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A recombinant E1-deleted porcine adenovirus-3 as an expression vector☆

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Abstract

Replication-defective E1-deleted porcine adenoviruses (PAVs) are attractive vectors for vaccination. As a prerequisite for generating PAV-3 vectors containing complete deletion of E1, we transfected VIDO R1 cells (fetal porcine retina cells transformed with E1 region of human adenovirus 5) with a construct containing PAV-3 $E1B^{large}$ coding sequences under the control of HCMV promoter. A cell line named VR1BL could be isolated that expressed $E1B^{large}$ of PAV-3 and also complemented PAV214 (E1A+$E1B^{small}$ deleted). The VR1BL cells could be efficiently transfected with DNA and allowed the rescue and propagation of recombinant PAV507 containing a triple stop codon inserted in the $E1B^{large}$ coding sequence. In addition, recombinant PAV227 containing complete deletion of E1 (E1A+$E1B^{small}$ + $E1B^{large}$) could be successfully rescued using VR1BL cell line. Recombinant PAV227 replicated as efficiently as wild-type in VR1BL cells but not in VIDO R1 cells, suggesting that $E1B^{large}$ was essential for replication of PAV-3. Next, we constructed recombinant PAV219 by inserting green fluorescent (GFP) protein gene flanked by a promoter and a poly(A) in the E1 region of the PAV227 genome. We demonstrated that PAV219 was able to transduce and direct expression of GFP in some human cell lines.

Keywords: E1, Replication-defective; PAV-3; VIDO R1; VR1BL1; Transduction

Adenovirus (Ad) vectors are frequently used for in vitro and in vivo gene transfer. They have several features that make them an attractive tool for gene transfer. Adenoviruses can be grown to high titers in cultured cells and can transduce both dividing and nondividing cells. To date, the mostly developed vectors are based on the closely related human adenovirus serotypes 2 and 5. Since HAV-2 or HAV-5 have naturally infected more than 50% of the adult human population (Foy and Grayston, 1976), immune responses to adenoviruses decrease the efficiency of gene transfer. Moreover, there are safety concerns regarding transcomplementation of human Ad vectors by wild-type human Ad infection (Rademaker et al., 2002). To overcome some of the problems associated with the use of human adenoviruses as a vector, we (Reddy et al., 1999a, 1999b; Zakhartchouk et al., 1998) and others (Klonjkowski et al., 1997; Michou et al., 1999; Sheppard et al., 1988; Xu et al., 1997; Tuboly and Nagy, 2001; Farina et al., 2001) are exploring the utility of nonhuman adenoviruses as expression vectors for human and animal genes transfer.

Since the E1 region encodes proteins involved in cell cycle regulation, the deletion of this region reduces the risk of oncogenic transformation. In addition, deletion of the E1 region reduces the expression of all other early genes to a larger extent, resulting in lower immune responses to adenovirus proteins and thus in long-term expression of a transgene. Moreover, E1-deleted adenovirus vector provides a significant safety profile to a vaccine as it reduces the potential for the spread of the vector to nonvaccinated contacts or to the general environment. Recombinant human adenovirus vectors currently used in gene transfer experiments have the E1 region of the viral genome replaced by a transgene. Since deletion of the E1 region renders the recombinant virus replication defective (Stratford-Perricaudet and Perricaudet, 1991), mutant viruses are grown in a cell line expressing E1 proteins.

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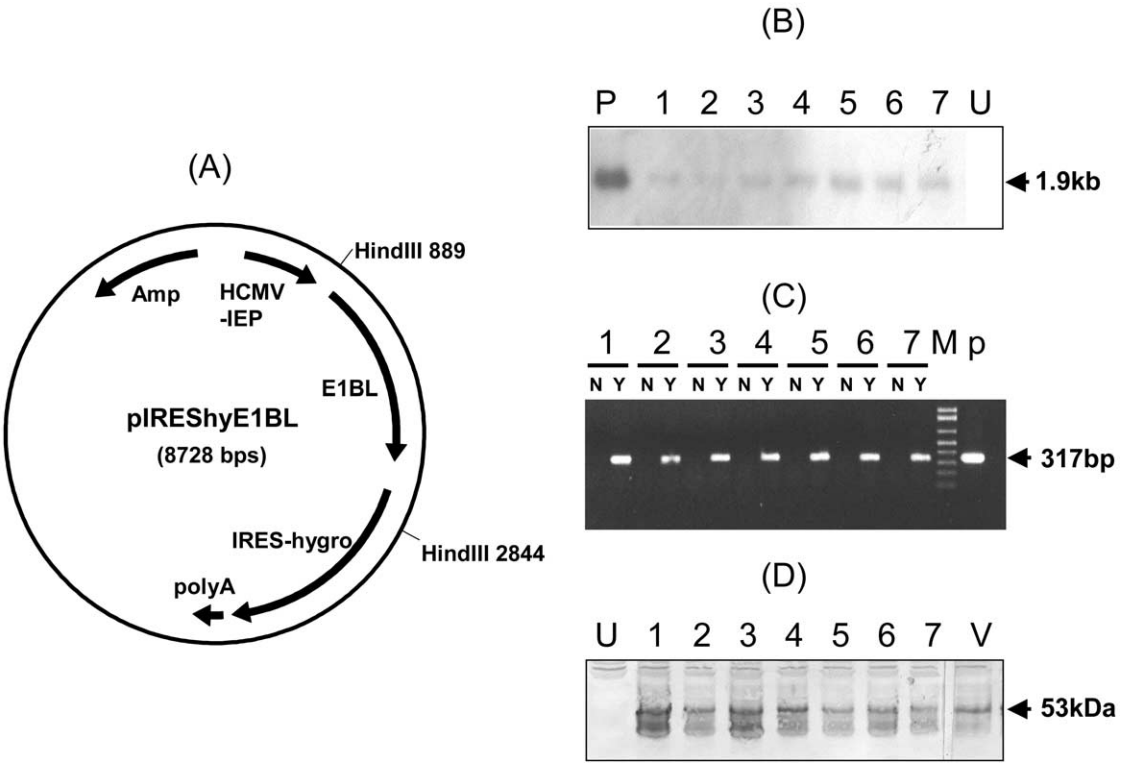

