Leucine residues in conserved region of 33K protein of bovine adenovirus – 3 are important for binding to major late promoter and activation of late gene expression

Vikas Kulshreshtha [a,b,1,2], Azharul Islam [a,2], Lisanework E Ayalew [a,b], Suresh K. Tikoo [a,b,c,*]

[a] VIDO-InterVac, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5E3
[b] Veterinary Microbiology University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5E3
[c] Vaccinology & Immunotherapeutics, School of Public Health[3] University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5E3

ABSTRACT

The L6 region of bovine adenovirus 3 (BAdV-3) encode 33K (spliced) and 22K (unspliced) proteins. Earlier, anti-33K serum detected five major and three minor proteins in BAdV-3 infected cells. Here, we demonstrate that anti-sera raised against L6-22K protein detected two proteins of 42 and 37 kDa in BAdV-3 infected cells and one protein of 42 kDa in transfected cells expressing splice-site variant 22K protein (pC.22K containing substituted splice acceptor/donor sequence). Unlike 22K, 33K stimulated the transcription from the major late promoter (MLP) by binding to the downstream sequence elements (DE). Analysis of the variant proteins demonstrated that amino acids 201–240 of the conserved C-terminus of 33K containing the potential leucine zipper and RS repeat are required for the activation of MLP. Furthermore, amino acid substitution analysis demonstrated that unlike arginine residues of RS repeat, the leucine residues (217, 224, 232 and 240) of the conserved leucine zipper appear required for the binding of 33K to the MLP.

© 2015 Elsevier Inc. All rights reserved.

Introduction

Adenoviruses contain a non-enveloped, double-stranded DNA genome of 26–45 kb 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; 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 (Kulshreshtha, 2009; Reddy et al., 1998). Earlier, using anti-33K serum we detected three proteins of 42 kDa, 38 kDa and 33 kDa (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 (Kulshreshtha, 2009; Reddy et al., 1998). The 33K and 22K proteins share a N-terminus region of 138 amino acids (Kulshreshtha, 2009) and play an important role in virus assembly (Kulshreshtha et al., 2004).

Recently, adenovirus 33K protein has been shown to be involved in different steps of virus infection (Wu et al., 2013) including early to late switch of major late transcription unit expression (Farley et al., 2004), alternative RNA splicing factor (Tormanen et al., 2006), a transcriptional activator (Ali et al., 2007) and packaging of viral genome in empty capsid (Finnen et al., 2001; Wu et al., 2013). In this report, we demonstrate that a highly conserved C-terminus region of BAdV-3 33K protein contains domain required for binding to bovine MLP sequences. Moreover, conserved leucine residues (amino acids 217, 224, 232, 240) located in this conserved region of bovine adenovirus-3 33K are important for binding of 33K to major late promoter.

---

* Corresponding author at: VIDO-InterVac, University of Saskatchewan, 120 Veterinary Road, Saskatoon, Saskatchewan, Canada S7N 5E3. Fax: +1 306 966 7478.
E-mail address: Suresh.tik@usask.ca (S.K. Tikoo).
[1] Present address: Department of Pathobiology, LSU School of Veterinary Medicine, Skip Bertman Drive, Baton Rouge, LA 70803, USA.
[2] Contributed equally to this work.

Results

Expression of BAdV-3 22K protein

The BAdV-3 22K protein (translated from unspliced form of 33K transcript) shares N-terminal 138 amino acids with 33K protein (translated from spliced form of 33K transcript) (Kulshreshtha et al., 2004). Earlier, we reported the characterization of protein (s) encoded by spliced form of BAdV-3 33K mRNA using anti-33Kp serum raised against peptides representing unique C-terminal region (amino acid 141–185 and 156–200) of 33K (spliced form of 33K) protein (Kulshreshtha et al., 2014). To characterize BAdV-3 22K protein, we raised rabbit antisera against a peptide representing unique region of (amino acids 191–241) of 22K protein. As expected, anti-33Kp sera detects protein of 42 kDa (Fig. 1, panel B) in cells transfected with pC.33K DNA (Fig. 1, panel B). Surprisingly, anti-33Kp serum also detected a major protein of 39 kDa in the cells (Fig. 1, panel B) transfected with plasmid pC.22K DNA (expressing unspliced form of 33K mRNA) (Fig. 1, panel A) or plasmid pC.22KS1 DNA (pC.22K containing stop codon in the unique region) (Fig. 1, panel A). No such protein could be detected in cells (Fig. 1, panel B) transfected with plasmid pC.22Kss DNA (pC.22K containing substitutions in splice acceptor/donor sequence) (Fig. 1 panel A) or plasmid pCDNA3 DNA (Fig. 1 panel A).

To analyze the expression of 22K protein, we raised antisera (anti-22Kp) against a peptide representing the unique C-terminal region (amino acid 91–241) of 22K protein. Interestingly, sera raised against L6-22K protein detected two major proteins of 42 kDa, and 37 kDa in BAdV-3 infected cells at 48 h post infection (Fig. 1, panel C). Anti-22Kp serum detected a protein of 42 kDa (Fig. 1, panel C) in cells transfected with plasmid pC.22Kss DNA (Fig. 1, panel A). No such protein(s) could be detected in cells (Fig. 1, panel C) transfected with plasmid pC.33K DNA, plasmid pC.22K DNA, plasmid pC.22KS1 DNA or plasmid pCDNA3 DNAs (Fig. 1 panel A).

