

**Characterization and nuclear localization
of the fiber protein encoded by the late
region 7 of bovine adenovirus type 3***

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Summary. To identify the protein encoded by the L7 region of bovine adenovirus-3 (BAdV-3), specific antisera were raised by immunizing rabbits with bacterial fusion proteins encoding the N-terminus or C-terminus of the BAdV-3 fiber protein. Immunoprecipitation and Western blot analysis confirmed that the fiber is expressed as a 102 kDa glycoprotein, which is localized to the nucleus of infected cells. To identify the nuclear localization signals (NLS), BAdV-3 fiber deletion mutants and GFP/ β -galactosidase fusion proteins were expressed in transfected cells, and subcellular localization was visualized by immunofluorescence microscopy. Analysis of deletion mutants localized the NLS to the N-terminal 41 amino acids. Analysis of the N-terminal 41 amino acids identified a cluster of basic residues between amino acid 14 and 20. Substitution of the basic residues ($^{16}\text{KAKR}^{19}$) with acidic residues ($^{16}\text{EAEE}^{19}$) resulted in the accumulation of fiber in the cytoplasm. However, $^{16}\text{KAKR}^{19}$ or $^{12}\text{VYPYKAKRPNI}^{22}$ were not sufficient for efficient transport of a cytoplasmic protein GFP/ β -galactosidase to the nucleus. The recombinant BAdV-3 expressing mutant fiber containing $^{16}\text{EAEE}^{19}$ instead of $^{16}\text{KAKR}^{19}$ was unable to replicate efficiently in Madin-Darby bovine kidney cells, suggesting that the NLS of fiber carries out important in vivo functions.

Introduction

The adenovirus (AdV) fiber is a structural protein, which forms antenna-like projections extending from the vertices of icosahedral virus capsid [3]. All known mammalian AdVs have one fiber per vertex, noncovalently attached to a penton base protein [21]. However, avian AdVs have two fibers per vertex [6]. The AdV

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fiber is a homotrimer [23] with each subunit consisting of three domains, namely, (a) the N-terminal tail associated with the penton base protein, (b) a slender shaft of variable length characterized by a repeating motif of about 15 residues, and (c) the C-terminal globular head, termed knob, which interacts with cellular receptors. After its synthesis in the cytoplasm, the human AdV (HAdV) fiber protein localizes to the nucleus for assembly into the virus particles. A nuclear localization signal (NLS) is required for the correct nuclear targeting of the fiber [7]. The fiber protein of HAdV-2 contains O-linked N-acetyl-glucosamine (O-GlcNAc) that may play a role in the assembly or stabilization of the HAdV-2 and HAdV-5 fiber trimer [12].

The fiber is responsible for the specific high-affinity attachment of virus to cellular AdV receptors, thus playing a major role in the determination of tissue tropism [5, 10, 22]. The fiber is involved in the intracellular trafficking of the virus [11] and in the correct assembly and/or stabilization of the virion [8]. Moreover, the fiber, together with another capsid protein, hexon, is also involved in the induction of the serotype-specific host immune response [5, 9].

The fiber protein of BAdV-3 is encoded by the late region 7 (L7) as a polypeptide of 976 amino acids, which shares 17% to 26% identities with the fiber proteins of other human and animal AdVs [18]. Sequence alignments revealed that the BAdV-3 fiber could also be subdivided into tail, shaft and knob domains [20]. Similar to the HAdV fiber, a hydrophobic sequence motif in the N-terminal tail region, which involves specific interaction with the penton base protein [1] and the TLWT motif near the shaft-knob junction, is present in the BAdV-3 fiber [18, 20]. However, unlike its AdV counterparts, the BAdV-3 fiber protein contains a very long shaft region which contains 46.5 repeat motifs [20]. In this report, we describe the characterization of the BAdV-3 fiber and identification of the putative region involved in nuclear localization. In addition, we describe the construction and characterization of recombinant BAdV-3 expressing fiber protein lacking putative NLS.

Materials and methods

Cell lines and virus

Madin-Darby bovine kidney (MDBK) cells, COS-7 (SV40-transformed African green monkey kidney) cells and VIDO R2 (HAdV-5 E1-transformed fetal bovine retina cells) [16] cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). The wild-type (WBR-1 strain) BAdV-3, BAV302 (E3 deleted BAdV-3; [24]) and BAV302EAEE (this study) were propagated in MDBK cells and purified by CsCl gradient. The viruses were titrated by a plaque assay as described [16].

Production of polyclonal antibodies

To raise antibodies against BAdV-3 fiber, plasmid pGB3FdC460 encoding the N-terminal 459 amino acids of BAdV-3 fiber protein was created by cloning a 1.4-kb *Bam*HI fragment isolated from plasmid P6-1 [18] into *Bam*HI-digested plasmid pGEX-5X-3 (Pharmacia Biotech). The coding region for the C-terminal 200 amino acids of the BAdV-3 fiber knob and the 46th repeat of the shaft was PCR-amplified from plasmid P6-1 using the primer QW5 and QW6 (Table 1). The PCR product was digested with *Bam*HI-*Xho*I and ligated to *Bam*HI – *Xho*I-digested pGEX-5X-3 to create plasmid pGB3FK. The competent *E. coli*

Table 1. List of primers

Primer	Sequence
QW5	5'-CGCGGATCCGCGTTAGGGTTAAATTGGGCGATGGC-3'
QW6	5'-TTGTTAGCAGCCGGATCAAG-3'
pBF1	5'-CACTGCTTACTGGCTTATCG-3',
pBF2	5'-CCGGAATTCCTAAAGCCCGTT TCCTAAACT-3'
YC7	5'-GGGGTACCGCCATGCTTGTGGAAAAGCC-3'
YC8	5'-GGGAGAGCTTTAACCGGAGG-3'
YCP11	5'-CTAGCTAGCTTCCAACGCCCTGACTTTA-3'
YCP12	5'-AACCAAGCCCT CCGATAG-3'
QW73	5'-CGCA AGAAATGGC TAGCA-3'
QW74	5'-CCGGAATTCGTTGTACAGTTCATCC ATGCC-3'
QW75	5'-CCGGAATTCGTG ACACCAGC AAGGAG-3'
QW76	5'-TAACCACCACGCT CATCG-3'
NLSoligo 1	5'-CGGGGTACCGAGCTCG GATCCCCAACATCA TGAAGAGAAGTGT GCCCCAGGACTTTAATCTTGTGTATCCATACGAgGCTGAgGAac CCAACATCATGCC-3'
NLSoligo 2	5'-GGCTGAG GAACCCAAGATCATGCCGCCCTTTTTTGACCGCAAT GGCTTTGTTGAAAACC AAGAAGCCAC GCTAGCCATGCTTGTGG AAAAGCCGCTCACG-3'
NLSoligo 3	5'-GGGCACCGGTGAAATGAAGGCTAAGAGGG-3'
NLSoligo 4	5'-CTAGCCCTCTTAGCCTTCATTCACCGGTG CCCGC-3'
NLSoligo 5	5'-GGGCACC GG TGAAATGGTGTAT CCGTACAAGGCTAAGAGG CCCAACATCG-3'
NLSoligo 6	5'-CTA GCGATGT TGGGCCTCTTAGCCTTGTACGGATACACCATT TCACCGGTGCCCG C-3'

The restriction endonuclease sites in pBF2, YC7, YCP11, QW74 and QW75 are underlined. The sequence encoding mutant NLS (NLSoligo 1), putative NLS (NLSoligo 3; NLSoligo 5) is underlined. The substituted sequences in NLSoligo 1 are shown in lower case

BL21 (DE3) (Novagen) was transformed with plasmid pGB3FdC460 or pGB3FK. The glutathione *S*-transferase (GST)-fiber fusion protein was induced by the addition of 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) and purified by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE). New Zealand white rabbits were subcutaneously injected with gel purified fusion protein in complete Freund's adjuvant followed by two immunizations in Freund's incomplete adjuvant at 4-week intervals.