Fig. 1. PAV-3 EIB^{large} expressing cell clones. (A) Schematic diagram of the plasmid pIREShyEIBL. (B) Southern blot analysis. Hygromycin-resistant clones (lanes 1 to 7) obtained after transfection of VIDO R1 cells with pIREShyEIBL were used to prepare genomic DNA for Southern blot analysis using 1.9-kb *Hin*dIII fragment as a probe. Lane P, 1.9-kb *Hin*d III fragment; Lane U, VIDO RI cells transfected with control plasmid. Expected band size is shown on the right. (C) Analysis of ethidium bromide-stained RT-PCR products. Products of RT-PCR using DNAse-treated RNA isolated from different hygromycin-resistant clones (lanes 1 to 7) or of PCR using DNA (lane P) as a template were synthesized using primer pair for PAV-3 EIB^{large} (described under Materials and methods). RNA with reverse transcriptase (Y); RNA without reverse transcriptase (N). Size marker (M) DNA and expected band size is shown on the right. (D) Western blot analysis of EIB^{large} protein. Proteins from uninfected VIDO R1 (lane U), PAV-3-infected VIDO R1 (lane V), or different EIB^{large} cell clones (lanes 1 to 7) were separated by SDS–PAGE (10% gel) under reducing conditions and transferred to nitrocellulose. The separated proteins were probed in Western blots using PAV-3 anti-EIB^{large} serum. Size of the expected band is shown on the right.

Among other nonhuman adenovirus species, which are under development as a vector, is porcine adenovirus type 3 (PAV-3). The complete nucleotide sequence and a transcriptional map for the whole genome has been reported (Reddy et al., 1998). This has facilitated the construction of the full-length genomic clone of the PAV-3 genome (Reddy et al., 1999b) and recombinant PAV-3s (Reddy et al., 1999a, 1999b; Zhou and Tikoo, 2001). Earlier, we reported the characterization of E1 proteins and construction of a recombinant PAV214 with a 1060-bp deletion in the E1 region (Zhou and Tikoo, 2001). In this report, we describe the construction of the recombinant PAV-3 with the E1 region (2750 bp) completely deleted. In addition, we constructed E1-deleted recombinant expressing green fluorescent protein (GFP) that was used to detect vector capability to infect human cells in vitro.

Results

Generation of E1-complementing cell line

Since the production of E1-deleted adenovirus vectors relies on transcomplementation of the E1 functions in helper cells, earlier we developed a cell line (VIDO-R1) by transformation of fetal porcine retina cells with the plasmid DNA containing the E1 sequence of HAV-5 (Reddy et al., 1999a). This cell line could complement the E1A and/or E1B^{small} defect of PAV-3 (Zhou and Tikoo, 2001). However, attempts to rescue the recombinant PAV-3 containing the insertional inactivation/deletion of E1B^{large} proved unsuccessful even on VIDO R1 cells. We hypothesized that the E1B^{large} protein of PAV-3 may be required for rescuing the E1B^{large}-deleted PAV-3.

To develop a cell line expressing PAV-3 E1B^{large} protein, subconfluent monolayers of VIDO R1 cells were transfected with plasmid pIREShyE1BL (Fig. 1A) using lipofectin (Invitrogen). The plasmid pIREShygE1BL contains the PAV-3 E1B^{large} coding sequence under the control of the constitutive human cytomegalovirus (HCMV) immediate early (IE) promoter. The plasmid also has a selection marker, the hygromycin B phosphotransferase gene fused to the internal ribosome entry site (IRES) sequence of the encephalomyocarditis virus at the 5′ end and bovine growth hormone (BGH) poly(A) signal at the 3′ end. IRES permits the translation of two open reading frames from one mRNA.