Activation of transcription from major late promoter by BAdV-3 33K protein

To investigate if BAdV-3 33K protein activate transcription from major late promoter (MLP), initially we used plasmid pC.33K expressing BAdV-3 33K (spliced form of 33K mRNA; Kulshreshtha et al., 2014) and plasmid pC.IVa2 expressing full
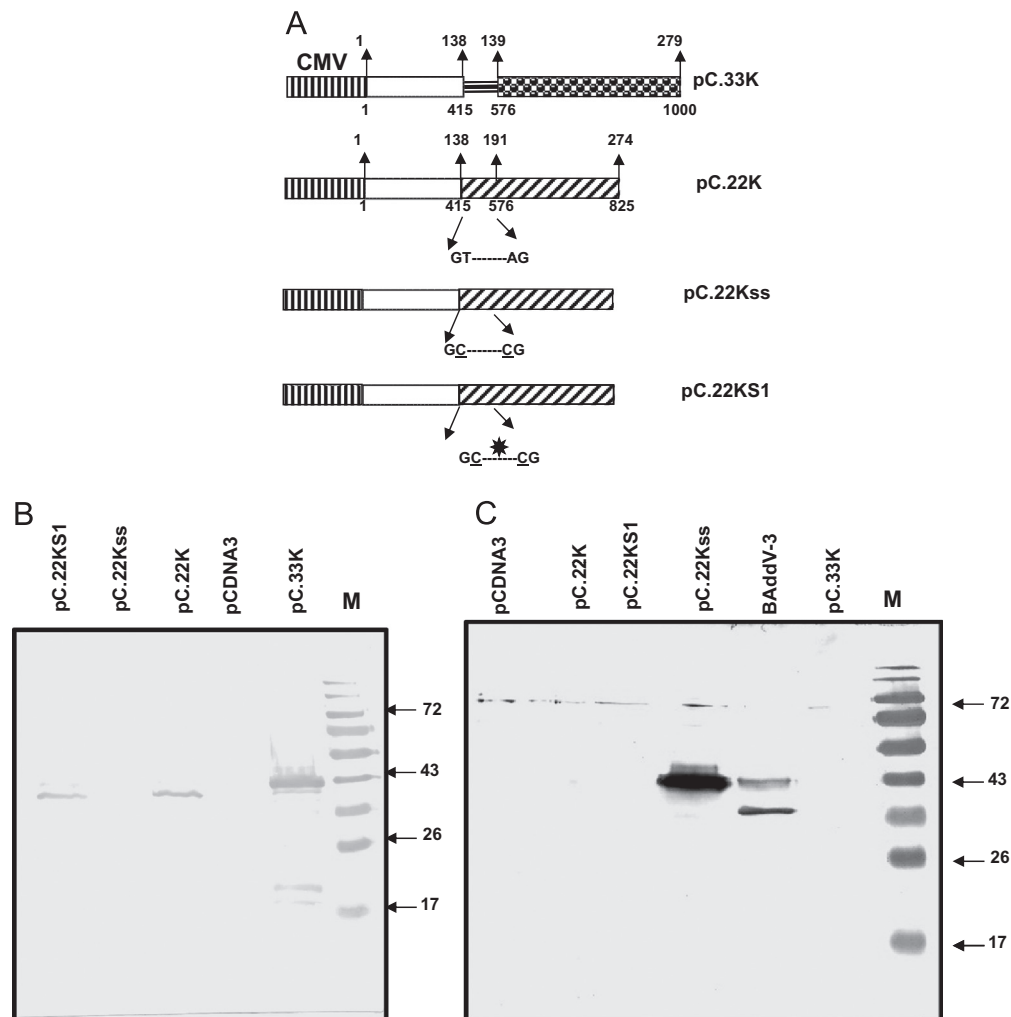
Fig. 1. Analysis of BAdV-3 22K protein. Schematic representation of BAdV-3 33K and 22K. The coding sequences shared by 33K and 22K (▭), unique region of 33K (▨), unique region of 22K (▨) and the spliced region of 33K (▬) are depicted. Human cytolmegalovirus immediate early promoter (CMV). The wild-type (GT—AG) and mutated (GC—CG) splice acceptor/donor sites are depicted. The amino acid numbers are shown on top while nucleotide numbers are shown at the bottom. The star represents the stop codon. The name of the plasmid is depicted on the right of the panel. (B, C) *Western blot*. Proteins from the lysates of BAdV-3 infected MDBK cells or plasmid transfected 293T cells were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes and probed with ant-33Kp sera (panel B) (Kulshreshtha et al., 2014) or anti-22Kp sera (panel C). The molecular weight (in kDa) of proteins is depicted on the right of the panel.
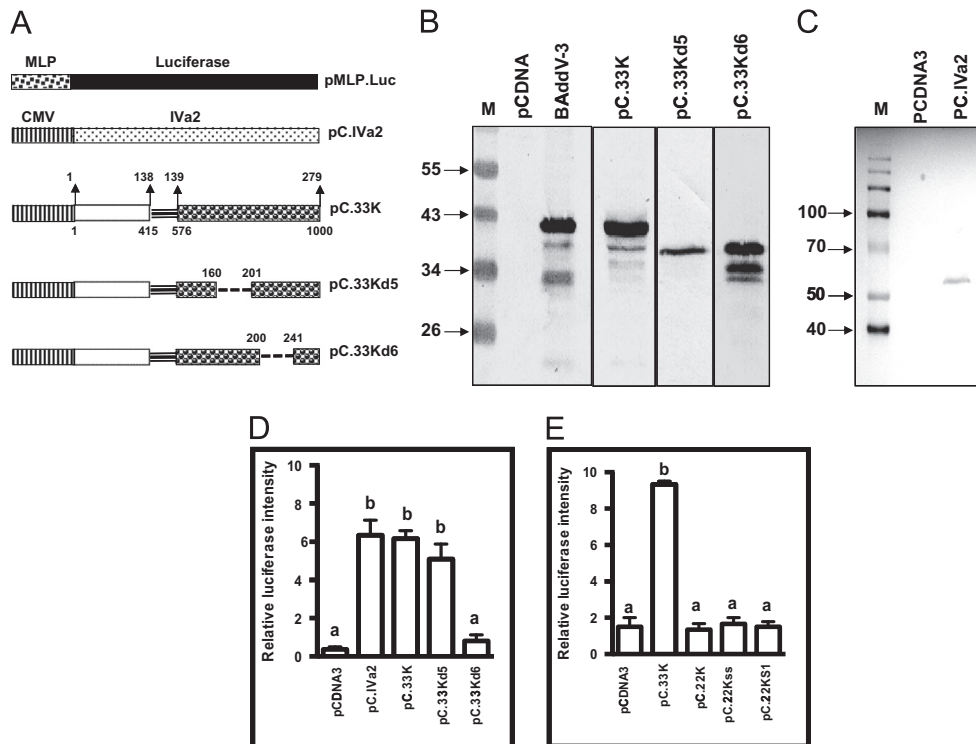
Fig. 2. Transactivation of MLP by BAdV-3 33K. (A) *Schematic representation of plasmids DNAs*. The coding sequences shared by 33K and 22K (▭), unique region of 33K (▨) and the spliced region of 33K (▬) are depicted. The human cytomegalovirus immediate early promoter (▮); major late promoter (MLP). The name of the plasmid is depicted on the right of the panel. (B, C) *Western blot.* Proteins from the lysates of the 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 sera (panel B) or anti-pIVa2 sera (panel C). The position of the molecular weight markers (M) in kDa is shown to the left of the each panel. (D, E) *Luciferase assay*. Monolayers of HeLa cells were co-transfected with plasmid pMLP.luc DNA, plasmid phRL-Renilla luciferase together with indicated individual plasmid DNAs. The cells were collected 48 h post transfection and analysed for firefly luciferase and renilla luciferase activity. The firefly luciferase activity value was normalized by the corresponding value of Renilla luciferase. Values are expressed as relative light units (RLU). Relative luciferase units (means from three independent experiments) are represented with corresponding standard deviations. Means with same letter are not significantly different *P < 0.05.

length BAdV-3 IVa2 (Tekele and Tikoo, unpublished data) as positive control (Lutz and Kedinger, 1996). Western blot analysis indicated that the cells transfected with individual plasmid pC.33K (Fig. 2, panel A) or pC.IVa2 (Fig. 2, panel A) DNAs synthesized proteins of expected molecular weight (Fig. 2, panels B,C). Next, plasmid pMLP.Luc containing a luciferase reporter gene under the control of MLP (Fig. 1A) was co-transfected into HeLa cells together with individual plasmid pC.33K or plasmid pC.IVa2 (Fig. 1A) DNA. As seen in Fig. 2D, luciferase expression increased 12 to 15-fold when the cells were co-transfected with plasmid pMLP. Luc+pC.33K or pMLP.Luc+pC.IVa2 DNAs (Fig. 2, panel D). However, there was no increase in the luciferase expression when the cells were co-transfected with plasmid pMLP.Luc+pCDNA3 DNAs (Fig. 2, panel D). These results suggested that BAdV-3 33K activate transcription from MLP.
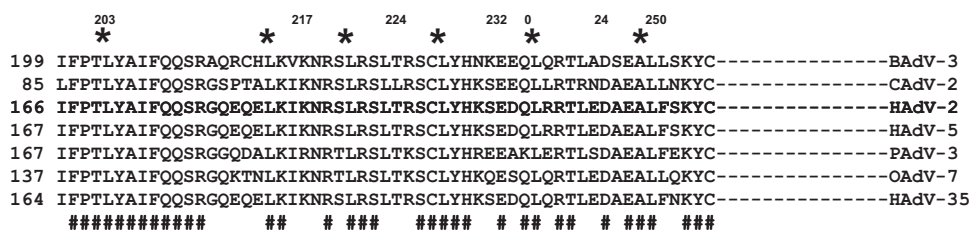
Analysis of 33K protein sequences of *Mastadenoviruses* infecting humans and different animals identified a highly conserved region in BAdV-3 33K spanning amino acid 199–255 (Fig. 3). Earlier, we constructed plasmids (Kulshreshtha et al., 2014; Fig. 2, panel A) containing deletion of amino acid 161–200 (PC.33Kd5) and amino acid 201–240 (PC.33Kd6). Western blot analysis confirmed the expression of variant protein in plasmid DNA transfected cells (Fig. 2, panel B). To determine the domain involved in the activation of MLP, the 33K mutant proteins (Fig. 2, panels A, B) were tested for the transactivation of MLP. As seen in Fig. 2 (panel D), deletion of amino acid 161–200 of 33K (pC.33Kd5) did not affect the transactivation of MLP. However, the deletion of amino acid 201–240 of 33K (pC.33Kd6) abolished the transactivation of MLP (Fig. 2, panel D).

Activation of transcription from major late promoter by BAdV-3 22K protein

To determine if 22K protein (unspliced form of 33K mRNA) activate transcription from MLP, we constructed plasmids pC.22K, pC.22Kss or pC.22KS1 (Fig. 1, panel A). The Hela cells were co-transfected with plasmid pMLP.Luc+pC.33K; pMLP.Luc+pC.22K; pMLP.Luc+pC.22Kss, pMLP.Luc+pC. 22KS1 or pMLP.Luc+pCDNA3 DNAs. As expected, there was a significant increase in the luciferase expression when cells were transfected with plasmid pMLP.Luc+pC.33K (Fig. 2, panel E) DNAs. However, no such increase was observed when cells were co-transfected with plasmid pMLP.luc and pC.22K, pC.22Kss, pC.22Ks1 or pCDNA3 (Fig. 2, panel E) DNAs.

