As seen in Fig 5.9A, B mock transfected cells treated with staurosporine showed 100 % of PARP cleaved to its 85 kDa form. Similarly, pCDNA3 transfected cells treated with staurosporine showed 80% of PARP cleaved to its 85 kDa form (Fig. 5.9A, B). In contrast, pC.33K transfected cells treated with staurosporine showed only 20% of PARP cleaved to its 85 kDa (Fig. 5.9A, B). These experiments revealed that 33K protein may have the ability to counteract the stauropsorine induced apoptosis.

5.4 Discussion

The 33K protein of adenovirus appears to be a multifunctional protein, which acts as alternative splicing factor (Tormanen et al., 2006) and plays an important role in viral assembly (Fessler and Young, 1999; Finnen et al., 2001; Kulshreshtha et al., 2004), stimulation of transcription from the MLP (Farley et al., 2004; Ali et al., 2007) and early to late switch in MLP transcription (Farley et al., 2004). Here, we report for the first time that 33K protein interacts with the protein similar to presenilin-1-associated protein and may inhibit apoptosis.

The screening of bovine retina cDNA library, using 33K as bait by yeast two hybrid analysis revealed that 33K protein may be interacting with bovine presenilin-1-associated protein (BoPSAP) and microtubule associated protein (BoMAP). This initial observation between 33K and BoPSAP is supported by the fact that these interactions were confirmed *in vitro* by GST pull down assay and colocalization assays. However, the interactions (observed by yeast two hybrid screening) between 33K protein and BoMAP were not

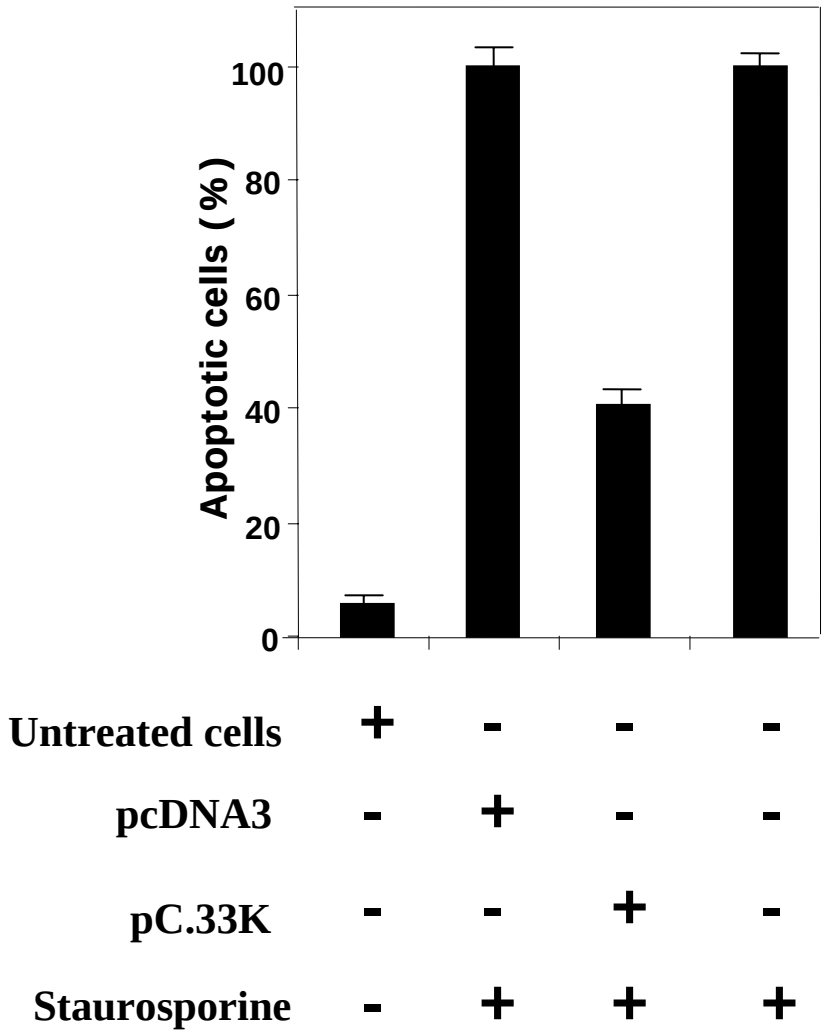


Fig. 5.8. Anti-apoptotic activity of 33K protein. 293 cells were cotransfected individually with plasmid pC.33K DNA, or pCDNA3 DNA. At 48h post-transfection, the cells were treated with 1μM concentration of staurosporine for 4 hrs. Finally, the cells were trypsized, washed and stained with annexin V / propidium iodide (PI). The Annexin V/PI stained cells were analyzed by flow cytometry.