Construction of plasmid pBAV3F

Initially, a 1.4-kb *HpaI-EcoRI* fragment containing the N-terminal half of the fiber protein was isolated from plasmid p6-1 [18], repaired by T4 DNA polymerase and ligated to *SmaI*-digested pSP64polyA creating plasmid pSPHE148. Similarly, a 1.5-kb *BamHI-XhoI* fragment containing the C-terminal half of the fiber protein was isolated from plasmid P6-1 [18] and ligated to *BamHI - XhoI*-digested plasmid pRSETC (Invitrogen) to create plasmid pRsetBX159. The entire coding sequence of BAdV-3 fiber was assembled by ligating a 1.3-kb *BamHI* fragment isolated from plasmid pSPHE148 to *BamHI*-digested plasmid pRsetBX159

creating plasmid pRB3F. A 2.8 kb *Bam*HI-*Xho*I fragment isolated from plasmid pRB3F was ligated to *Bam*HI – *Xho*I-digested plasmid pcDNA3 to create plasmid pBAV3F.

Construction of plasmids encoding mutated BAdV-3 fiber protein

A 1.4-kb DNA fragment was amplified by PCR using primers pBF1 and pBF2 (Table 1), and plasmid pBAV3F DNA as a template. An *Eco*RI site (underlined) and TAG stop codon (bold) were incorporated into the antisense primer pBF2. The PCR product was digested with *Kpn*I-*Eco*RI and ligated to *Kpn*I-*Eco*RI-digested plasmid pBAV3F creating plasmid pBAV-3FdC491. Similarly, a 0.8-kb DNA fragment was amplified by PCR using primers YC7 and YC8 (Table 1), and plasmid pBAV3F DNA as a template. A *Kpn*I site (underlined) and an ATG start codon (bold) were incorporated into the sense primer YC7. The PCR product was digested with *Kpn*I-*Bsp*EI and inserted into *Kpn*I-*Bsp*EI-digested pBAV3F to create pBAV-3FdN42.

The plasmid pBAV-3Fd42-109 was made by deletion of a 0.2-kb *Nhe*I fragment from pBAV3F and in-frame re-ligation of the large *Nhe*I fragment. To construct plasmid pBAV-3Fd42-478, a 0.3-kb DNA fragment was amplified by PCR using primers (YCP11 and YCP12) (Table 1), and plasmid pBAV-3Fd42-109 DNA as a template. The primer YCP11 contains a *Nhe*I site (underlined). The PCR product was digested with *Nhe*I-*Eco*NI and ligated to *Nhe*I-*Eco*NI-digested pBAV3Fd42-109 creating plasmid pBAV-3Fd42-478.

A 1.4-kb *Kpn*I-*Eco*RI fragment was isolated from plasmid pBAV-3FdC491 and ligated to *Kpn*I-*Eco*RI-digested plasmid pUC19 creating plasmid pUKE150, which was used as a template to introduce point mutations into the putative NLS of the BAdV-3 fiber. Two synthetic overlapping oligonucleotides NLSoligo1 and NLSoligo 2 (Table 1) designed to introduce point mutations into the BAdV-3 fiber coding sequence (sequence encoding K-16-K-18-R-19 is underlined with the substituted sequences in lower case letters) were annealed and the single-stranded ends were filled in with the Klenow fragment of *E. coli* DNA polymerase I. The duplex DNA fragment was digested with *Kpn*I – *Bst*XI and ligated to *Kpn*I-*Bst*XI-digested pUKE150 creating plasmid pUEAEE. A 1.0-kb *Kpn*I-*Spl*I fragment was isolated from plasmid pUEAEE and ligated to *Kpn*I-*Spl*I-digested plasmid pBAV3F creating plasmid pK16E-KR18EE.

Construction of plasmids encoding GFP-β-galactosidase – fiber fusion proteins

A 1.082 kb *Eco*RI- *Sna*BI DNA fragment isolated from plasmid pBAV-301.gfp [16] was treated with T4 DNA polymerase and ligated to *Eco*RV-digested plasmid pcDNA3 creating plasmid pGFP containing the green fluorescent protein (GFP) coding region downstream of the human cytomegalovirus (HCMV) immediate early (IE) promoter. A 3.4-kb *Not*I fragment containing the coding sequence for β-galactosidase was isolated from plasmid pCMVβ (Clontech) and ligated to *Not*I-digested plasmid pGFP to create plasmid pCN347. The coding regions of GFP and β-galactosidase were fused in-frame by PCR. Product 1 was amplified by PCR using primers QW73 and QW74 (Table 1), and pGFP DNA as a template. Product 2 was amplified by PCR using primers QW75 and QW76 (Table 1), and plasmid pCMVβ DNA as template. An *Eco*RI site was incorporated into primers QW74 and QW75 (underlined). The PCR products 1 and 2 were digested with *Nhe*I-*Eco*RI and *Eco*RI-*Cl*aI, respectively, and ligated to *Nhe*I-*Cl*aI-digested plasmid pCN347 to create plasmid pGFP/β-gal.

Synthetic oligonucleotides NLSoligo 3 and NLSoligo 4 (Table 1) encoding for the putative NLS (¹⁶KAKR¹⁹ is underlined) were annealed and ligated to *Sac*II-*Nhe*I- digested plasmid pGFP/β-gal to create plasmid pNLS1GFP/β-gal. Similarly, synthetic oligonucleotides NLSoligo 5 and NLSoligo 6 (Table 1) encoding NLS ¹²VYPYKAKRPNI²² (underlined) were annealed and ligated to *Sac*II-*Nhe*I-digested plasmid pGFP/(βgal creating plasmid pNLS2GFP/β-gal.

Construction of plasmid pFBAV302EAEE

To generate a full-length pFBAV302EAEE infectious clone containing point mutations in the putative NLS of the BAdV-3 fiber protein, a 3.7-kb *KpnI*-*EcoRI* fragment isolated from plasmid pBAV301 [17] was ligated to the *KpnI* – *EcoRI*-digested plasmid pUC19 to create plasmid pUKE375. A 1 kb *Bam*HI (Klenow treated)-*Rsr*II DNA fragment was isolated from plasmid pK16E-KR18EE and ligated to *Age*I (Klenow treated) and *Rsr*II-digested plasmid pUKE375 creating plasmid pUKEAEE. A 2.9 kb *KpnI* – *Xba*I DNA fragment isolated from plasmid pUKEAEE was ligated to the *KpnI* – *Xba*I-digested plasmid pBAV301 creating plasmid pBAV-301EAEE. A 7.650 kb *EcoRV*-*Swa*I fragment containing the fiber with a modified NLS was isolated from pBAV-301EAEE and recombined with *Srf*I-*Rsr*II-digested pFBAV302 [24] DNA in *E. coli* BJ5183 [2], creating plasmid pFBAV302EAEE.

Construction of recombinant BAdV-3

VIDO R2 cell monolayers in 60 mm dishes were transfected with 5 to 10 μ g of *Pac*I-digested pFBAV302EAEE plasmid DNA using Lipofectin (GIBCO/BRL). After incubation at 37 °C, the transfected cells showing cytopathic effects were collected and freeze-thawed two times, and the recombinant virus was plaque-purified and propagated on MDBK cells.

Virus-neutralization assay

Rabbit antiserum was heat-inactivated at 56 °C for 30 min. Aliquots of diluted serum (in MEM) were incubated with 100 plaque forming units (pfu) of wild-type BAdV-3 for two hours at room temperature in a total volume of 50 μ l. A total of 100 μ l of media was added to the antiserum/virus mixture and incubated with MDBK cells in 48-well tissue culture plates. After one hour of adsorption, the mixture was removed. The cells were washed twice with PBS and overlaid with MEM containing 2% FBS and 0.7% low melting temperature agarose. Viral plaques were counted 14 days later. The assay was performed in duplicate. The titers are expressed as reciprocals of the highest antibody dilution that caused 50% reduction in the number of plaques relative to the control cells that were infected with untreated virus.