DNA binding activity of BAdV-3 33K

Earlier reports have suggested that human adenovirus (HAdV)-5 33K binds to intragenic sequences of MLP and stimulates transcription (Ali et al., 2007). In particular, 33K alone bind to DE1 sequence and in the presence of IVa2 binds to DE2A sequence of MLP (Ali et al., 2007). The sequence alignment using CLUSTAL W2 program demonstrated that DE1 and DE2a sequences of intragenic regions of MLP are highly and partially conserved, respectively between BAdV-3 and HAdV-5 (Fig. 4, panel A). To determine if BAdV-3 33K binds to similar sequences of BAdV-3 MLP, we performed electrophoretic mobility shift assay (EMSA). The GST alone or GST tagged 33K or 22K proteins were produced in *Escherichia coli* (Kulshreshtha, 2009) and purified using

```
           203              217      224      232   0        24    250
            *               *        *        *     *         *     *
     199 IFPTLYAIFQQSRAQRCHLKVKNRSLRSLTRSCLYHNKEEQLQRTLADSEALLSKYC--------------BAdV-3
      85 LFPTLYAIFQQSRGSPTALKIKNRSLRSLLRSCLYHKSEEQLLRTRNDAEALLNKYC--------------CAdV-2
     166 IFPTLYAIFQQSRGQEQELKIKNRSLRSLTRSCLYHKSEDQLRRTLEDAEALFSKYC--------------HAdV-2
     167 IFPTLYAIFQQSRGQEQELKIKNRSLRSLTRSCLYHKSEDQLRRTLEDAEALFSKYC--------------HAdV-5
     167 IFPTLYAIFQQSRGGQDALKIRNRTLRSLTKSCLYHREEAKLERTLSDAEALFEKYC--------------PAdV-3
     137 IFPTLYAIFQQSRGQKTNLKIKNRTLRSLTKSCLYHKQESQLQRTLEDAEALLQKYC--------------OAdV-7
     164 IFPTLYAIFQQSRGQEQELKIKNRSLRSLTRSCLYHKSEDQLQRTLEDAEALFNKYC--------------HAdV-35
         ###########      ##   # ###   ##### # ## ##  # ###  ###
```

Fig. 3. Amino acid homology of BAdV-3 33K. Alignment of deduced amino acid sequences of BAdV-3 33K (Genbank Accession # AF030154) homologs with those of canine adenovirus (CAdV)-2 (GeneBank Accession # AC_000020), HAdV-2 (GenBank Accession # AC_000007), HAdV-5 (GenBank Accession # AC_000008), porcine adenovirus-3 (GenBank Accession # AF083132), Ovine adenovirus-7 (GenBank Accession # NC_004037.2), HAdV-35 (GenBank Accession # AC_000019). The identical residues (#) and Leucine residues (*) are indicated.

glutathione Sepharose beads (Fig. 4, panel B). The purified proteins were used with [^{32}P]-labeled 31 bp double stranded DNA MLP oligonucleotide (Fig. 4, panel A) containing nucleotide 5671–5701 of BAdV-3 genome representing intragenic regions of bovine MLP (Reddy et al., 1998) in EMSA analysis. As seen in Fig. 4, purified 33K.GST protein decreased the migration of labeled oligonucleotide and formed distinct band in 4% native acrylamide gel (panel C). No such complex was formed when purified GST alone was used in the reaction mixture (panel C). The interaction appears specific as increasing concentration of cold 31 bp double stranded oligonucleotide used as a competitor decreased the binding of purified GST-33K protein to labeled oligonucleotide (panel D).

The BAdV-3 22K protein translated from unspliced version of 33K transcript shares N-terminal 138 amino acids with 33K protein translated from spliced 33K transcript. To determine if 22K protein bind to MLP, purified GST, 33K.GST or 22K.GST proteins were used with [^{32}P]-labeled 31 bp double stranded DNA MLP oligonucleotide (Fig. 4, panel A) in EMSA. As expected, purified 33K.GST formed complex with labeled oligonucleotide (Fig. 4, panel E). No such complex was formed when 22K.GST or GST alone was used in the reaction mixture (Fig.4, panel E)

DNA binding domain of BAdV-3 33K

Unlike 22K protein, since MLP activation function of 33K appeared conserved in *Mastadenoviruses*, we focused on BAdV-3 33K protein particularly the region found to be highly conserved among 33K proteins encoded by different *Mastadenoviruses* (Fig. 3). To determine the MLP DNA binding domain of BAdV-3 33K, we constructed two GST tagged 33K deletions namely 33Kd6.GST and 33Kd7.GST (Fig. 5, panel A). The tagged proteins were expressed in *Escherichia coli* and individual variant 33K.GST fusion proteins were purified using glutathione sepharose beads (Fig. 5, panel B). Protein 33Kd6.GST contains a deletion of 201–240 amino acids while protein 33Kd7 contains deletion contains a deletion of amino acid 242–274. The purified, GST, 33K.GST, 33Kd6.GST, or 33Kd7d.GST proteins were used to determine their interaction with [^{32}P] labeled 31 bp double stranded oligonucleotide (Fig. 4, panel A) using electrophoretic mobility shift assay (EMSA). As expected, GST.33K formed a complex with labeled probe (Fig.5, panel C). Similar complex was detected when 33Kd7 protein was used with labeled probe (Fig. 5, panel C). No such complex formation was observed in the presence of purified 33Kd6.GST or GST alone (Fig. 5, panel C).

To confirm these results, C- terminus of BAdV-3 33K encoding amino acids 195–255 was amplified by PCR and fused in frame to C-terminus of GAL4 activation domain containing SV40 NLS (GADT7; Clontech) creating plasmid pC.33K.GalAD (Fig. 5, panel D). The plasmid pMLP.Luc DNA containing a luciferase reporter gene under the control of MLP (Fig. 5, panel D) was co-transfected into HeLa cells together with individual plasmid pcDNA3, pC.IVa2 or pC.33K.GalAD DNAs and effect was determined using luciferase

assay. As seen in Fig. 5 (panel E), luciferase expression increased 5–6 fold when HeLa cells were co-transfected with plasmid pMLP.Luc+pC33K. GalAD or pMLP.Luc+pC.IVa2 DNAs. However, there was no increase in the luciferase expression when plasmid pMLP.Luc DNA was co-transfected into HeLa cells with the plasmid pcDNA3 DNA (Fig. 5, panel E).

Leucine residues at 217, 224, 232 and 240 of BAdV-3 33K are important for DNA binding activity.

Analysis of the highly conserved C-terminal region of BAdV-3 33K revealed that amino acids 199–255 contain 6 conserved leucine residues (L203, L217, L224, L232, L240 and L250) (Fig. 3). Since four of these conserved leucines (L217, L224, L232 and L240) have spacing consistent with requirement and prediction of a potential leucine zipper, initially we focused on these residues and constructed mutant BAdV-3 33K containing point mutations. To determine the importance of leucine residues of the potential leucine zipper, leucine residue at 217, 224, 232 and 240 were individually mutated to alanine residues. All 33K leucine variant proteins were expressed and transported to the nucleus in transfected cells (data not shown). Next, we constructed plasmids containing GST tagged 33K219A, 33K224A, 33K232A or 33K240A (Fig. 6, panel A). The individual tagged proteins were expressed in *Escherichia coli* and purified using glutathione sepharose beads (panel B). The GST-tagged variant 33K proteins were used to determine the formation of complexes with [^{32}P] labeled 31 bp double stranded oligonucleotide (Fig. 4, panel A) using EMSA. As expected 33K.GST formed complex with labeled MLP DNA probe (Fig. 6, panel C). In contrast, like GST or 33Kd6.GST, all variant 33K proteins generated by mutating leucine to alanine at 217, 224, 232 or 240 did not form any complex with the labeled MLP DNA probe (Fig. 6, panel C)

To further confirm these results, we amplified region spanning the amino acids 195-255 of individual mutant 33K protein and fused in-frame to C-terminus of GAL4 activation domain containing SV40 NLS (GADT7; Clontech) (Fig. 7, panel A). The plasmid pMLP.Luc containing a luciferase reporter gene under the control of major late promoter (MLP) was co-transfected into HeLa cells together with individual plasmid pcDNA3 or pC33K.GalAD DNA and effect was determined using Luciferase assay. As seen in Fig. 7B, the cells co-transfected with plasmid pMLP.Luc DNAs+pC33K.GalAD DNAs showed 2.5 fold increase in the luciferase expression compared to the cells transfected with plasmid pCDNA3 DNA. In contrast, there was no increase in the luciferase expression when cells were co-transfected with plasmid, pMLP.Luc+pC33K217A.GalAD, pMLP.Luc+pC33K223A.GalAD, pMLP.Luc+pC33K230A.GalAD or pMLP.Luc+pC33K240A.GalAD compared to cells co-transfected with plasmid pMLP.Luc+pcDNA3 DNAs.

*Leucines (203,250) and arginines (211,222,225) of BAdV-3 33K are not required for DNA binding*

To determine the role of conserved leucines (203,250) which does not appear to be part of the potential leucine zipper, we constructed variant 33K ORFs expressing 33K containing alanine residue in place of leucine residue at amino acid 203 (33K203A) or at amino acid 250 (33K250A). Earlier, we also constructed a variant 33K (33Ksr) protein where arginine residues (211, 222, 225) in the conserved RS repeat were mutated to glycine residues (Kulshreshtha et al., 2014). To determine the role of these conserved residues of BAdV-3 33K in binding to MLP, we constructed GST tagged variant 33K (Fig. 8, panel A) proteins. The purified GST-tagged variant proteins were produced (Fig. 8, panel B) and used to determine the formation of complexes with [^{32}P] labeled 31 bp double stranded oligonucleotide (Fig. 4, panel A) using EMSA. As expected, GST (Fig. 8, panels, C, D, E), 33Kd6.GST (Fig. 8, panel C) or 33K217A (Fig. 8, panel D) fusion proteins did not retard the migration of the labeled DNA probe while 33K.GST (Fig. 8, panels, C, D, E) fusion proteins retarded the migration of the labeled DNA probe (Fig. 8, panel D). Interestingly, 33K203A.GST (Fig. 8, panel C), 33K250A.GST (Fig. 8, panel D) or 33Ksr.GST (Fig. 8, panel E) fusion proteins also retarded the migration of the labeled probe when used in the reaction mixture.