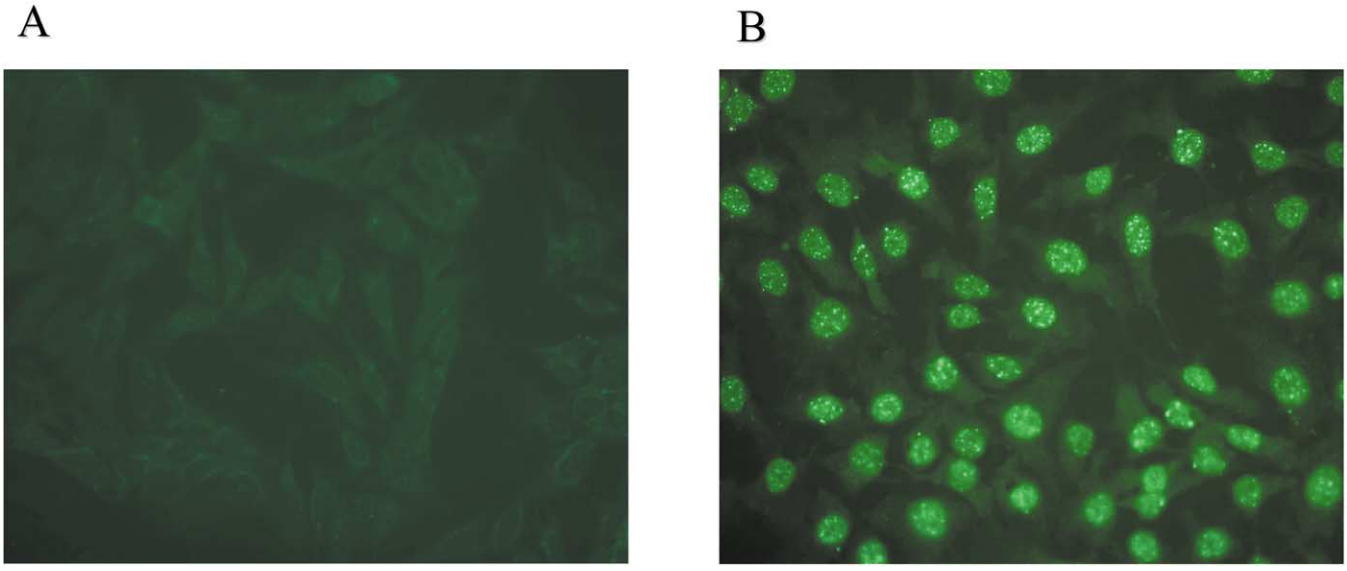

Fig. 2. Immunofluorescence staining. Confluent monolayers of VIDO R1 (A) or VR1BL (B) were fixed and analyzed by indirect immunofluorescence using PAV-3 anti-E1B^{large} serum and FITC-labeled goat anti-rabbit antibodies.

Several hygromycin-resistant colonies appeared 20 days after transfection. Potential clones were expanded and analyzed for the presence of E1B^{large} gene. Total genomic DNA was extracted from the cells, digested with *Hin*dIII, and analyzed by Southern blot using ^{32}P-labeled DNA of E1B^{large} gene as a probe. As seen in Fig. 1B, an E1B^{large}-specific fragment of expected length (1.9 kb; Fig. 1B, lane P) was detected in genomes of different clones (Fig. 1B). No such band could be detected in cells transfected with control plasmid (Fig. 1B, lane U)

Initially, the expression of E1B^{large} in these clones was analyzed by reverse transcription (RT)-PCR using primers specific for PAV-3 E1B^{large}. Using specific primers, an E1B^{large}-specific RT-PCR product of expected size (Fig. 1C, lane P) was detected in different cell clones (Fig. 1C, lanes 1Y to 7Y). The RNA samples with "no RT" control did not show any bands (Fig. 1C, lanes 1N to 7N), indicating that the 317-bp fragment was amplified from E1B^{large} mRNA and not from the residual DNA. To confirm the expression of PAV-3 E1B^{large} protein, Western blot analysis was performed. Anti-E1B^{large} serum detected a major band of 53-kDa protein in different cell clones (Fig. 1D, lanes 1 to 7). A similar band could be detected in PAV-3-infected VIDO R1 cells (Fig. 1D lane V) but not in uninfected VIDOR1 (Fig. 1D, lane U) cells.