Virus growth

MDBK cells were infected with mutant or wild-type BAdV-3 at an MOI of 5. The infected cells harvested at the indicated times post-infection were lysed by three rounds of freezing-thawing and virus titers were determined in MDBK cells in a plaque assay [16].

Immunofluorescence

COS-7 cells were seeded overnight at 10^6 cells per well in a six-well tissue culture plate containing microscope coverslips. The cells were transfected with 2 μ g of purified plasmid DNA by Lipofectamine Reagent (GIBCO-BRL). The DNA-Lipofectamine mixture was removed after 5 h incubation at 37 °C, and the cells were incubated for an additional 72 h. The cells were fixed in methanol at –20 °C for 20 min and blocked with 2% normal goat serum for 15 min at room temperature or overnight at 4 °C. Similarly, the MDBK cells were infected with wild-type or mutant BAdV-3 at different MOIs. At different times post-infection, the infected cells were fixed in methanol at –20 °C for 20 min and blocked with 2% normal goat serum for 15 min at room temperature. The subcellular location of fiber proteins was determined by staining with appropriate rabbit anti-BAdV-3 fiber polyclonal antibodies for one hour followed by incubation with the Fab fragment of goat anti-rabbit IgG (H + L) conjugated with fluorescein isothiocyanate (FITC; Jackson Immuno Research). Finally, the

samples were viewed with a fluorescence microscope and representative photographs were taken with Tmax 100 film (Kodak).

Western blotting

MDBK cells were infected with wild-type BAdV-3 at an MOI of 5. At indicated times post-infection, the cells were collected and analysed by Western blot analysis as described [16].

Immunoprecipitation

Immunoprecipitation was carried out as previously described [16]. Briefly, MDBK cells grown in six-well plates were infected with the wild-type BAdV-3 at an MOI of 10. The cells were preincubated for two hours in glucose-free RPMI1640 medium before labeling with 100 μ Ci of [³H]glucosamine for 12 hours, or preincubated for two hours in MEM free of methionine and cysteine before labeling with 50 μ Ci of [³⁵S] methionine for four hours. Proteins were immunoprecipitated from cells lysed with modified RIPA and analysed by SDS-PAGE as described earlier [24].

Results

In-vivo expression of BAdV-3 fiber

In order to identify and characterize protein encoded by the L7 region of BAdV-3, we made two anti-fiber sera [99-48 against the N- (amino acid 1-459) and 99-44 against the C- (amino acid 776-976) terminus] by immunizing rabbits with 250 μ g of purified individual GST-protein fusions. Sera collected after the final boost were analysed by radioimmunoprecipitation assay. The 99-44 antisera detected two proteins of 102 kDa and 62 kDa in BAdV-3 infected cells (Fig. 1A, lane 3) but not in mock infected cells (Fig. 1A, lane 1). Similarly, 99-48 antisera also detected two proteins of 102 kDa and 62 kDa in BAdV-3 infected cells (Fig. 1A, lane 4) but not in mock infected cells (Fig. 1A, lane 2).

To determine the time course of fiber expression and to confirm whether two proteins observed in the immunoprecipitation studies represented post-translationally modified forms of fiber or cellular/viral proteins that coimmunoprecipitate with fiber protein, we carried out a Western blot assay. As seen in Fig. 1B, 99-44 (Fig. 1B, lane 6) or 99-48 (Fig. 1B, lane 4) antisera detected a protein of 102 kDa in BAdV-3 infected cells but not in mock (Fig. 1B, lanes 3,5) infected cells. Similarly, no such protein could be detected in BAdV-3 infected cells using normal rabbit serum (Fig. 1B, lanes 1,2). The 102 kDa protein was similar to the 102 kDa protein detected by immunoprecipitation. Moreover, the 102 kDa protein could be detected at 24 h post-infection and continued to be produced up to 96 h post-infection (Fig. 1C). The continued presence of the fiber protein late in the infection cycle can be attributed to the stability of the protein, and/or the continued synthesis of new protein throughout the virus replication cycle.

To determine whether the BAdV-3 fiber protein was glycosylated, [³H]glucosamine -labeled proteins from lysates of BAdV-3 infected cell extracts were immunoprecipitated with antisera and separated by SDS-PAGE. As seen in Fig. 1D, 99-44 antisera detected two proteins of 102 kDa and 62 kDa in BAdV-3 infected