To further confirm these results, we amplified region spanning the amino acids 195-255 of individual mutant 33K protein and fused in-frame to C-terminus of GAL4 activation domain containing SV40 NLS (GADT7; Clontech) (Fig. 9, panel A). The plasmid pMLP.Luc containing a luciferase reporter gene under the control of major late promoter (MLP) was co-transfected into HeLa cells together with individual plasmid pcDNA3 or pC33K.GalAD DNA and effect was determined using Luciferase assay. As seen in Fig. 9B, the cells co-transfected with plasmid pMLP.Luc DNAs+pC33K.GalAD DNAs showed 3 fold increase in the luciferase expression compared to the cells transfected with plasmid pC33K224A.GalAD DNA. Similar results were observed when plasmid pMLP.Luc DNA was co-transfected with plasmid pC33K203A.GalAD or pC33K250A.GalAD DNAs.

Discussion

Viral transcription regulators can act by recognizing specific DNA sequences (Lutz et al., 1997), by interacting with cellular DNA binding proteins, and/or by acting as cellular co-factors (Lutz and Kedinger, 1996; Perez-Romero et al., 2005). Adenovirus intermediate protein IVa2 has been shown to bind DE sequences downstream of MLP and trans-activate the MLP (Lutz and Kedinger, 1996). Earlier reports have also suggested that adenovirus 33K protein (Ali et al., 2007) is involved in the activation of MLP by direct binding to MLP. Interestingly, adenovirus 22K protein has also been shown to bind DE elements (Ostapchuk et al., 2006) and stimulate transcription from MLP (Backstrom et al., 2010). In this report, we demonstrate that (a) BAdV-3 33K activates the transcription from MLP by binding to DE sequences. The DNA binding
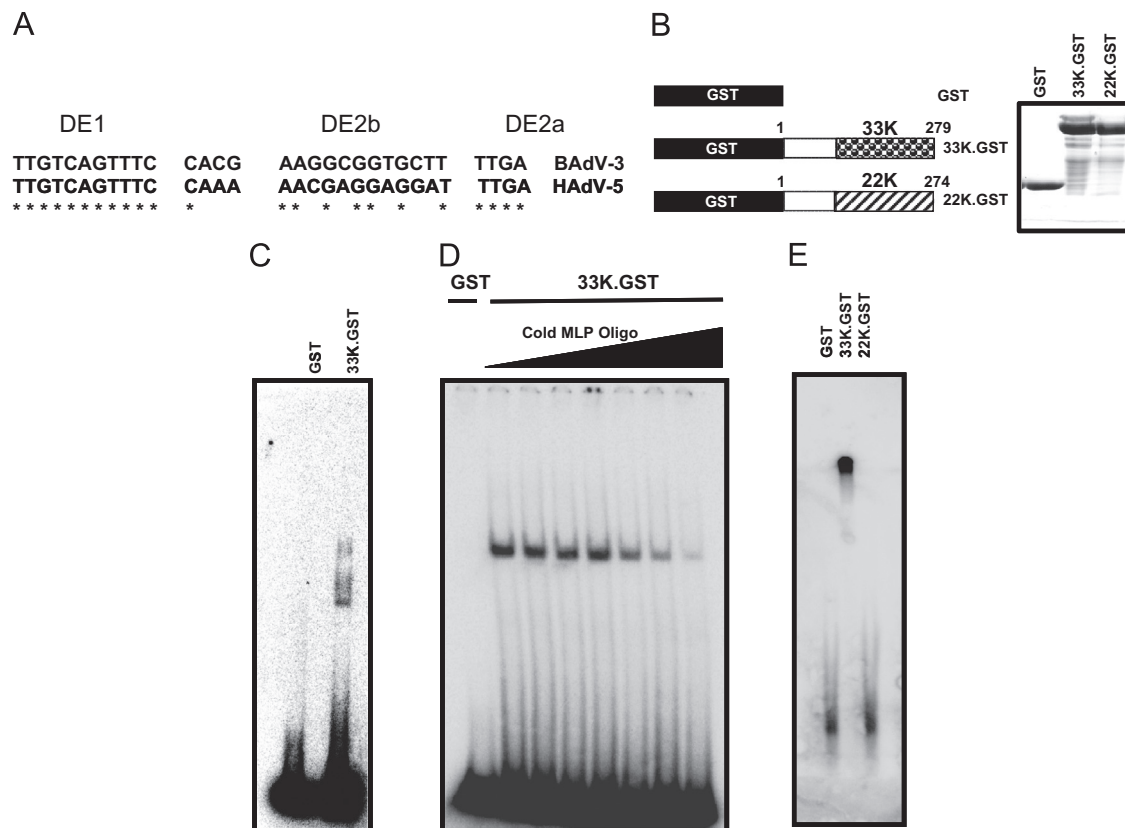
Fig. 4. BAdV-3 33K binds to DE elements. (A) *DE sequence homology*. Comparison of nucleotide sequence at the position of DE sequence of BAdV-3 (GenBank Accession # AF030154) and HAdV-5 (GenBank Accession # AC_000008). The identical residues are indicated (*). (B) *Schematic diagram of BAdV-3 33K and 22K fusion proteins*. The GST sequences are represented by black box. The coding sequences shared by 33K and 22K (☐) and unique region of 33K (▨) are depicted. The amino acid numbers are shown on top. The name of the proteins is depicted on the right of the panel. Coomassie blue staining of purified proteins. Purified GST, 33K.GST and 22K.GST fusion proteins were separated by 10% SDS-PAGE and stained with 0.25 Coomassie blue stain. (C, D, E) *Electrophoretic mobility shift assay*. A 31 bp [^{32}P] labeled double stranded DNA containing DE binding sites (A) was incubated with GST (panels C, D, E), 33K.GST fusion protein (panels C, D, E) or 22K.GST fusion protein (panel E) with increasing amount of cold competitor oligonucleotides as indicated (panel D). The complexes were separated by 4% acrylamide native gel and exposed over night on a phosphor-imaging screen and scanned using molecular imager (BioRad).
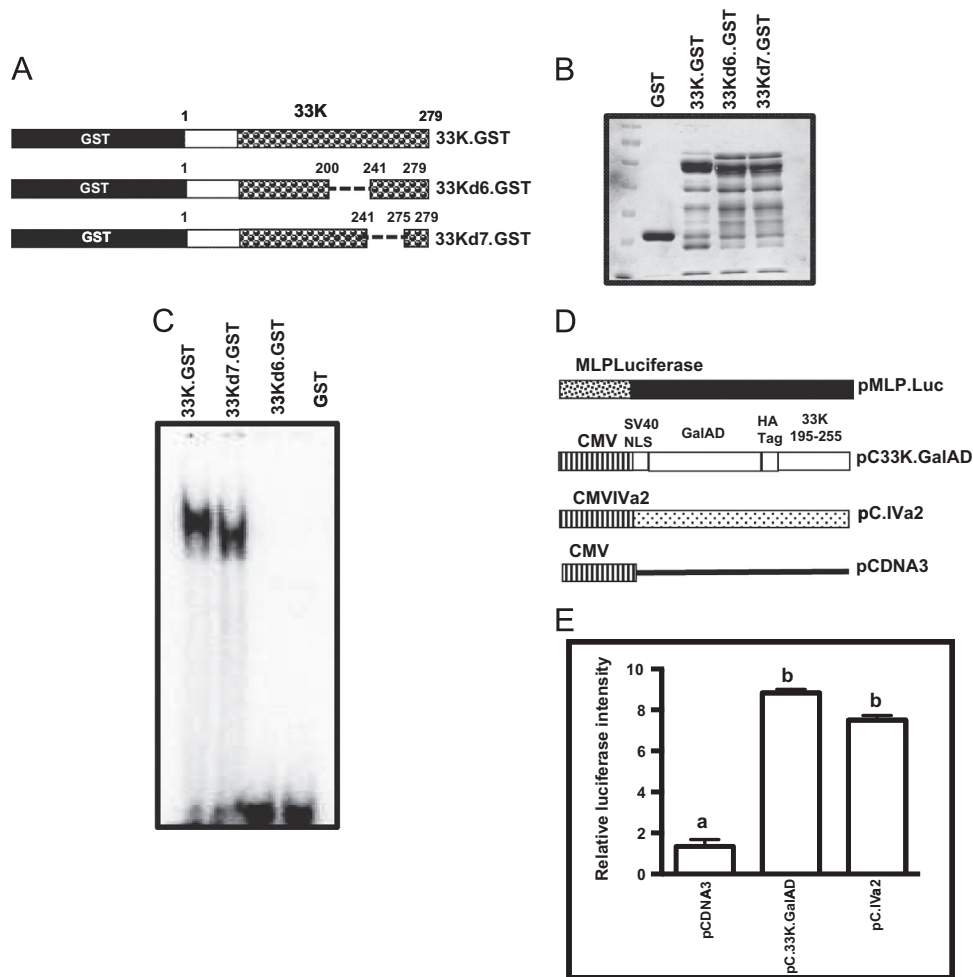
Fig. 5. Mutational analysis of BAdV-3 33K. (A) *Schematic representation of BAdV-3 33K.* Schematic representation of plasmids DNAs. The coding sequences shared by 33K and 22K ( ) and unique region of 33K ( ) are depicted. The dotted line represents deleted region. The amino acid numbers are shown on top. The name of the fusion protein is shown on the right of the panel. (B) *Coomassie blue staining of purified proteins.* Purified GST, 33K.GST, 33Kd6.GST and 33Kd7.GST proteins were separated by 10% SDS-PAGE and stained with 0.25 Coomassie blue stain. (C) *Electrophoretic mobility shift assay.* A 31 bp [^{32}P] labeled double stranded DNA containing DE binding sites (Fig. 4A) was incubated with purified GST, 33K.GST, 33Kd6.GST or 33Kd7.GST fusion proteins. The complexes were separated by 4% acrylamide native gel and exposed over night on a phosphor-imaging screen and scanned using molecular imager (BioRad). (D) *Schematic representation of plasmids DNAs.* Major late promoter (MLP), Human cytomegalovirus immediate early promoter (CMV), SV40 large T antigen nuclear localization signal (SV40 NLS); Yeast transcriptional activator GAL4 activation domain (GalAD); Heamagglutinin epitope (HA tag), BAdV-3 33K amino acids 195–255 (33K 195-255). The thin line represents plasmid DNA. (E) *Luciferase expression.* The cells were co-transfected with plasmid pMLP.luc, plasmid phRL-Renilla luciferase and individual plasmid (pC33K.GalAD, pC.IVa2 or pCDNA3) DNAs. After 48 h, Firefly luciferase and Renilla luciferase activity were measured. The Firefly luciferase activity value was normalized by the corresponding value of Renilla luciferase. Values are expressed as relative light units (RLU). Relative luciferase units (means from three independent experiments) are represented with corresponding standard deviations. Means with same letter are not significantly different *P < 0.05.