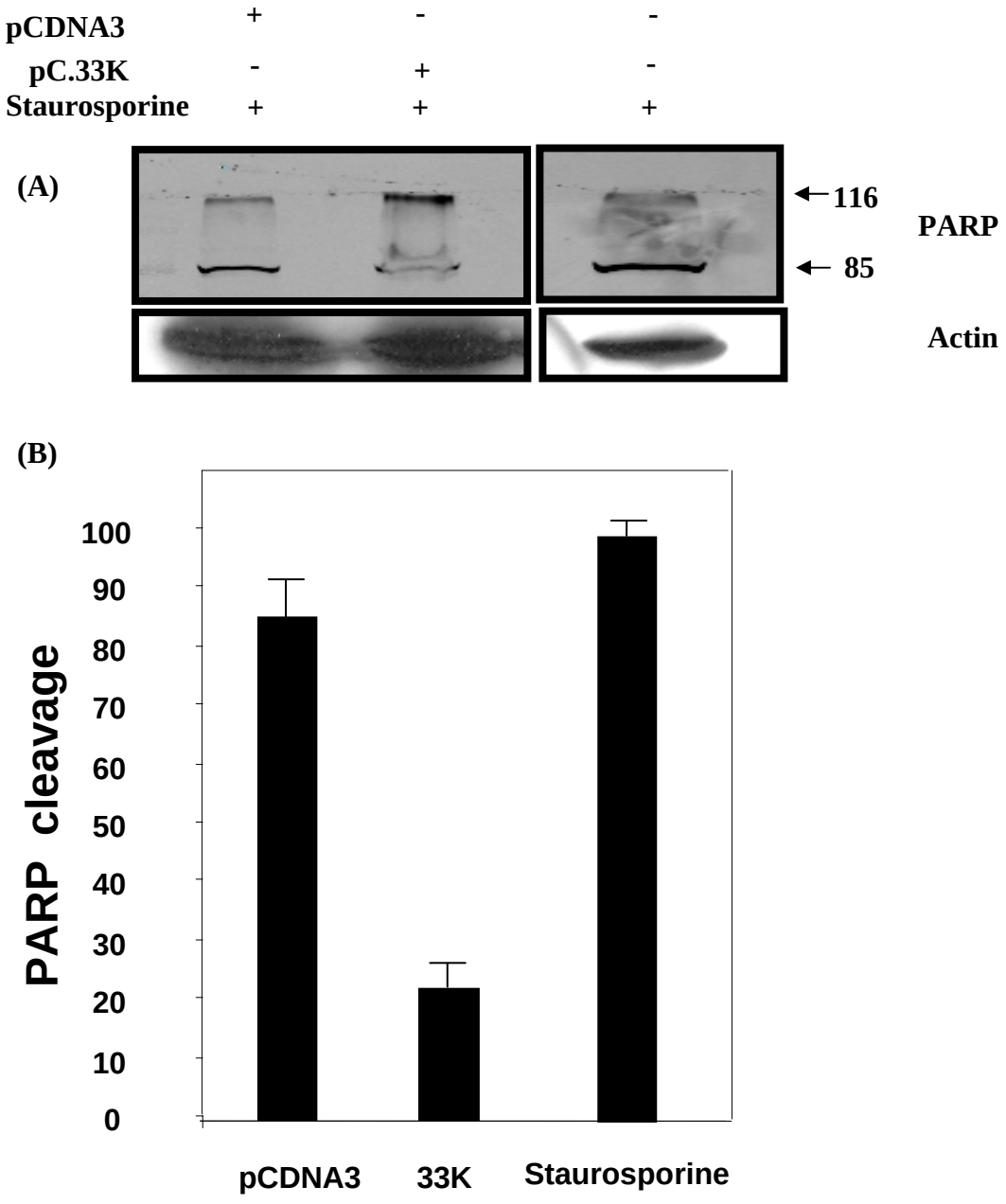


Fig. 5.9. PARP cleavage assay. (A) 293 cells were transfected with indicated plasmid DNA. After 48 hrs, the transfected cells were treated with 1μm staurosporine (Sigma S6942). After 4hrs of treatment, proteins from lysates of cells were separated by 10% SDS-PAGE under reducing conditions, transferred to nitrocellulose membranes and analyzed by Western blotting using anti-actin serum (Sigma) or monoclonal antibody against PARP (BD PharMingen). The position of specific bands is shown to the right of the panels. **(B)** Graphic representation of the results described in A. SDs were calculated from three independent experiments.

confirmed by GST pulldown assay suggesting that 33K-BoMAP interaction was a false observation.

The detection of specific interaction between 33K and BoPSAP suggested that such interactions have a significant role in the virus life cycle. However, the exact function of these interactions during the viral infection is currently unclear. The HuPSAP protein a human homolog of BoPSAP has been shown to induce apoptosis when over expressed in the cells (Xu et al., 2002; Lamarca et al., 2007). Since BoPSAP shows significant homology to HuPSAP there is a possibility that BoPSAP may also be involved in inducing apoptosis. In fact, BoPSAP contain domains, which show complete homology to domains of HuPSAP involved in inducing apoptosis (Lamarca et al., 2007).

Mitochondria is an important organelle involved in regulating the apoptosis cascade (Han et al., 2006; Feng et al., 2008). To counter the effect of apoptosis, viruses encode mitochondria localized proteins, which can inhibit apoptosis (Hart et al., 2007; Radke et al., 2008). Several evidence suggests that BAdV-3 33K proteins may act as anti-apoptotic protein. First, confocal studies revealed that in addition to nucleus, a portion of 33K localizes to the mitochondria. Secondly, BAdV-3 33K colocalizes with BoPSAP in mitochondria. Thirdly, expression of 33K significantly reduced the presence of apoptotic cells in Staurosporine treated cells. Finally, 33K significantly reduced the cleavage of PARP.

Thus, the interaction of 33K with BoPSAP may inactivate BoPSAP and induce an anti-apoptotic effect. This property of 33K protein may help in successful propagation of BAdV-3 by overcoming apoptosis, a part of innate and acquired immune responses (Jung et al., 2004; Cardoso et al., 2008). Further studies are in progress to identify the domain of 33K protein involved in interaction with BoPSAP protein.