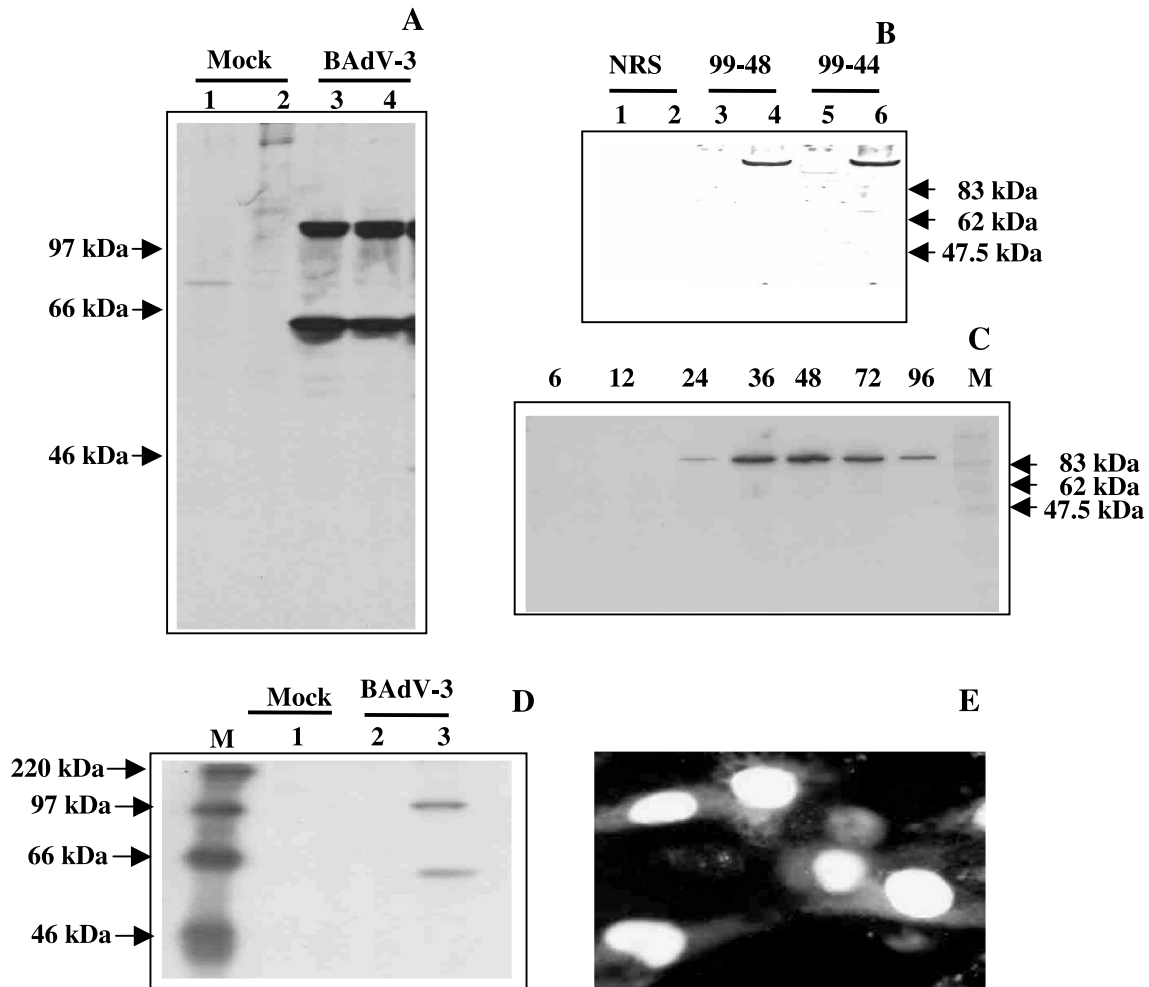


Fig. 1. Expression of fiber in wild-type BAdV-3 infected cells. **A** [^{35}S]methionine labeled proteins from lysates of mock- or BAdV-3-infected MDBK cells were immunoprecipitated with polyclonal antibody 99-44 (1 and 3) or 99-48 (2 and 4) specific for BAdV-3 fiber. The proteins were separated by 10% SDS-PAGE and visualized by autoradiography. The position of the molecular weight markers is shown to the left of the panel. **B** Proteins from mock (3, 5) or wild-type BAdV-3 (1, 2, 4, 6) infected MDBK cells were separated by 10% SDS-PAGE under reducing conditions and transferred to nitrocellulose. The separated proteins were probed with normal rabbit sera (NRS) (1, 2), 99-48 antisera (3, 4) and 99-44 antisera (5, 6) in a Western blot. **C** Proteins from lysates of mock-infected (M) or BAdV-3-infected MDBK cells harvested at different times post-infection were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane and probed with antiserum 99-44 in a Western blot. The position of the molecular weight markers is shown to the right of the panel. **D** [^3H]glucosamine labeled proteins from lysates of mock-infected (1) or BAdV-3-infected (2, 3) MDBK cells were immunoprecipitated with 99-44 antisera (1 and 3) or normal rabbit serum (2), separated by 10% SDS-PAGE and visualized by autoradiography. **E** MDBK cells were grown on coverslips and infected with BAdV-3. The subcellular locations of the fiber were determined by indirect immunofluorescence using rabbit polyclonal antibody 99-44

(lane 3) but not in mock infected (lane 1) cells which were labeled with [^3H]glucosamine (lane 3). No such proteins could be detected in BAdV-3 infected cells immunoprecipitated with normal rabbit sera (lane 2). These results indicated that BAdV-3 fiber protein is a glycoprotein.

To determine the intracellular distribution of fiber protein, MDBK cells were infected with wild-type BAdV-3 and examined by indirect immunofluorescence staining using 99-44 antisera. At 21 h post-infection, fiber protein was localized predominantly in the nucleus of infected cells (Fig. 1E).

Neutralization of BAdV-3

The knob domain of Ad fiber protein is responsible for the specific high-affinity binding of virions to the host cell receptors and thus represents one of the major targets of neutralizing antibodies [9]. As fiber protein is a target of neutralizing antibodies against adenoviruses [5], we determined whether different antisera raised against the BAdV-3 fiber could neutralize BAdV-3 infectivity in vitro. Duplicate aliquots containing 100 PFU of wild-type BAdV-3 were incubated at room temperature for two hours with serial two-fold dilutions of different polyclonal antibodies. MDBK cells were then infected with a pre-incubated mixture of BAdV-3 and antisera and observed for 14 days for the development of viral plaques. The wild-type BAdV-3 was neutralized by 99-44 (1:800), but not by either 99-48 or normal rabbit serum, even at the lowest dilution of 1:50. These results suggested that 99-44 antiserum raised against the knob domain of BAdV-3 fiber was neutralized BAdV-3.

Identification of NLS(s) in fiber

To determine whether fiber protein is transported to nucleus in the absence of other viral proteins, we used indirect immunofluorescence to determine distribution of fiber protein in COS7 cells transfected with the plasmid pBAV3F (Fig. 2A), carrying the gene encoding wild-type BAdV-3 fiber. Like BAdV-3 infected cells (Fig. 1E), fiber protein was predominantly localized in the nucleus of transfected cells (Fig. 3A). These results suggested that the transport of BAdV-3 fiber protein to the nucleus is independent of other viral proteins and can be investigated using the transient expression of fiber protein in transfected cells.

To locate the nuclear localization signals, a panel of plasmids encoding mutant fiber proteins was constructed (Fig. 2A). The distribution of mutant fiber proteins in plasmid-transfected COS-7 cells was examined by immunofluorescence staining using fiber specific sera. The deletion of amino acids 491–976 of BAdV-3 fiber protein did not affect its nuclear localization (Fig. 3B). However, compared to the full length fiber (Fig. 3A), the truncated protein showed granular distribution within the nucleus (Fig. 3B). Similarly, mutant fiber protein lacking amino acids 42–109 (Fig. 3C) or amino acids 42–478 (Fig. 3D) also localized to the nucleus. However, compared to the full length (Fig. 3A), the mutant containing a deletion between 42–478 showed granular distribution within the nucleus (Fig. 3D). In