activity is located in the highly conserved region of BAdV-3 33K protein, and involves leucine residues of a potential leucine zipper.

Earlier, we characterized BAdV-3 33K produced in infected or transfected cells (Kulshreshtha et al., 2014). To characterize BAdV-3 22K, sera raised against L6-22K produced against a peptide representing unique region of BAdV-3 22K was used in Western blots. Anti-22Kp serum detected two proteins of 42 kDa and 37 kDa in BAdV-3 infected cells, which could be produced due to different post-translational modification or initiation of translation from different ATG codons. Surprisingly, anti-22Kp serum did not detect such proteins in cells transfected with plasmid pC.22K DNA with the potential to express 22K. 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 pC.22K DNA. Secondly, anti-33Kp detects a similar protein of 39 kDa in cells transfected with 22K expression plasmid pC.22KS1 containing insertion of a stop codon in the region spliced out in 33K. Third, anti-22Kp serum detects a protein of 42 kDa in cells

transfected with 22K expression plasmid pC.22Kss containing mutated splice accepter/donor sites but not in cells transfected with 22K expression plasmid pC.22K. Fourth, as expected anti-22Kp serum did not detect any protein in cells transfected with 22K expression plasmid pC.22KS1 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.

Our MLP activation and EMSA results suggest that BAdV-3 22K does not appear to bind DE sequence and activate transcription from MLP. In contrast, BAdV-3 33K activates transcription from MLP by binding to DE sequences. Earlier, conflicting observations
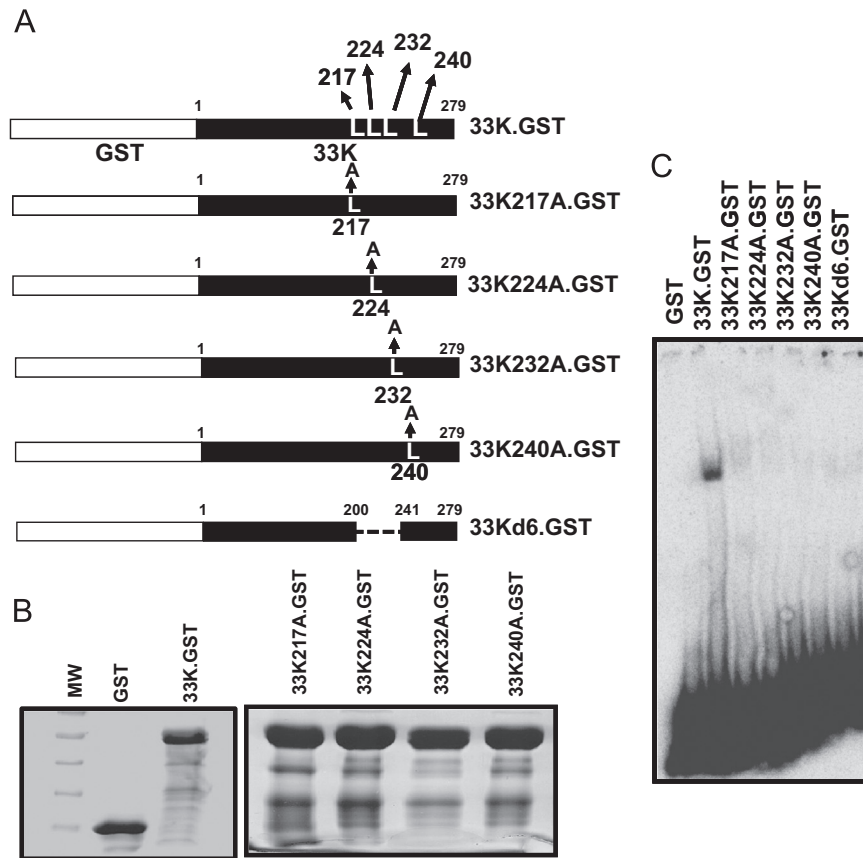
Fig. 6. EMSA with 33K leucine substitutions. (A) *Schematic representation of plasmid DNAs.* The 33K (black box), GST (white box) and deleted region (dotted line) is depicted. Leucine residues (L) are indicated. The substitution of leucine (L) with alanine (A) is shown by arrow on the top. The amino acid numbers of 33K are depicted. The name of the proteins is depicted on the right of the panel. (B) *Coomassie blue staining of purified proteins.* Purified GST, 33K.GST, 33K217A.GST, 33K224A.GST, 33K232A. GST, 33K240A. GST and 33Kd6.GST proteins were separated by 10% SDS-PAGE and stained with 0.25 Coomassie blue stain. (C) *Electrophoretic mobility shift assay.* A 31 bp [^{32}P] labeled double stranded DNA containing DE binding sites (Fig. 4A) was incubated with indicated purified GST or GST fusion proteins. The complexes were separated by 4% acrylamide native gel and exposed over night on a phosphor-imaging screen and scanned using molecular imager (BioRad).
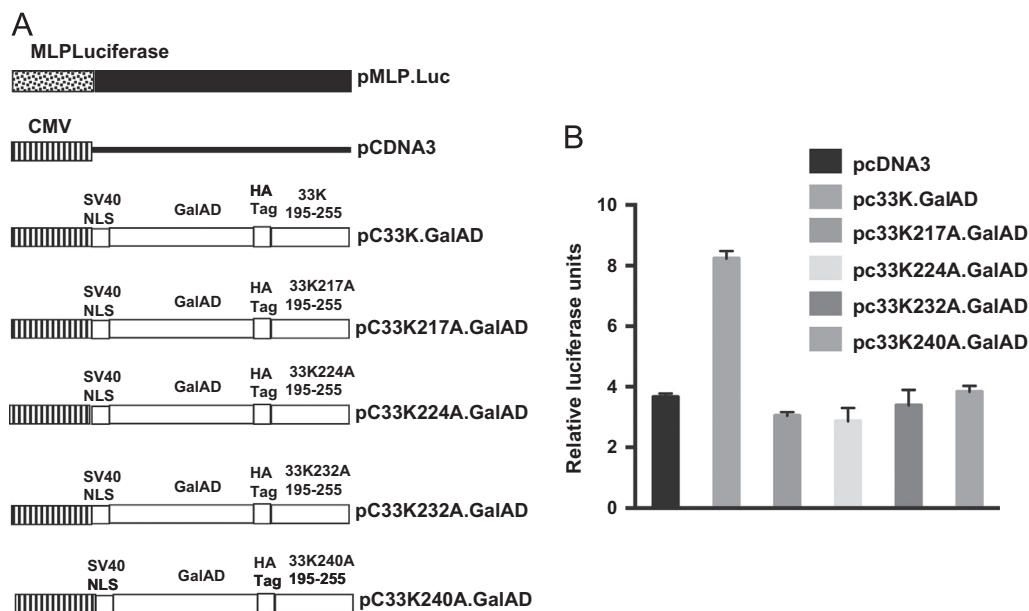
Fig. 7. Luciferase expression. (A) *Schematic representation of plasmid DNAs.* Major late promoter (MLP), human cytomegalovirus immediate early promoter (CMV), SV40 large T antigen nuclear localization signal (SV40 NLS); Yeast transcriptional activator GAL4 activation domain (GalAD); Heamagglutinin epitope (HA tag), BAdV-3 33K amino acids 195–255 (33K 195–255). The thin line represents plasmid DNA. The name of the plasmids is depicted on the right of the panel. (B) *Luciferase expression.* The cells were co-transfected with plasmid pMLP.luc, plasmid phRL-Renilla luciferase and individual plasmid DNAs (pc33K.GalAD, pC33K217A.GalAD, pC33K224A. GalAD, pC33K232A.GalAD, pC33K240A. or pCDNA3). After 48 h, Firefly luciferase and Renilla luciferase activity were measured. The Firefly luciferase activity value was normalized by the corresponding value of Renilla luciferase. Values are expressed as relative light units (RLU). Relative luciferase units (means from three independent experiments) are represented with corresponding standard deviations. Means with same letter are not significantly different *$P < 0.05$.
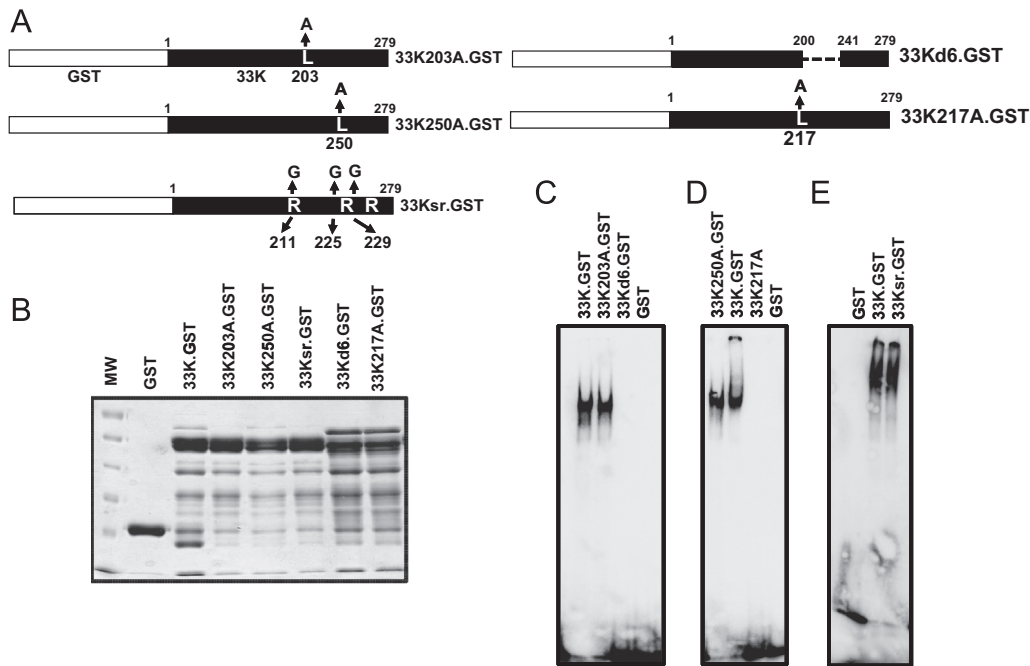
Fig. 8. EMSA with mutant 33K. (A) *Schematic representation of plasmid DNAs.* The 33K (black box), GST (white box) and deleted region (dotted line) is depicted. Leucine residues (L) are indicated. The substitution of leucine (L) with alanine (A) and arginine (R) with glycine (G)is shown by arrow on the top. The amino acid numbers of 33K are depicted. The name of the proteins is depicted on the right of the panel. (B) *Coomassie blue staining of purified proteins.* Purified GST, 33K.GST, 33K203A.GST, 33K250A.GST, 33Ksr.GST, 33K217A.GST and 33Kd6.GST proteins were separated by 10% SDS-PAGE and stained with 0.25 Coomassie blue stain. (C) *Electrophoretic mobility shift assay* . A 31 bp [^{32}P] labeled double stranded DNA containing DE binding sites (Fig. 4A) was incubated with indicated purified GST or individual GST fusion proteins. The complexes were separated by 4% acrylamide native gel and exposed over night on a phosphor-imaging screen and scanned using molecular imager (BioRad).