Based on these results, a cell line named VR1BL was established. To determine if VR1BL cells homogenously express PAV-3 E1B^{large} protein, the VR1BL cell line was subjected to immunofluorescence analysis, using rabbit antibody against PAV-3 anti-E1B^{large} protein (Zhou and Tikoo, 2001). As seen in Fig. 2, more than 95% of VR1BL cells showed bright fluorescence in the nucleus of the cells (panel B). No such fluorescence was detected in VIDO R1 cells (panel A). These results suggest that VR1BL cells expresses E1B^{large} protein which is similar to native E1B^{large} expressed in PAV-3-infected cells.

To determine the stability of E1B^{large} expression, VR1BL cells were cultured 20 times; split one in four, twice weekly; and tested for the ability to support the replication of PAV507 (described below). The results suggest that VR1BL retained the ability to support the replication of PAV507. To determine whether the VR1BL could support the plaque formation, cells cultured in 35-mm-diameter dishes were infected with wild-type (Fig. 3A) or recombinant (Fig. 3B) PAV-3 and incubated in MEM containing 5% FBS and 0.75% low melting agarose. Clear plaque formation was evident on day 8 postinfection.

Transfectability of VR1BL cell line

To determine the transfection efficiency, VIDO R1 and VR1BL cells in 35-mm-diameter dishes were transfected with 5 μg of plasmid pQB125 by using lipofectin (Invitrogen). This plasmid contains green fluorescent protein gene under the control of a human cytomegalovirus immediate early promoter. At 48h posttransfection, the cells were collected and the percentage of GFP expressing cells were determined by FACS analysis. Our results suggest that VR1BL cells (10.5% GFP positive) were transfected as efficiently as VIDO R1 cells (10% GFP positive).

To test the ability of the cells to take up large DNA, VIDO R1 and VR1BL cells were transfected with 8 μg of *Pac*I-digested pFPAV300 (35.6 kb) (Reddy et al., 1999b) DNA using lipofectin (Invitrogen). After 7–9 days of incubation at 37°C, recombinant PAV300 could be isolated from both cell lines. These observations suggest that VR1BL is as efficient as VIDO R1 for the generation of recombinant PAV-3. In addition, VR1BL could also com-

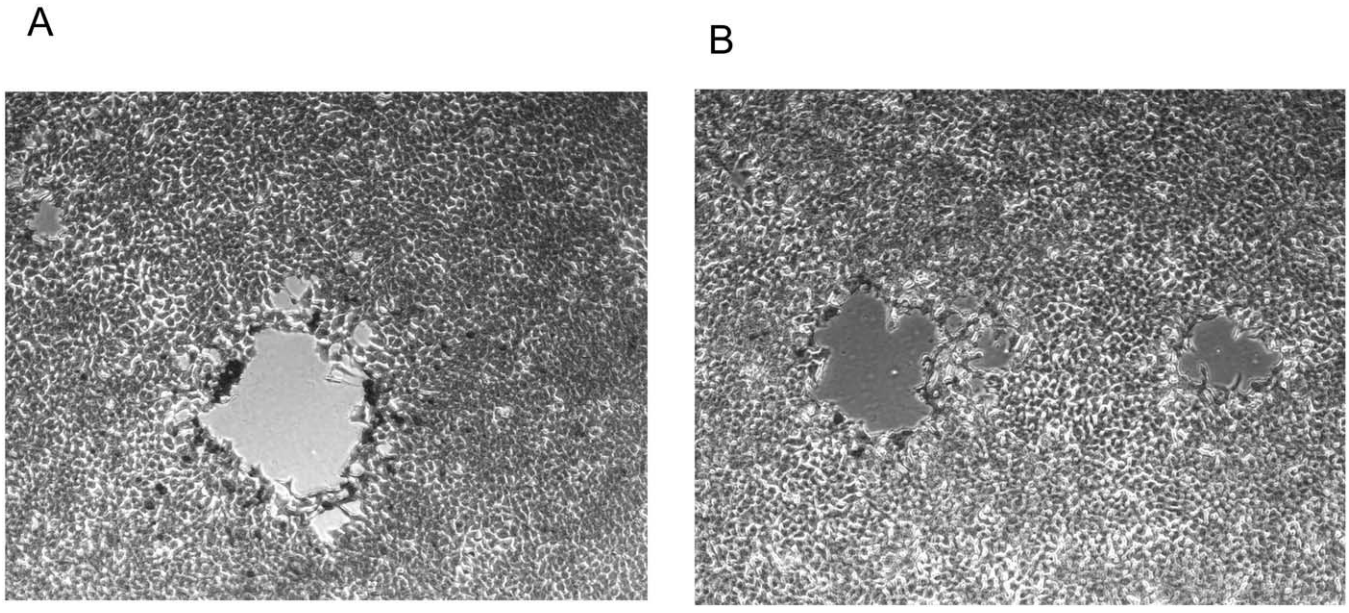

Fig. 3. Plaque formation. Confluent monolayers of VRIBL were infected with wild-type PAV-3 (panel A) or recombinant PAV227 (B) and incubated under an agarose overlay. The appearance of plaques was examined using an Axiovert 25 inverted microscope equipped with a digital camera.

plement (data not shown) the replication defect (E3+E1A+E1B^{small} deletion) of recombinant PAV214 (Zhou and Tikoo, 2001).