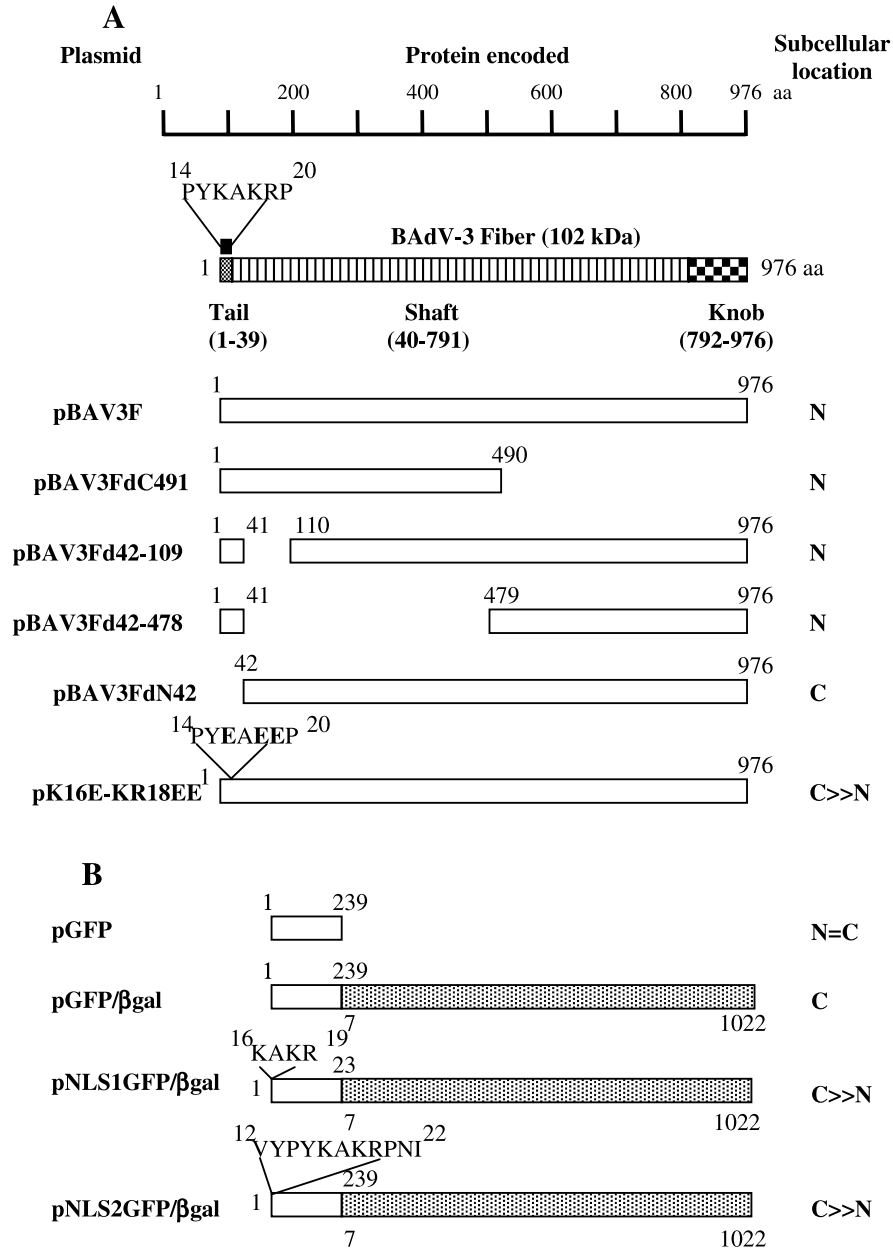


Fig. 2. Schematic representation of BAdV-3 fiber. **A** Hollow bars represent coding sequences for BAdV-3 fiber protein with gaps showing where sequences have been deleted. Numbers above the bars denote residue numbers for BAdV-3 fiber. The N-terminal tail, the long shaft with predicted 46.5 repeat, and the knob region of fiber are depicted. The name given to each plasmid encoding a mutant protein is shown on the left. Subcellular locations [*N* (nuclear), *C* (cytoplasmic), *C* >> *N* (mostly cytoplasmic)] are shown on the right. **B** Open bars represent GFP sequences, whereas hatched bars represent β-galactosidase sequences. The numbers above each bar represent amino acid numbers of GFP, whereas those below each bar represent amino acid numbers of β-galactosidase. The amino acid residues shown above the bar denote the fused putative nuclear localization signal. The name given to each plasmid encoding a mutant protein is shown on the left. Subcellular locations [*N* = *C* (nuclear and cytoplasmic), *C* (cytoplasmic), *C* >> *N* (mostly cytoplasmic)] are shown on the right

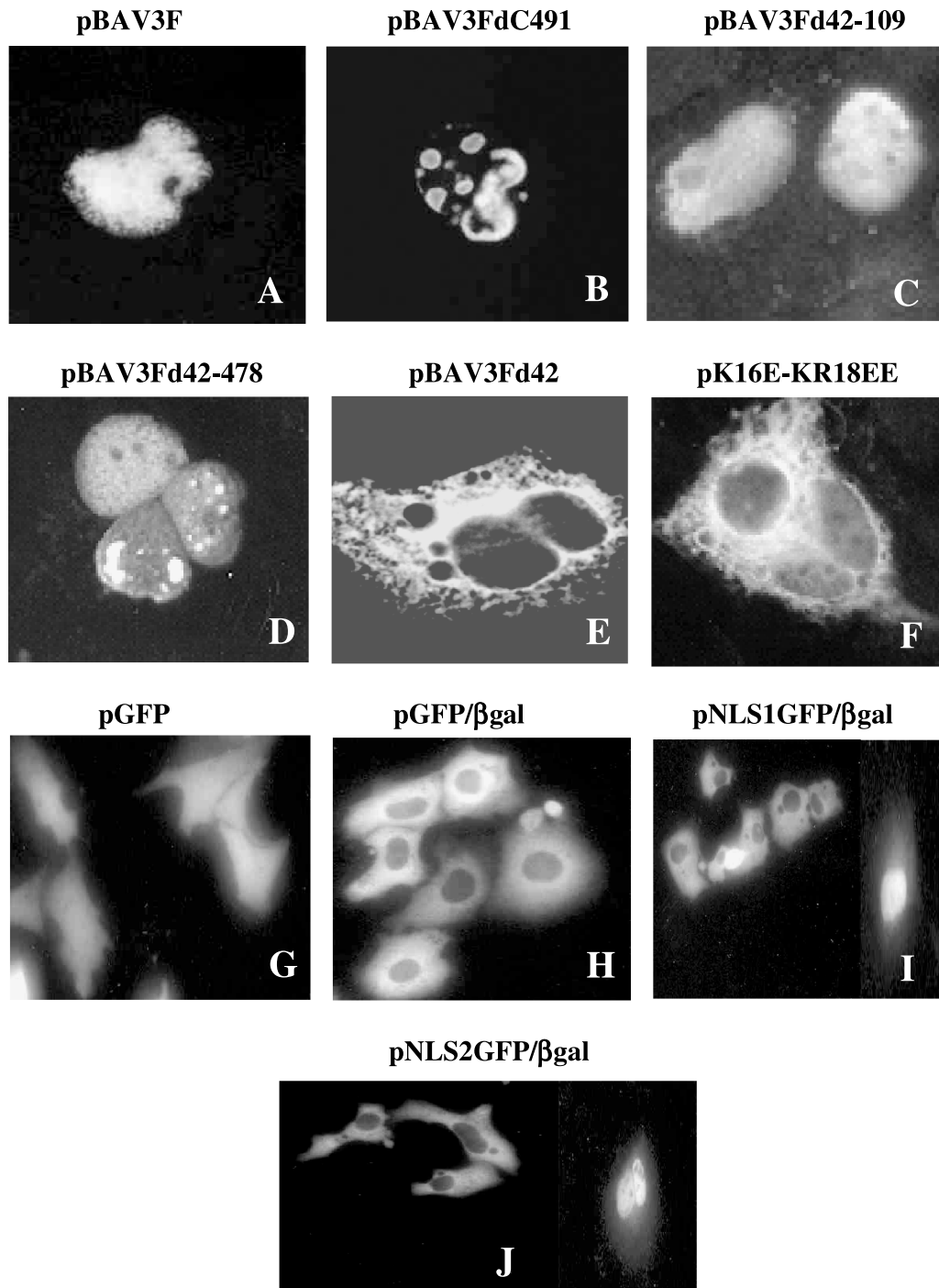


Fig. 3. Subcellular localization of mutant and NLS-GFP-β gal – fiber fusion proteins. The COS-7 cells were grown on coverslips and transfected with plasmids encoding wild-type (A) or mutant (B–J) BAdV-3 fiber protein. The subcellular locations of the proteins were determined by indirect immunofluorescence using rabbit polyclonal antibody 99-44 (A, C, D, E, and F), or 99-48 (B). GFP (G) and fusion proteins GFP/βgal (H), NLS1GFP/βgal [¹⁶KARR¹⁹ fused to GFP/βgal] (I) and NLS2 GFP/βgal [¹²VYPYKAKRPNI²²] fused to GFP/βgal (J) were directly observed by fluorescence microscopy

contrast, mutant fiber protein with the deletion of amino acids 1–42, localized predominantly in the cytoplasm (Fig. 3E). Taken together, these results suggested that the N-terminal 41 amino acids may contain the NLS for nuclear translocation of BAdV-3 fiber protein.

Analysis of the N-terminal 41 amino acid sequence of BAdV-3 fiber protein did not reveal stretches of basic amino acid residues that resemble the classical