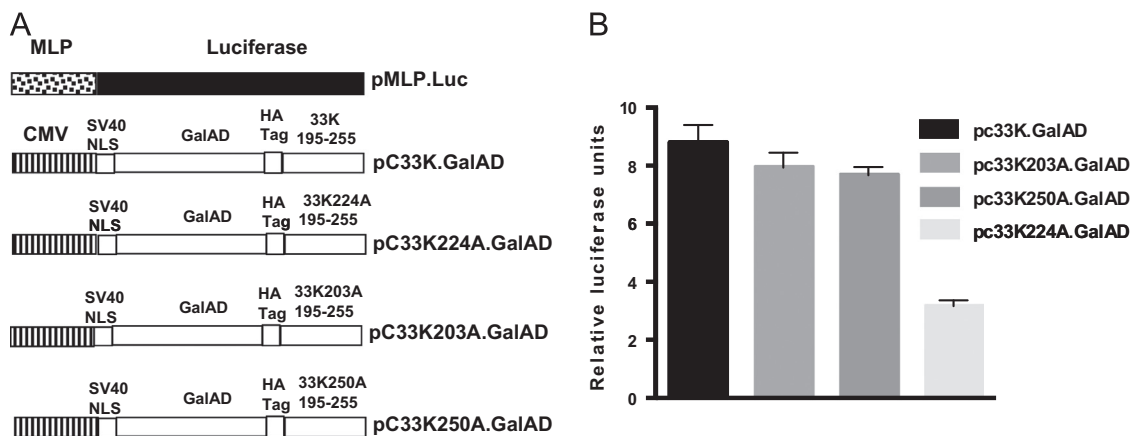
Fig. 9. *Luciferase expression.* (A) *Schematic representation of plasmid DNAs.* Major late promoter (MLP), human cytomegalovirus immediate early promoter (CMV), SV40 large T antigen nuclear localization signal (SV40 NLS); Yeast transcriptional activator GAL4 activation domain (GalAD); Heamagglutinin epitope (HA tag), BAdV-3 33K amino acids 195–255 (33K 195–255). The name of the plasmids is depicted on the right of the panel. (B) *Luciferase expression.* The cells were co-transfected with plasmid pMLP.luc, plasmid phRL-Renilla luciferase and individual plasmid DNAs (pC33K. GalAD, pC33K203A. GalAD, GalAD, pC33K250A.GalAD or pC33K224A. GalAD). After 48 h, Firefly luciferase and Renilla luciferase activity were measured. The Firefly luciferase activity value was normalized by the corresponding value of Renilla luciferase. Values are expressed as relative light units (RLU). Relative luciferase units (means from three independent experiments) are represented with corresponding standard deviations. Means with same letter are not significantly different *$P < 0.05$.

have been reported regarding the role of HAdV-5 33K and 22K in stimulating transcription from MLP (Ali et al., 2007; Backstrom et al., 2010) in transfected cells. Although unspliced transcript was not detected by RT-PCR (Ali et al., 2007), it was postulated that the plasmid expressing wild -type 33K may have produced both spliced or unspliced transcripts expressing 33K and 22K proteins, respectively in the transfected cell (Backstrom et al., 2010). Several lines of evidence suggest that BAdV-3 22K protein produced from unspliced 33K transcript is not expressed in plasmid pC.22K DNA transfected cells. First, protein expressed in cells transfected with plasmid pC.22K or pC.22KS1 DNA is recognized by anti-33K but not by anti-22K serum, Secondly, no such proteins could be

detected in cells transfected with plasmid pC.22Kss DNA or plasmid pC22KssS1 DNAs using anti-33K serum. Thirdly, 22K specific proteins could be detected in cells transfected with plasmid pC.22Kss DNA (where splice acceptor/donor site(s) are altered) by anti-22K serum. Our results support the notion (Ali et al., 2007) that protein produced in cells transfected with plasmid with potential to express unspliced form of 33K efficiently produce spliced form of protein (33K), which is stimulating transcription from MLP.

The inability of 22K protein to bind MLP and, conservation of the DNA binding and activation of MLP transcription function among 33K proteins of HAdV-5 and BAdV-3 suggested that DNA

binding domain may be present in the unique C-terminal conserved region of 33K proteins. Analysis of 33K proteins of different animal and human adenoviruses revealed a highly conserved domain located between amino acid 199-255. Analysis of mutant BAdV-3 33K proteins demonstrated that amino acids 201–240 contain MLP DNA binding domain and are critical for transcriptional activity of 33K.

The leucine zipper motif has been shown to be required for (a) 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). The identified conserved domain of BAdV-3 33K protein contains 7 conserved leucines, four (217, 224, 232, 240) of which appear to have configuration consistent with a potential leucine zipper motif (amino acids 217–240; Fig.3). Our results suggest that individual leucine residues of the putative leucine zipper motif appear essential for DNA binding and subsequent activation of transcription from MLP. Moreover, the function of activation of MLP by binding to DE elements appears conserved among 33K proteins of *Mastadenoviruses*.

Material and methods

Production of antisera

A peptide (amino acid [191]CRPEADQNRHSEQKEPPECQRGAPSPSSS SSQACSG APPPQRPAPSGRRRK[241]) representing 22K was 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 (500 µg/rabbit) emulsified with Freund's 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.