Complementation of PAV-3 E1B^{large} insertion mutant

Earlier attempts to isolate a mutant PAV-3 containing insertional inactivation of E1B^{large} expression were unsuccessful using VIDO R1 cells (Reddy et al., 1999a). In order to determine if VR1BL cells could support the replication of E1B^{large} inactivated mutant PAV-3, cells were transfected with *Pac*I-digested pFPAV507 (Zhou and Tikoo, 2001) DNA which contains full-length PAV-3 genomic DNA with a triple phase stop codon inserted in E1B^{large} (nt 2109) and deletion of the E3 region (nt 28,112–28,709). After 14 days of transfection, cells showing cytopathic effects were freeze–thawed twice and the recombinant virus named PAV507 was plaque purified and propagated. The viral DNA was isolated from virus-infected cells by Hirt's extraction method (Hirt, 1967) and analyzed by agarose gel electrophoresis after digestion. As pFPAV507 contains an additional *Spe*I enzyme site (Fig. 4A), the recombinant viral DNA was digested with *Spe*I. As seen in Fig. 4B, the PAV507 genome contains an extra band of 2190 bp (lane 4) which is not present in wild-type PAV-3 (lane 1).

Construction of the E1-deletion mutant of PAV-3

Taking advantage of homologous recombination in *Escherichia coli* strain BJ5183, we constructed the plasmid pFPAV227, containing the full-length genome of PAV-3 with the deletion of E1 (nt 524–3274) and a partial deletion of E3 (nt 28,112–28,709). Transfection of VR1BL cells with *Pac*I-digested pFPAV227 DNA produced a cytopathic effect in 14 days. The virus named PAV227 was amplified in VR1BL cells and the viral DNA was extracted from the infected cells. The DNA was analyzed after digestion with restriction enzyme *Spe*I. The wild-type PAV-3 had *Spe*I fragments of 724, 3510, and 29,860 bp (Fig. 4, lane 1). In contrast, PAV227 had *Spe*I fragments of 527, 724, 3510, and 26,000 bp (Fig. 4, lane 2). This is consistent with the expected *Spe*I fragments of wild-type and the deletion mutant PAV227 (Fig. 4A).

Construction of E1 deletion based recombinant

To determine if E1-deleted PAV-3 can be used to express a foreign gene, we constructed a recombinant PAV-3 expressing GFP. Using homologous recombination machinery of *E. coli* (Chartier et al., 1996), the full-length GFP gene (flanked by HCMV IE promoter and BGH poly(A) signal) was inserted into the E1 region of PAV227 genome in the same orientation as the E1 creating plasmid pFPAV219. The *Pac*I-digested pFPAV219 DNA transfected into VR1BL cells produced a cytopathic effect in 14 days. The virus named PAV219 was amplified in VR1BL cells and virion DNA was extracted from infected cells. The presence of a foreign gene (e.g., GFP) in the viral genome was confirmed by *Spe*I restriction enzyme analysis. The wild-type PAV-3 had *Spe*I fragments of 724, 3510, and 29,860 bp (Fig. 4, lane 1). In contrast, PAV219 had *Spe*I fragments of 547, 724, 828, 849, 3510, and 26,630 bp (Fig. 4, lane 3). This suggested that recombinant PAV219 contains GFP gene in the E1 region of PAV-3. To demonstrate