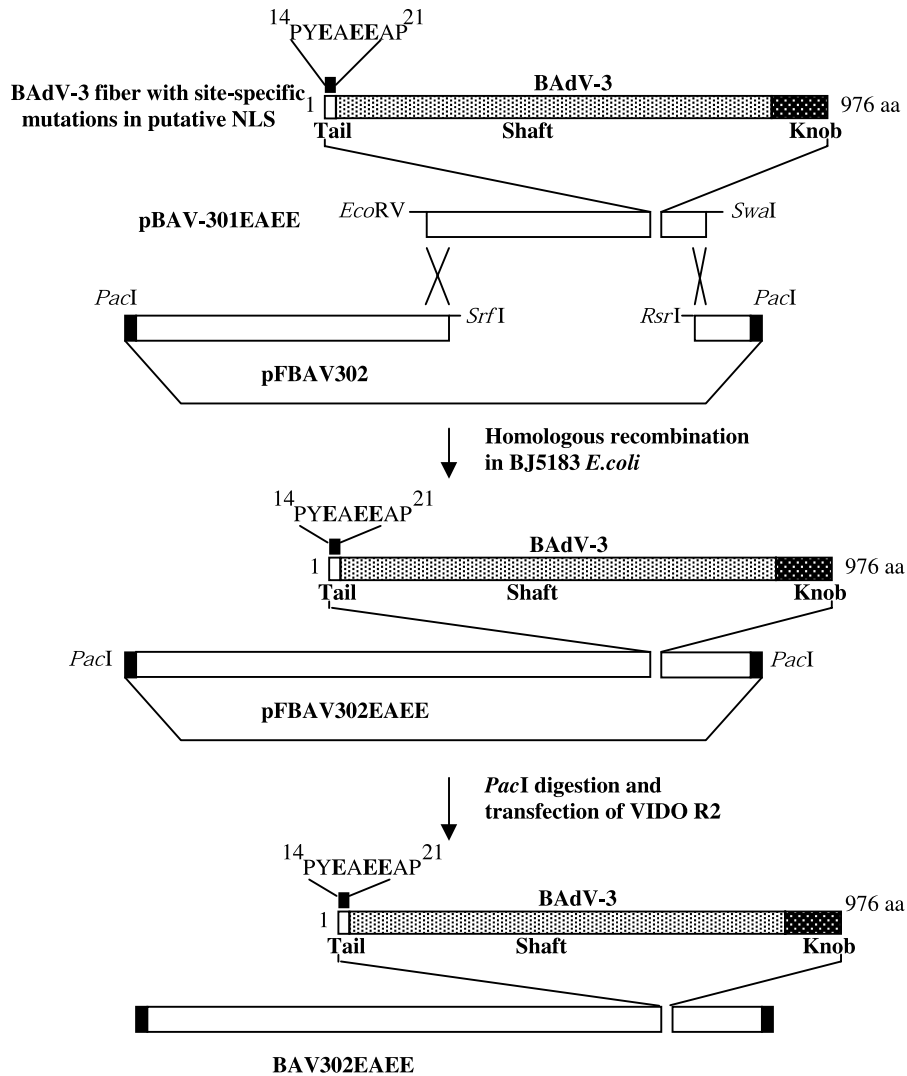


Fig. 4. Schematic representation of the strategy used for the construction of recombinant virus BAV302EAEE. Plasmid pFBAV302EAEE was generated by homologous recombination in *E. coli* between *SrfI*-digested pFBAV302 and an *EcoRV* – *SwaI* fragment containing mutant fiber. Recombinant BAV302EAEE was obtained following transfection of VIDO R2 cells with *PacI*-digested pFBAV302EAEE. Plasmid DNA is denoted by the thin line, BAdV-3 genomic DNA by the hollow box and inverted terminal repeats by the black box. Specific amino acid substitutions in the mutated BAdV-3 fiber are shown in bold face. The plasmid maps are not drawn to the scale

NLSs [4]. However, one cluster of basic amino acid residues was found between residues 14 to 20 of the BAdV-3 fiber protein. The cluster of basic residues ($^{14}\text{PYKAKRP}^{20}$) starts with proline followed by a basic segment where three out of six residues are lysines/arginines. To determine whether these basic residues play a role in localizing the fiber protein to the nucleus, we simultaneously substituted the three basic residues (KKR) with three glutamic acid residues (EEE) (Fig. 2A). As shown in Fig. 3F, the mutant protein encoded by plasmid pK16E-KR18EE was detected predominantly in the cytoplasm, suggesting that these basic residues ($^{16}\text{K}^{18}\text{K}^{19}\text{R}$) are essential elements of the NLS of BAdV-3 fiber protein.

Next, we evaluated whether these basic residues could direct the import of a heterologous cytoplasmic protein into the nucleus. A chimeric GFP/ β -gal protein was produced by fusing the GFP to the N-terminus of β -galactosidase (β -gal)

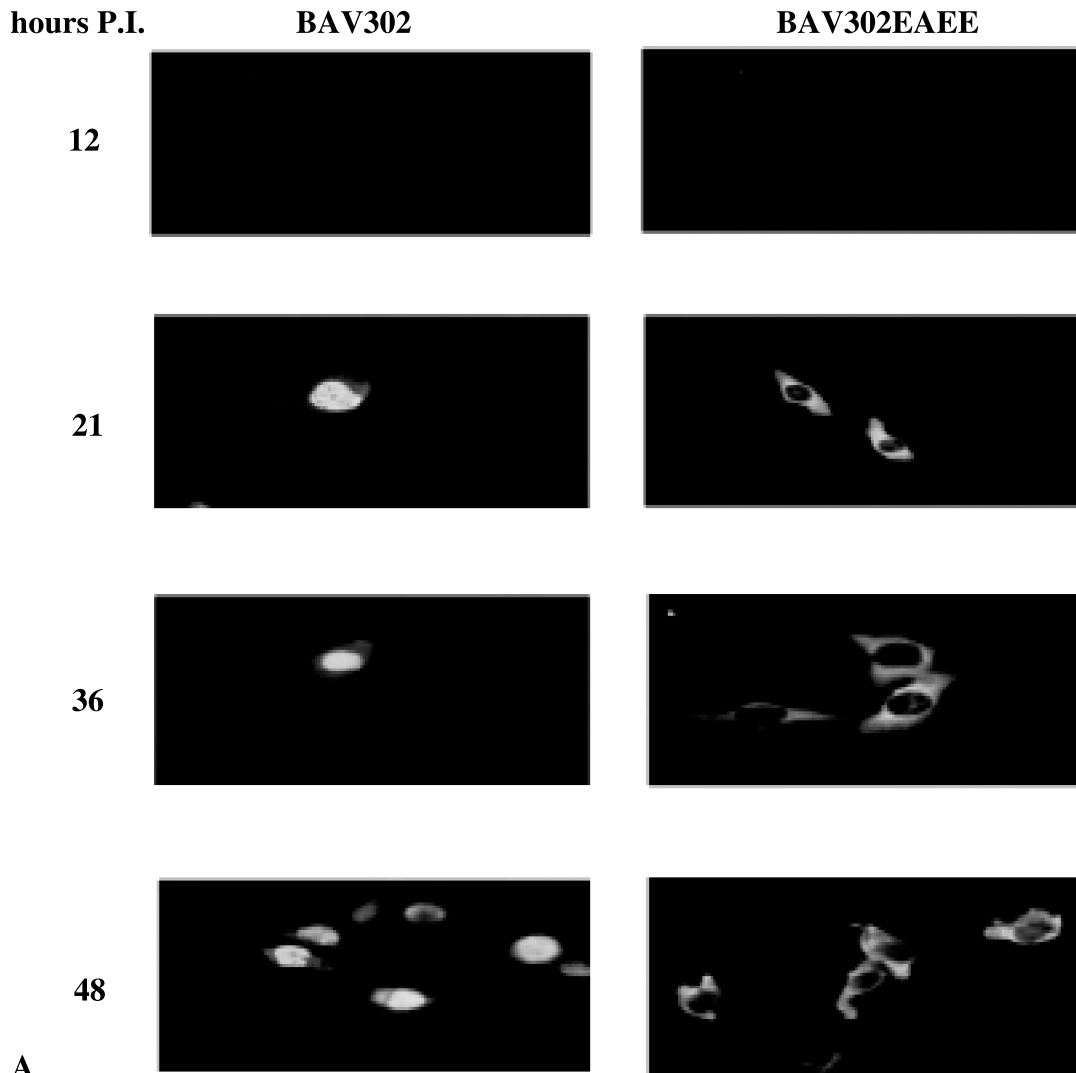


Fig. 5 (continued)