Construction of plasmids

The construction of plasmids pC.33K (spliced 33K), pC.22K, (unspliced 33K), pC.33KD5, pC.33Kd6 and pC.33Ksr is described elsewhere (Kulshreshtha et al., 2014). The construction of pC.IVA2 (contain IVa2 gene under the control of CMV promoter), and pMLP. Luc (contains firefly luciferase gene under the control of adenovirus major late promoter) will be described elsewhere.

Plasmids expressing 22K.

(i) *pC.22K*. A 835 bp fragment was amplified by PCR using primers P20 and P37 Table 1), and plasmid DNA pSM12 (Kulshreshtha 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 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 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 1), and plasmid pC.22Ks DNA as a

Table 1
List of primers.

P1	5′-ACTGAC*GAATTC*ATGAAACCCCGCAGCATGTC-3′
P2	5′-GGAATATGGCATAAGCAGTGGGAAAGATC-3′
P3	5′-GATCTTTCCCACTGCTTATGCCATATTCC-3′
P4	5′-ACTGACGTCGACAGGGACGGATTCGTCCTT-3′
P5	5′-CAGTATTTACTGAGAGCCGCCTCGGAGTCTG-3′
P6	5′-AGACTCCGAGGCGGCTCTCAGTAAATACTG-3′
P7	5′-ACTGACGCGGCCGCTCAAGGGACGGATTCGTCCTT-3′
P20	5′-G*GAATTC*CATATGATGAAACCCCGCAGCATGTCG-3′
P21	5′-G*GAATTC*CTTAGGCGGGTCCGGATTCG-3′
P22	5′-GAAGGCACA*GTCGAG*GCT-3′
P35	5′-GCTCCTCTTGCCTCTAGCGCC-3′
P36	5′-GCTAGAGGCAAGAGGAGCTAC-3′
P37	5′-G*GAATTC*CTATTTTTCACTTTGAGGTG-3′
P38	5′-TTCGGGTCGGCAGTAAGAATT-3′
P39	5′-TCTTACTGCCGACCCGAAGC-3′
P40	5′-TTTCACTTTGGCGTGACACCGC-3′
P41	5′-CGGTGTCACGCCAAAGTGAAAAAT -3′
P42	5′-CAGTGAACGTGCGGATCTATTTTTC-3′
P43	5′-AATAGATCCGCACGTTCACTGACG-3′
P44	5′-GTTGTGGTAGGCGCAGCTGCG-3′
P45	5′-CGCAGCTGCGCTACCCACAAC-3′
P46	5′-GGTTCGCTGGGCCTGTTCCTC-3′
P47	5′-GAGGAACAGGCCCAGCGAACC-3′
P48	5′-CG*GAATTC*CTTCGGGAGCTGATCTTTCCC-3′
P49	5′-ACTGAC*GTCGAC*AGGGACGGATTCGTCCTT-3′
P50	5′-CG*GAATTC*CTTCGGGAGCTGATCTTTCCC-3′
P51	5′-CCG*CTCGAG*TCAGCAGTATTTACTGAG-3′
P52	5′-ACTGAC*GAATTC*ATGAAACCCCGCAGCAT
P53	5′-ACTGAC*GTCGAC*ACTTTGAGGTGACACCG

Restriction enzyme recognition sites are underlined and in italics.

template. Similarly, a 427 bp fragment was amplified from by PCR using the primers P22 and P39 (Table 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 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.22Kss (splice donor and splice acceptor site mutated).

(iii) *pC.22KS1*. A 835 bp *Nde*I–*Eco*RI fragment amplified by PCR using primers P20 and P37 (Table 1), and plasmid pSM12 (Kulshreshtha et al., 2004) DNA as a template and ligated to 7.2 kb *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 pGBKT7.22 K 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.

Plasmids expressing mutant 33K

(i) *Plasmid pC.33K203A*. A 1 kb DNA fragment was amplified using primers P1 and P7 (Table 1), and plasmid pGEX.33K203A DNA as a template. The PCR product was digested with EcoRI–NotI and ligated to *Eco*RI–*Not*I digested pCDNA3 creating plasmid pC.33K203A.

(ii) *Plasmid pC.33K217A*. A 650 bp fragment was amplified from by PCR using the primers P20 and P40 (Table 1) and plasmid pC.33K DNA as a template. Similarly, a 356 bp fragment was amplified by PCR using the primers P21 and P41 (Table 1) and plasmid pC.33K DNA as a template. In a third PCR reaction, two PCR fragments that have 21 bp of internal overlap were annealed and external primers P1 and P21 (Table 1) were used to PCR across to give a 1 kb amplicon. This PCR product

was digested with SacII–EcoRI and ligated to SacII–EcoRI digested pC.33K creating plasmid pC.33K217A (Fig. 6A).

(iii) *Plasmid pC.33K224A.* A 672 bp fragment was amplified from by PCR using the primers P20 and P42 (Table 1), and plasmid pC.33K DNA as a template. Similarly, a 334 bp fragment was amplified by PCR using the primers P21 and P43 (Table 1), and plasmid pC.33K DNA as a template. In a third PCR reaction, two PCR fragments that have 21 bp of internal overlap were annealed and external primers P20 and P21 (Table 1) were used to PCR across to give a 1 kb amplicon. This PCR product was digested with SacII–EcoRI and ligated to SacII–EcoRI digested plasmid pC.33K creating plasmid pC.33K224A (Fig. 6A).

(iv) *Plasmid pC.33K232A.* A 695 bp fragment was amplified from by PCR using the primers P20 and P44 (Table 1), and plasmid pC.33K DNA as a template. Similarly, a 311 bp fragment was amplified by PCR using the primers P45 and P21 (Table 1), and plasmid pC.33K DNA as a template. In a third PCR reaction, two PCR fragments that have 19 bp of internal overlap were annealed and external primers P20 and P21 (Table 1) were used to PCR across to give a 1 kb amplicon. This PCR product was digested with SacII–EcoRI and ligated to SacII–EcoRI digested plasmid pC.33K creating plasmid pC.33K232A (Fig. 6A).

(v) *Plasmid pC.33K240A.* A 717 bp fragment was amplified from by PCR using the primers P20 and P46 (Table 1), and plasmid pC.33K DNA as a template. Similarly, a 289 bp fragment was amplified by PCR using the primers P21 and P47 (Table 1), and plasmid pC.33K DNA as a template. In a third PCR reaction, two PCR fragments that have 21 bp of internal overlap were annealed and external primers P20 and P21 (Table 1) were used to PCR across to give a 1kp amplicon. This PCR product was digested with SacII–EcoRI and ligated to SacII–EcoRI plasmid pC.33K digested plasmid pC.33K creating plasmid pC.33K240A (Fig. 6A).

(vi) *Plasmid pC.33K250A.* A 1 kb DNA fragment was amplified using primers P1 and P7 (Table 1), and plasmid pGEX.33K250A DNA as template. The PCR fragment was digested with EcoRI–NotI and ligated to EcoRI–NotI digested plasmid pCDNA3 creating plasmid pC.33K250A

Plasmids expressing GST fusion

The construction of plasmid pGEX.33K has been described earlier (Kulshreshtha and Tikoo, 2008). A 850 bp DNA fragment amplified by PCR using primers P48 and P49 (Table 1), and individual plasmid (pC.33K217A; pC.33K224A; pC.33K232A; pC.33K240A, pC.33Kd6, pC.33Kd7; pC.33Ksr) DNA a template. The PCR amplified DNA fragments were individually digested with EcoRI–SalI and ligated to EcoRI–SalI digested plasmid pGEX-5X-1 creating plasmids pGEX.33K217A, pGEX.33K224A, pGEX. 33K232A, pGEX.33K240A, pGEX.33Kd6, pGEX33Kd7 and pGEX.33Ksr, respectively.

A 839 bp DNA fragment amplified by PCR using primers P52 and P53, and plasmid pC.22K DNA as a template. The PCR amplified DNA fragment was digested with EcoRI–SalI and ligated to EcoRI–SalI digested plasmid pGEX-5X-1 creating plasmid pGEX.22K.

A 622 bp DNA fragment was amplified by PCR using the primers P1 and P2 (Table 1), and plasmid pGEX.33K DNA as a template. Similarly, 241 bp fragment was amplified by PCR using primers P3 and P4 (Table 1), and plasmid pGEX.33K DNA as a template. In third PCR, two PCR fragments that have 29 bp of internal overlap were annealed and external primers P1 and P4 (Table 1) were used to PCR across to give 1 kb amplicon. This PCR

product was digested with EcoRI–SalI and ligated to EcoRI–SalI digested plasmid pGEX.33K creating plasmid pGEX.33K203A.

A 764 bp DNA fragment was amplified by PCR using the primers P1 and P5 (Table 1), and plasmid pGEX.33K DNA as a template. Similarly, 100 bp fragment was amplified by PCR using primers P4 and P6 (Table 1), and plasmid pC.33 K DNA as a template. In third PCR, two PCR fragments that have 30 bp of internal overlap were annealed and external primers P1 and P4 were used to PCR across to give 1 kb amplicon. This PCR product was digested with EcoRI–SalI and ligated to EcoRI–SalI digested plasmid pGEX.33K creating plasmid pGEX.33K250A.