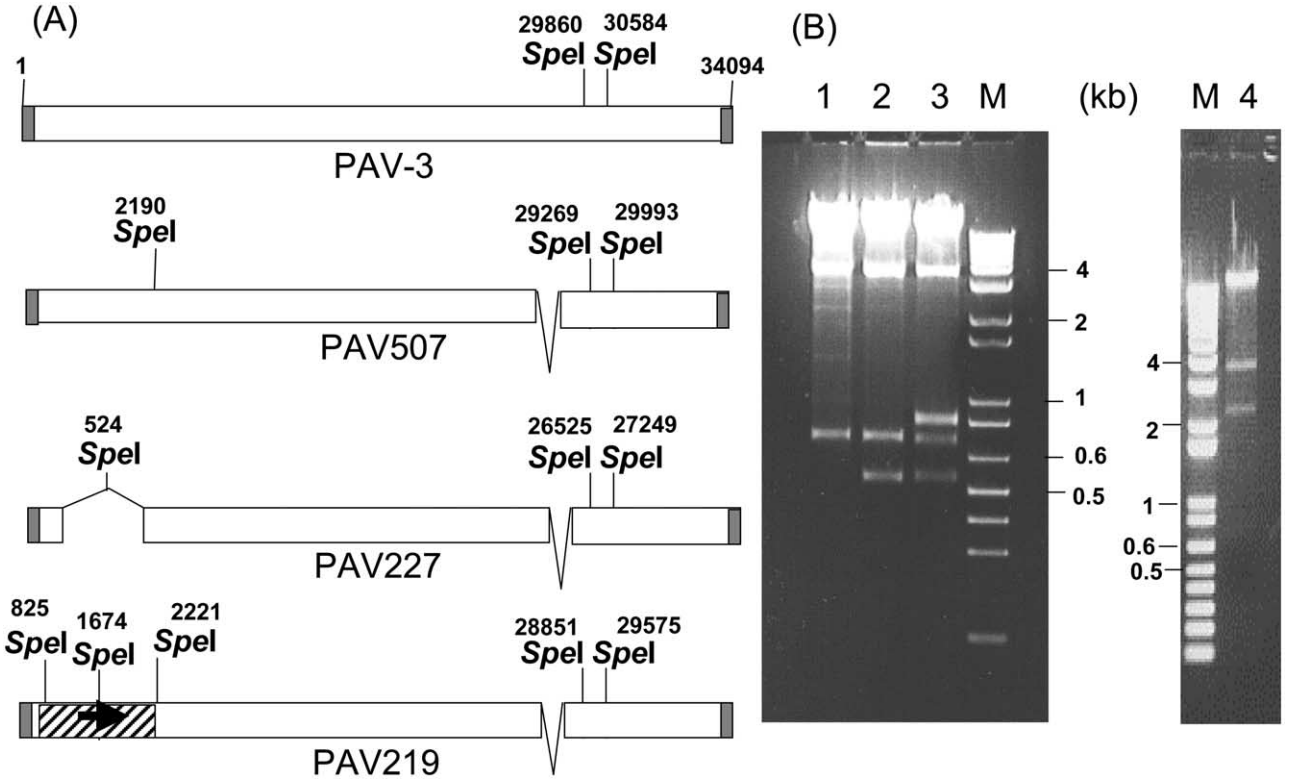

Fig. 4. Restriction enzyme analysis of recombinant PAV-3 genome. (A) Schematic representation of full-length genomes of PAV-3s depicting *Spe*I restriction enzyme sites. (B) Viral DNAs were extracted from VR1BL cells infected with wild-type PAV-3 (lane 1), PAV227 (lane 2), PAV219 (lane 3), or PAV507 (lane 4) and digested with *Spe*I. Sizes of markers (M) are shown in kilobases.

that PAV219 expresses GFP, swine testicular (ST) cells were infected with an m.o.i. of 1 $TCID_{50}$/cell or 100 $TCID_{50}$/cell. At 24 h postinfection, the cells were harvested and analyzed by flow-activated cell sorting (FACS). As seen in Fig. 5, the infected cells expressed GFP protein in a dose-dependent manner (panels B and C). No GFP expression was detected in mock-infected cells (panel A). This suggested that recombinant PAV219 expresses GFP protein.

Growth kinetics of PAV227

To determine the importance of $E1B^{large}$ in viral replication, the growth kinetics of mutant and wild-type PAV-3 were compared in ST, VIDO R1, and VR1BL cells. Monolayers of different cells were infected with mutant PAV227 or wild-type PAV-3 and cells were harvested at 12, 24, 36, 48, 60, and 72 h postinfection. Virus from each sample was released by freeze–thawing and titered on VR1BL cells by

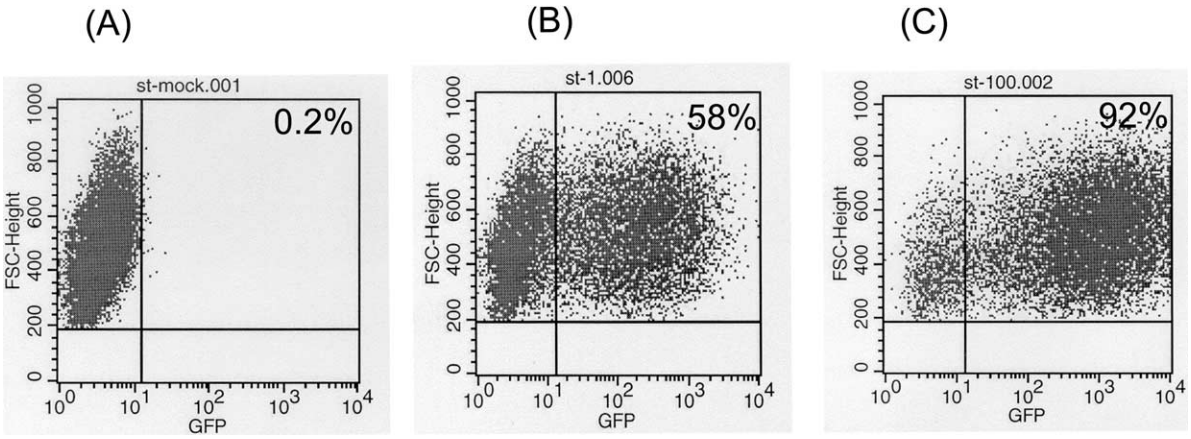

Fig. 5. Quantitation of GFP expression using FACS analysis. VR1BL cells mock infected (A) or infected with an m.o.i. of 1 (panel B) or 100 (panel C) of recombinant PAV219. At 24 h postinfection, the cells were collected, fixed, and analyzed by flow cytometry.