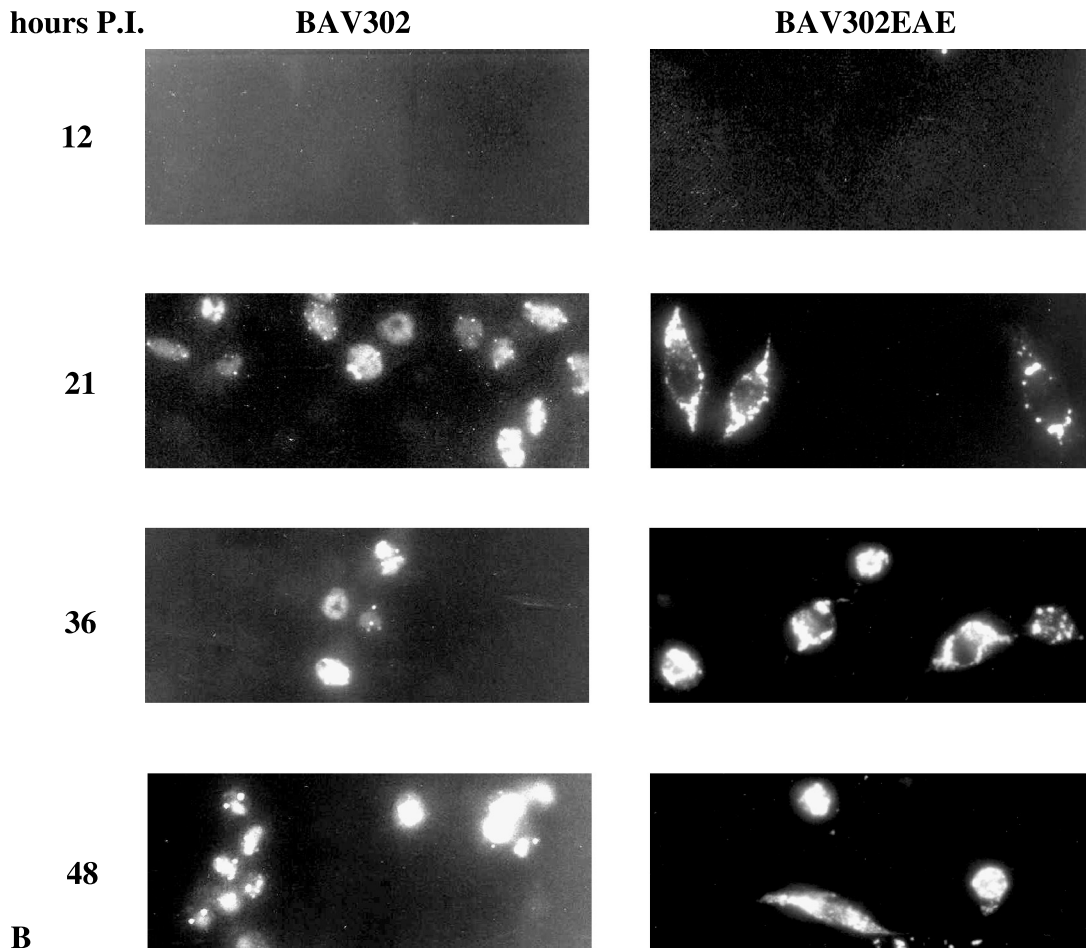


Fig. 5. Detection of mutant fiber in BAV302EAE infected cells. MDBK cells were infected with BAV302 or BAV302EAE at an MOI of 0.1 (A) or 5 (B). At 12, 21, 36, and 48 h post-infection, the infected cells were stained for immunofluorescence microscopy using a polyclonal antibody 99-44

(Fig. 2B). As expected, GFP localized to both the cytoplasm and nucleus (Fig. 3G). However, fusion of GFP with β -gal resulted in localization of GFP predominantly in the cytoplasm (Fig. 3H). When the basic sequence ($^{16}\text{KAKR}^{19}$) was fused to the chimeric GFP/ β -gal protein, the majority of transfected cells showed primarily cytoplasmic staining with few cells showing both nuclear and cytoplasmic staining (Fig. 3I). Moreover, the addition of adjacent residues to the $^{16}\text{KAKR}^{19}$ sequence ($^{12}\text{VYPYKAKRPNI}^{22}$) had little impact on the predominantly cytoplasmic distribution of GFP/ β -gal fusion protein (Fig. 3J).

Construction of BAV302EAE virus

To determine if point mutations in the NLS also affected the nuclear transportation of the fiber protein during viral infection, we constructed a full length plasmid, pF-

BAV302EAEE, in which the three basic residues $^{16}\text{K}^{18}\text{K}^{19}\text{R}$ in the putative NLS of wild-type BAdV-3 fiber were replaced with three glutamic acid residues (EEE) (Fig. 4). The *PacI*-digested pFBAV302EAEE plasmid DNA was transfected into VIDO R2 cells. The transfected monolayers showing 50% cytopathic effects were collected and freeze-thawed and the recombinant virus, named BAV302EAEE (Fig. 4) was plaque-purified and propagated in MDBK cells. The identity of BAV302EAEE was confirmed by sequence analysis of virion DNA.

Nuclear transport of mutant fiber protein during viral infection

MDBK cells were infected with wild-type BAdV-3 or mutant BAV302EAEE and subcellular location of the fiber protein was studied by immunofluorescence staining (Fig. 5). When cells were infected with an MOI of 0.1 (Fig. 5A), the mutant fiber protein could be detected predominantly in the cytoplasm of BAV302EAEE infected cells from 21 to 48 h post infection. Similarly, when cells were infected with an MOI of 5 (Fig. 5B), mutant fiber protein was primarily localized in the cytoplasm of infected cells at 21 h post-infection. However at 48 h post infection, the mutant fiber protein could be detected in the nucleus of BAdV-3 infected cells. In contrast, irrespective of MOI (Fig. 5A, B) used, the wild-type BAdV-3 fiber protein was primarily localized in the nucleus from 21 h to 48 h post infection.

Growth kinetics of BAV302EAEE

To determine whether BAV302EAEE was defective for growth in MDBK cells, a single step growth experiment was performed. As seen in Fig. 6, the virus yield of BAV302EAEE in MDBK cells was lower compared to that of BAdV-3. At 72 h post infection, BAdV-3 grew to a titer of 9.8×10^9 PFU/ml. In contrast, BAV302EAEE grew to a titer of to 6.8×10^6 PFU/ml.

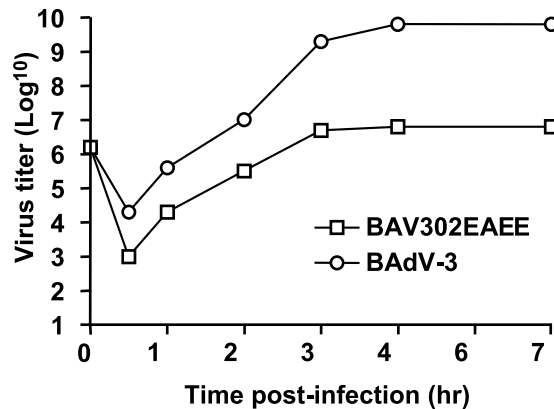


Fig. 6. Replication of BAV302EAEE in MDBK cells. MDBK cells were infected with BAV302EAEE or wild-type BAdV-3 at an MOI of 5 and cells were harvested at different times post-infection. Virus from each sample was released by freeze-thawing and titered on MDBK cells by plaque assay as described [16]

Discussion

Several studies have shown that fiber encoded by members of all adenovirus genera is one of the major capsid proteins involved in the initial attachment of virus to the cell surface receptor(s) [3], intracellular trafficking and possibly virus maturation [8]. Although fiber is a structural component of all adenoviruses examined so far, the structure and the nature of cell surface receptors recognized by fiber differs amongst different adenoviruses suggesting that fiber is the major determinant of host cell tropism.

The L7 mRNA of BAdV-3 has the potential to code for fiber protein of 976 amino acids in length. Antisera directed against the fiber protein immunoprecipitated two glycoproteins of 62 kDa and 102 kDa from BAdV-3 infected cells but not from mock infected cells. However, Western blot analysis suggested that fiber protein is expressed as a 102 kDa protein in BAdV-3 infected cells. Moreover, the 62 kDa protein is not a precursor and/or cleavage product of 102 kDa protein, but a co-immunoprecipitating protein (penton base protein; unpublished data). Like HAdV-2 [7], the BAdV-3 fiber protein is detected predominantly in the nucleus and contains epitopes in the knob domain for the production of neutralizing antibodies against BAdV-3.