Plasmids expressing Gal activation domain fusions

A 510 bp HindIII–EcoRI fragment (containing Gal activation domain fused to SV40 NLS and HA epitope) of plasmid pGADT7 was isolated and ligated to 5.4 kb HindIII–EcoRI digested plasmid pcDNA3 creating plasmid pC.galAD. DNA fragments (190 bp each) were amplified by PCR using primers P50 and P51 (Table 1), and individual plasmid (pC.33K, pC.33K203A, pC.33K217A, p33K.224A, pC.33K232A, pC.33K240A or pC.33K250A) as DNA templates. The individual PCR fragment was digested with EcoRI–XhoI and ligated to EcoRI–XhoI digested pC.GalAD creating plasmids pC33K.GalAD, pC33K203A, pC33K217A.GalAD, pC33K224A.GalAD, pC33K232A. GalAD, pC33K240A.GalAD and pC33K250A.GalAD.

Production of GST fusion proteins

The expression of individual GST fusion proteins was induced in *Escherichia coli* by IPTG (1 M) for 5 h and fusion proteins were purified using GST-beads following the manufacturer's protocol. The purity of GST fusion proteins was checked by Coomassie staining of SDS-PAGE gels. Protein concentration was determined by Bradford protein assay.

Transcriptional activation assay.

Cells in one well (1.2×10^5 cells/well) of a 6 well plate were co-transfected with 20 ng of plasmid phRL-Renilla luciferase DNA, 1.5 μg/well of pMLP.Luc DNA and 500 ng of individual effector plasmid using lipofectamine 2000 (Invitrogen) as suggested by the manufacturer. Cells were lysed after 48 h of transfection and firely luciferase and Renilla luciferase activity were measured by TD 20/20 luminometer (Turner Designs Instrument) using the kit (Promega) according to the manufacturer's protocol. The firefly luciferase activity value was normalized by the corresponding value of Renilla luciferase.

Electrophoretic mobility shift assay

Two complementary oligonucleotides 31bases long containing 5′ overhangs (5′-ctagg TTGTCAGTTTCCAC GAAGGCGGTGCTTT TGA-3′; 5′-ctaggTCAAAAGCAC CGCCTTCGTGGAAACTGACAA-3′) representing BAdV-3 major late promoter sequences were annealed. The 5′ overhang was filled with dNTPs (dATP, dGTP, dTTP and α-dCTP[32]) using T4 DNA polymerase. Un-incorporated nucleotides were removed using phenol-chloroform extraction and labeled oligos were recovered by ethanol precipitation. The intensity of labeled oligos was determined by liquid scintillation counter (Backman). About 10,000 cpm labeled oligo was applied in this experiment to explore the binding of BAdV-3 33K protein in the MLP region. EMSA was performed following the protocol described earlier with little modification (Ali et al., 2007). The labeled oligo (1000 cpm) and 500 ng protein were incubated in the binding buffer for 20–30 min. The reaction mixture was run on 4% acrylamide native gel for 3 h at 150 V. The gel was dried on

Whatman paper and exposed over night on a phosphor-imaging screen. The image was analyzed by Molecular Imager (BioRad). Empty GST fusion protein was used as a control in this experiment. The binding of 33K protein in the MLP region was confirmed by competitive binding assay using unlabeled oligonucleotides.

Acknowledgment

References

Ali, H., Leroy, G., Bridge, G., Flint, S.J., 2007. The adenoviral L4 33 kDa protein binds to intragenic sequences of the major late promoter required for late phase-specific stimulation of transcription. J. Virol. 8, 1327–1338.

Backstrom, E., Kaufmann, K.B., Lan, X., Akusjarvi, G., 2010. Adenovirus L4-22K stimulates major late transcription by a mechanism requiring the intragenic late specific transcription factor binding site. Virus Res. 151, 220–228.

Baxi, M.K., Robertson, J., Babiuk, L.A., Tikoo, S.K., 2001. Mutational analysis of early region 4 of bovine adenovirus type 3. Virology 290, 153–163.

Davison, A.J., Wright, K.M., Harrach, B., 2000. DNA sequence of frog adenovirus. J. Gen. Virol. 81, 2431–2436.

Farley, D.C., Brown, J.L., Leppard, K.N., 2004. Activation of the early-late switch in adenovirus type 5 major late transcription unit expression by L4 gene products. J. Virol. 78, 1782–1791.

Finnen, R.L., Biddle, J.F., Flint, J., 2001. Truncation of the human adenovirus type 5 L4 33-kDa protein: evidence for an essential role of the carboxy-terminus in the viral infectious cycle. Virology 289, 388–399.

Geisberg, J.V., Lee, W.S., Berk, A.J., Ricciardi, R.P., 1994. The zinc finger region of the adenovirus E1A transactivating domain complexes with TATA box bindig protein. Proc. Natl. Acad. Sci. USA 91, 2488–2492.

Goodwin, D.J., Hall, K.T., Giles, M.S., Calderwood, M.A., Markham, A.F., Whitehorse, A., 2000. The carboxy terminus of the herpesvirus saimiri ORF57 gene contains domains that are required for transactivation and transrepression. J. Gen. Virol. 81, 2253–2265.

Jansen-Durr, P., Mondesert, G., Kedinger, C., 1989. Replication-dependent activation of the adenovirus major late promoter is mediated by the increased binding of a transcription factor to sequences in the first intron. J. Virol. 63, 5124–5132.

Kulshreshtha, V., 2009. Molecular Characterization of 33K Protein of Bovine Adenovirus-3 Ph.D. thesis. University of Saskatchewan, Saskatoon, SK, Canada.

Kulshreshtha, V., Ayalew, L.E., Islam, A., Tikoo, S.K., 2014. Conserved arginines of bovine adenvirus-3 33K protein are important for transportin-3 mediated transport and viral replication. PLOS1 9 (7), e101316.

Kulshreshtha, V., Babiuk, L.A., Tikoo, S.K., 2004. Role of bovine adenovirus-3 33K protein in viral replication. Virology 323, 59–69.

Kulshreshtha, V., Tikoo, S.K., 2008. Interaction of bovine adenovirus-3 33K protein with other viral proteins. Virology 381, 29–35.

Landschulz, W.H., Johnson, P.F., McKnight, S.L., 1988. The leucine Zipper: a hypothetical structure common to a new class of DNA binding proteins. Science 240, 1759–1764.

Lutz, P., Kedinger, C., 1996. Properties of the adenovirus IVa2 gene product, an effector of late-phase-dependent activation of the major late promoter. J. Virol. 70, 1396–1405.

Lutz, P., Rosa-Calatrava, M., Kedinger, C., 1997. The product of the adenovirus intermediate gene pIX is a transcriptional activator. J. Virol. 71, 5102–5109.

Ostapchuk, P., Anderson, M.E., Chandrasekhar, S., Hearing, P., 2006. The L4 22-kilodalton protein plays a role in packaging of the adenovirus genome. J. Virol. 80, 6973–6981.

Perez-Romero, P., Tyler, R.E., Abend, J.R., Dus, M., Imperiale, M.J., 2005. Analysis of the interaction of the adenovirus L1 52/55-kilodalton and IVa2 proteins with the packaging sequence in vivo and in vitro. J. Virol. 79, 2366–2374.

Rasmussen, U.B., Benchaibi, M., Meyer, V., Schlesinger, Y., Schughart, K., 1999. Novel human gene transfer vectors: evaluation of wild-type and recombinant animal adenoviruses in human-derived cells. Hum. Gene Ther. 10, 2587–2599.

Reddy, P.S., Idamakanti, N., Zakhartchouk, A.N., Baxi, M.K., Lee, J.B., Pyne, C., Babiuk, L.A., Tikoo, S.K., 1998. Nucleotide sequence, genome organization, and transcription map of bovine adenovirus type 3. J. Virol. 72, 1394–1402.

Sanchez, T.A., Habib, I., Leland, B.J., Evetts, S.M., Metcalf, J.P., 2000. Zinc finger and carboxyl regions of adenovirus E1A 13S CR3 are important for transactivation of the cytomegalovirus major immediate early promoter by adenovirus. Am. J. Respir. Cell Mol. Biol. 23, 670–677.

Thomas, G.P., Mathews, M.B., 1980. DNA replication and the early to late transition in adenovirus infection. Cell 22, 523–533.

Tormanen, H., Backstrom, E., Carlsson, A., Akusjarvi, G., 2006. L4-33K, an adenovirus-encoded alternative RNA splicing factor. J. Biol. Chem. 281, 36510–36517.

Wu, K., Guimet, D., Hearing, P., 2013. The adenovirus L4-33K protein regulates both late gene expression and viral DNA packaging. J. Virol. 87, 6739–6747.

Zakhartchouk, A.N., Pyne, C., Mutwiri, G.K., Papp, Z., Baca-Estrada, M.E., Griebel, P., Babiuk, L.A., Tikoo, S.K., 1999. Mucosal immunization of calves with recombinant bovine adenovirus-3: induction of protective immunity to bovine herpesvirus-1. J. Gen. Virol. 80, 1263–1269.

Zakhartchouk, A., Connors, W., van Kessel, A., Tikoo, S.K., 2004. Bovine adenovirus type 3 containing heterologous protein in the C-terminus of minor capsid protein IX. Virology 320, 291–300.