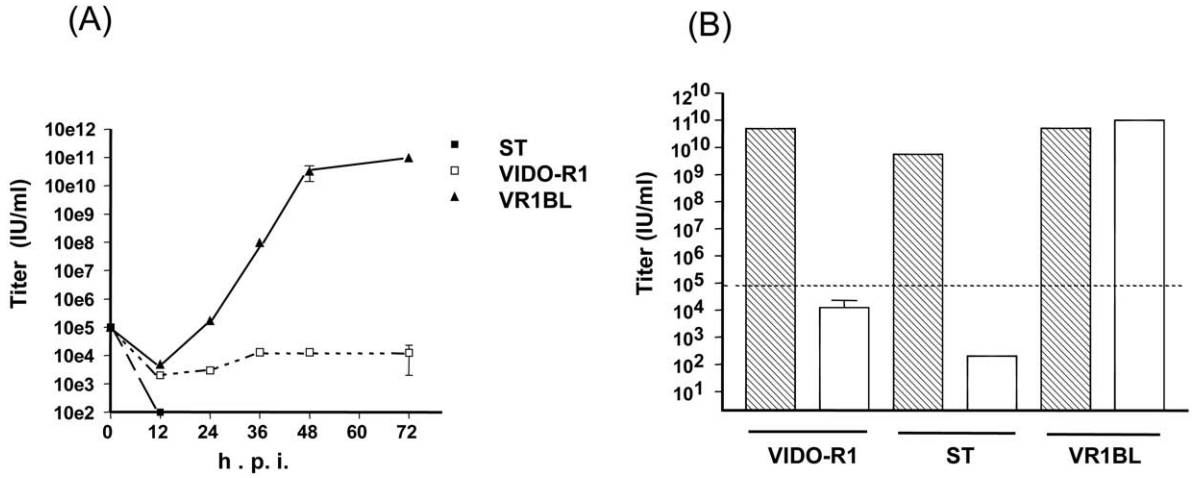

Fig. 6. Virus titer of recombinant and wild-type. (A) Near confluent monolayers of VR1BL, VIDO R1, or ST cells were infected with PAV227. At different time points postinfection, the cell pellet was freeze–thawed and the virus was titrated on VR1BL cells as described in the text. (B) Production of recombinant PAV-3 by different cell lines. Near confluent monolayers were infected with wild-type PAV-3 or recombinant PAV227. After 3 days, the cells were collected and freeze–thawed, and the virus was titrated as described in the text. Titers were expressed as infectious units (IU) in which 1 IU was defined as one positive focus of DBP stained cells at 3 days postinfection. The dotted line shows the amount of input virus.

DBP detection assay (Zhou et al., 2001). The wild-type PAV-3 titer was 5×10^{10} infectious units (IU)/ml at 72 h postinfection (hpi) on VR1BL cells (Fig. 6B). The titer of PAV227 was quite similar to that of wild-type PAV-3 virus (Figs. 6A and B). This suggests that complete deletion of E1 (E1A+E1B^{small}+E1B^{large}) in PAV227 did not have any effect on its ability to propagate in VR1BL cells (E1-complementing cells). In contrast, we could not observe any progeny virus in PAV227-infected VIDO R1 cells or ST cells (Fig. 6B)

Infection of human cell lines with PAV219

To determine if human cell lines could successfully be transduced with recombinant PAV-3 vector, human cell lines of different origins were infected with PAV219 at an m.o.i. of 100 $TCID_{50}$. At 24 hpi, the cells were harvested and the percentage of GFP expressing cells was determined by FACS analysis. As seen in Fig. 7, the transduction efficiency of PAV219 varied in different human cell lines. The human embryo kidney (293) cells were transduced as efficiently as porcine ST cells. However, the transduction efficiency of PAV219 was 76, 53, 31, 30, and 25% in SAOS-2 osteosarcoma, HeLa, MRC-5 lung fibroblasts, U118-MG glioblastoma, and Hep2 carcinoma cells. The transduction of A549 lung carcinoma, SK-N-MC neuroblastoma, and K562 myologenous leukemia cells with PAV219 was negligible.