6.0 GENERAL DISCUSSION AND CONCLUSION

To date, all reported members of *Mastadenovirus* genus contain homologs of 22K ORF and 33K ORF originating from an unspliced and a spliced form of a late transcript, respectively. Recent reports indicate that 33K acts as an alternative RNA splicing factor (Tormanen et al., 2006) and a transcriptional activator (Ali et al., 2007), and plays a role in viral capsid assembly as well as efficient capsid DNA interaction (Fessler and Young, 1999; Finnen et al., 2001; Kulshreshtha et al, 2004). Recently, adenovirus 22K protein has been shown to be involved in the packaging of adenovirus genome (Ostapchuk et al, 2006). Based on the nucleotide sequence of genomic DNA, similar genomic organization has been proposed for BAdV-3 (Kulshreshtha et al., 2004). However, our earlier data about structural and functional study could not resolve the issue of the existence of both 33K protein and 22K protein in BAdV-3 infected cells (Kulshreshtha et al., 2004).

Thus, the primary objective of this investigation was to further characterize 33K protein, determine its role in BAdV-3 infection and elucidate its interaction (if any) with other viral /cellular proteins.

Using RT-PCR analysis, we also detected spliced and unsliced mRNAs encoding 33K and 22K proteins, respectively in BAdV-3 infected cells. This was confirmed using rabbit polyclonal antisera raised against peptides representing unique C- terminal regions of the proteins. Anti-33Kp serum detected five major proteins of 42 kDa, 39 kDa, 37 kDa, 21 kDa and 19 kDa and three minor proteins of 35 kDa, 25 kDa, and 23 kDa in BAdV-3 infected cells or 33K transfected cells. The 21 kDa band appears to be a doublet. Similarly, anti-22Kp serum detected three proteins of 41kDa, 39kDa and 37kDa in BAdV-3 infected cells. However, only two proteins of 39kDa and 37kDa were detected in 22K (having splice sites removed) transfected cells. The 33K protein is predominantly localized to the nucleus of BAdV-3 infected cells and is involved in stimulating the transcription from major late promoter. Analysis of mutant 33K proteins demonstrated that amino acids 201-240 and amino acid 213-232 are required for nuclear localization and MLP transactivation, respectively.