Proteins less than 40 kDa in size can diffuse passively into the cell nucleus through nuclear pore complexes [14]. Therefore, it is very unlikely that BAdV-3 fiber protein enters the nucleus by a simple diffusion mechanism due to its large molecular mass (102 kDa). Furthermore, if diffusion accounts for subcellular location of the fiber, one would expect the protein to be equally distributed throughout the cell rather than accumulating predominantly in the nucleus. Although the presence of a non-conventional motif can lead to nuclear import of a protein [15], numerous studies have demonstrated that active import of large proteins to the nucleus requires a distinct amino acid sequence known as NLS [4]. The ability of BAdV-3 fiber to localize to the nucleus in the absence of other viral proteins indicated that the NLS(s) may be present within the protein.

Analysis of mutant BAdV-3 fiber proteins demonstrated that the NLS is located in the N-terminus 41 amino acids, which contain a cluster of basic residues, ¹⁴PYKAKRP²⁰. Substitution of the basic residues (KKR) with acidic residues (EEE) was sufficient to abolish translocation of fiber to the nucleus in transfected cells suggesting that KKR residues are essential for targeting of fiber to the nucleus. Surprisingly, ¹⁶KAKR¹⁹ or ¹²VYPYKAKRPNI²² motifs were not able to direct predominantly cytoplasmic GFP/ β -galactosidase fusion protein efficiently to the nucleus. It is possible that other sequences may be required for efficient nuclear transport of fusion protein. Alternatively, it is possible that the capacity of the NLS of BAdV-3 fiber protein for nuclear translocation may depend on the protein to which it is linked or the context of the NLS within the fusion protein. For example, the NLS of GAL4, a yeast DNA-binding protein, functions efficiently when fused to normally cytoplasmic invertase, but not when fused to *E. coli* β -gal [13]. Moreover, an NLS inserted into several sites within the polypeptide chain of pyruvate kinase could not function in some locations, as its activity was masked [19].

Analysis of the localization of mutant fiber in BAV302EAEE-infected cells suggested that NLS ¹⁶KAKR¹⁹ function in its natural context is required for highly efficient nuclear localization of fiber. However, although recombinant BAV302EAEE expressing mutant fiber protein was isolated, the virus yield was reduced by 3 logs compared to BAV302, suggesting that the sequence encoding NLS is required for optimum viral growth. Efficient HAdV-5 particle formation occurs in the absence of fiber, however the infectivity of such fiberless viruses is severely reduced [8]. Analysis by electron microscopy revealed no significant differences in the formation of virus particles in wild-type BAdV-3 or BAV302EAEE-infected cells. It is possible that the defective growth of BAV302EAEE results directly from the reduced level of nuclear localized fiber, which may affect the efficiency of the formation of infectious virus particles. Alternatively, the defective transport of fiber in BAV302EAEE-infected cells may alter other viral functions, which may in turn affect the efficiency of formation of infectious virus particles.

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References

1. Caillet-Boudin ML (1989) Complementary peptide sequences in partner proteins of the adenovirus capsid. *J Mol Biol* 208: 195–198
2. Chartier C, Degryse E, Gantzer M, Dieterle A, Pavirani A, Mehtali M (1996) Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*. *J Virol* 70: 4805–4810
3. Chroboczek J, Ruigrok RW, Cusack S (1995) Adenovirus fiber. *Curr Top Microbiol Immunol* 199: 163–200
4. Dingwall C, Laskey RA (1991) Nuclear targeting sequences—a consensus? *Trends Biochem Sci* 16: 478–481
5. Dmitriev I, Krasnykh V, Miller CR, Wang M, Kashentseva E, Mikheeva G, Belousova N, Curiel DT (1998) An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. *J Virol* 72: 9706–9713
6. Hess M, Cuzange A, Ruigrok RW, Chroboczek J, Jacrot B (1995) The avian adenovirus penton: two fibres and one base. *J Mol Biol* 252: 379–385
7. Hong JS, Engler JA (1991) The amino terminus of the adenovirus fiber protein encodes the nuclear localization signal. *Virology* 185: 758–767
8. Legrand V, Spehner D, Schlesinger Y, Settelen N, Pavirani A, Mehtali M (1999) Fiberless recombinant adenoviruses: virus maturation and infectivity in the absence of fiber. *J Virol* 73: 907–919
9. Liebermann H, Mentel R, Bauer U, Pring-Akerblom P, Dolling R, Modrow S, Seidel W (1998) Receptor binding sites and antigenic epitopes on the fiber knob of human adenovirus serotype 3. *J Virol* 72: 9121–9130

10. Louis N, Fender P, Barge A, Kitts P, Chroboczek J (1994) Cell-binding domain of adenovirus serotype 2 fiber. *J Virol* 68: 4104–4106
11. Miyazawa N, Crystal RG, Leopold PL (2001) Adenovirus serotype 7 retention in a late endosomal compartment prior to cytosol escape is modulated by fiber protein. *J Virol* 75: 1387–1400
12. Mullis KG, Haltiwanger RS, Hart GW, Marchase RB, Engler JA (1990) Relative accessibility of N-acetylglucosamine in trimers of the adenovirus types 2 and 5 fiber proteins. *J Virol* 64: 5317–5323
13. Nelson M, Silver P (1989) Context affects nuclear protein localization in *Saccharomyces cerevisiae*. *Mol Cell Biol* 9: 384–389
14. Pante N, Aebi U (1996) Toward the molecular dissection of protein import into nuclei. *Curr Opin Cell Biol* 8: 397–406
15. Pillet S, Annan Z, Fichelson S, Morinet F (2003) Identification of a nonconventional motif necessary for the nuclear import of the human parvovirus B19 major capsid protein (VP2). *Virology* 306: 25–32
16. Reddy PS, Idamakanti N, Chen Y, Whale T, Babiuk LA, Mehtali M, Tikoo SK (1999) Replication-defective bovine adenovirus type 3 as an expression vector. *J Virol* 73: 9137–9144
17. Reddy PS, Idamakanti N, Zakhartchouk AN, Babiuk LA, Mehtali M, Tikoo SK (2000) Optimization of bovine coronavirus hemagglutinin-esterase glycoprotein expression in E3 deleted bovine adenovirus-3. *Virus Res* 70: 65–73
18. Reddy PS, Idamakanti N, Zakhartchouk AN, Baxi MK, Lee JB, Pyne C, Babiuk LA, Tikoo SK (1998) Nucleotide sequence, genome organization, and transcription map of bovine adenovirus type 3. *J Virol* 72: 1394–1402
19. Roberts BL, Richardson WD, Smith AE (1987) The effect of protein context on nuclear location signal function. *Cell* 50: 465–475
20. Ruigrok RW, Barge A, Mittal SK, Jacrot B (1994) The fibre of bovine adenovirus type 3 is very long but bent. *J Gen Virol* 75: 2069–2073
21. Shenk T (1996) *Adenoviridae: the viruses and their replication*. In: Knipe DM, Fields BN, Howley PM (ed), *Fields virology*. Lippincott-Raven, Philadelphia
22. Stevenson SC, Rollence M, White B, Weaver L, McClelland A (1995) Human adenovirus serotypes 3 and 5 bind to two different cellular receptors via the fiber head domain. *J Virol* 69: 2850–2857
23. van Oostrum J, Burnett RM (1985) Molecular composition of the adenovirus type 2 virion. *J Virol* 56: 439–448
24. Zakhartchouk AN, Reddy PS, Baxi M, Baca-Estrada ME, Mehtali M, Babiuk LA, Tikoo SK (1998) Construction and characterization of E3-deleted bovine adenovirus type 3 expressing full-length and truncated form of bovine herpesvirus type 1 glycoprotein gD. *Virology* 250: 220–229

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