Since the assembly of mature adenovirus is a multi-step process involving protein-protein (homotypic / heterotypic) and protein-DNA interactions, the involvement of 33K

in various steps of adenovirus replication and assembly may require the direct protein-protein interactions. Earlier, reports have demonstrated protein –protein interactions among several adenovirus proteins in virus infected cells (Ewing et al., 2007; Ostapchuk and Hearing, 2008; Perez-Romero et al., 2005; Singh et al., 2005). As adenovirus 33K protein appears to be a multifunctional protein performing different roles in viral infection, the involvement of 33K protein in different steps of adenovirus replication may require protein-protein interaction. Thus, the next step was to determine if 33K protein interacts with other BAdV-3 proteins.

Using 33K proteins a bait in the yeast two hybrid system, we screened open reading frames (ORFs) of bovine adenovirus (BAdV)-3 for potential interactions with 33K protein. Interestingly, 33K protein showed specific interaction with 100K and pV proteins. The yeast two-hybrid findings were validated by *in vitro* binding using *in vitro* synthesized transcription-translation products, GST-pull down assay and *in vitro* co-immunoprecipitation assay using protein-specific antibodies. We demonstrated, that the interaction of 33K protein with 100K and pV proteins take place during BAdV-3 infection. Finally, our data suggests that the stretch of amino acids 81-120 and 161-200 in 33K protein are critical for the interaction with pV and 100K proteins, respectively.
The existence of such interactions in other adenoviruses and exploring the functional consequences of these interactions will help in determining the importance of such interactions.

A number of adenoviral proteins have been shown to interact with other cellular proteins during different stages of viral replication (Green et al., 2008; Xue et al., 2005; Yondola and Hearing, 2007). These interactions may alter the activities of cellular regulators of gene expression (Miura et al., 2007), cell cycling (Berk et al., 2005; Seifried et al., 2008) and apoptosis (Seifried et al., 2008). In addition, these interactions may help in sequestering (Fleisig et al., 2007) or redistribution (Yondola and Hearing, 2007) of important cellular proteins thus helping to defend against antiviral cellular responses (Weitzman and Ornelles, 2005). At different stage of virus replication, different adenovirus protein may be involved, in interacting with the same cellular protein(s) or altering the same cellular process (Berk, 2005) suggesting that adenovirus proteins are functionally redundant. Based on these reports, it is clear that these interactions are

critical for setting up the host for efficient adenovirus production during a lytic cycle (Weitzman and Ornelles, 2005).

Using bovine retina cDNA library, we demonstrated that BAdV-3 33K protein appeared to interact with bovine presenilin-1-associated protein / mitochondrial carrier homolog 1 (BoPSAP / BoMtch1) and bovine microtubule associated protein (BoMAP). However, subsequent analysis by various *in vitro* and *in vivo* assays could only confirm the interaction between 33K protein and BoPSAP/BoMtch1. False positive results can be detected using yeast two hybrid systems. As such yeast two hybrid results should always be confirmed using different *in vitro* and *in vivo* assays.

Human presenilin-1-associated protein / mitochondrial carrier homolog 1 (hPSAP / Mtch1) is a pro apoptotic outer mitochondrial protein (Lamarca et al., 2007; Xu et al., 2002). Two isoforms of hPSAP / Mtch1 have been identified (Lamarca et al., 2007) in human tissues, which share two pro apoptotic domains and multiple internal signals for import into the mitochondrial outer membrane (Lamarca et al., 2007). Human PSAP protein induces apoptosis when over expressed in cells in culture (Lamarca et al., 2007; Xu et al., 2002), However, we could isolate and identify only one form of BoPSAP protein from bovine cells. Our results suggest that 33K protein co-localizes with BoPSAP in the mitochondria and can modulate the apoptotic effect of Stauroporine. Further experiments are in progress to determine if BoPSAP can induce apoptosis in cells and the role 33K can play in modulating the pro apoptotic effect of BoPSAP.

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