Discussion

Since adenoviruses are considered one of the most efficient viral vectors for gene delivery (Imler, 1995, Russell, 2000), we (Reddy et al., 1999a, 1999b; Zakhartchouk et al., 1988; Zhou and Tikoo, 2001) and others (Farina et al., 2001; Klonjkowski et al., 1997; Michou et al., 1999; Sheppard et al., 1998; Xu et al., 1997) are developing adenoviruses of animal origin for use in human gene delivery and vaccination. In addition, these species-specific adenoviral vectors are ideal candidates for developing novel recombinant vaccines for animals. Earlier, we described the construction, characterization, and use of E3+E1A+E1B^{small}-deleted PAV-3 vector for the expression of foreign genes (Zhou and Tikoo, 2001). Here, we report (a) the construction of a cell line expressing PAV-3 E1B^{large} protein and (b) the isolation and use of E3+E1A+E1B^{small}+E1B^{large}-deleted PAV-3 vector for the expression of a foreign gene.

Earlier, we established the VIDO R1 cell line (fetal bovine retina cells expressing HAV-5 E1 proteins; Reddy et al., 1999a) which could support the replication of PAV 214 (E3+E1A+E1B^{small} deleted; Zhou and Tikoo, 2001). How-

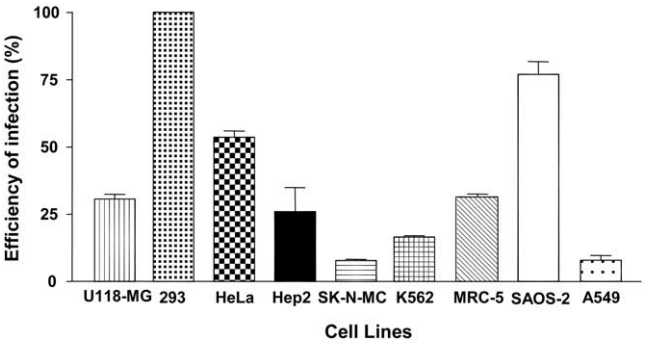

Fig. 7. Transduction of human cell lines. Confluent monolayers of different human cell lines were infected with 100 TCID50 of PAV219. At 24 h postinfection, cells were collected and GFP expression was monitored by FACS analysis.

ever, our repeated attempts to isolate a PAV-3 vector with either gene deletion or insertional inactivation of $E1B^{large}$ were not successful even in VIDO R1 (E1 complementing) cells. It is possible that the human $E1B^{large}$ protein does not complement the porcine $E1B^{large}$ defect of PAV-3. Since the $E1B^{large}$ gene product of HAV-5 interacts with both host and viral proteins (White, 1995), the absence of HAV-5 $E1B^{large}$-specific interactions with HAV-5-specific proteins in VIDO R1 cells may have been responsible for not isolating $E1B^{large}$-defective PAV-3.

To develop a porcine cell line for the generation of $E1B^{large}$-defective PAV-3, we transfected VIDO R1 cells with a plasmid pIREShyE1BL containing PAV-3 $E1B^{large}$ coding sequence and hygromycin B phosphotransferase gene (separated by IRES sequence of EMC [endomyocarditis] virus) under the control of HCMV promoter and BGH poly(A). This construct is expected to be very effective for stable transfection because the selective marker and gene of interest is translated from the same mRNA. A number of hygromycin-resistant clones were selected. As expected, all clones contained the integrated $E1B^{large}$ sequences of PAV-3 in their genomic DNA. In addition, all clones were positive for PAV-3 $E1B^{large}$-specific transcript and expression of $E1B^{large}$ protein. The resulting cell line, named VR1BL, expresses $E1B^{large}$ protein homogenously even after 20 passages, suggesting that the cell line is stable.

Recombinant PAV227 lacking the E1 (nt 524–3274) and part of the E3 (nt 28,112–28,709) coding regions grew to titers similar to those of wild type PAV-3 in VR1BL (E1A+ $E1B^{small}$ +$E1B^{large}$ complementing). In contrast, recombinant PAV227 failed to produce infectious progeny virus in VIDO R1 (E1A+$E1B^{small}$ complementing) or ST cells. This confirmed our earlier observation (Zhou and Tikoo, 2001) and further suggests that $E1B^{large}$ protein is essential for replication of PAV-3. Similar results have been reported for $E1B^{large}$ of HAV-7 (Abrahamsen et al., 1997). Although $E1B^{large}$ appears to be essential for the efficient replication of HAV-5 in HeLa cells (Babiss et al., 1985), it appears to be dispensable for the replication of HAV-5 in A549 and human embryonic retina cells (Imler et al., 1996) and human 143 cells (Klessig et al., 1982).

The construction of PAV227 is a step further toward the development of a PAV-3 vector containing complete deletion of E1 (nt 524–3274) and partial deletion of E3 (nt 28,112–28,709), and therefore should be able to accommodate 4.7-kb foreign DNA, an improvement over the capacity of the currently available PAV-3 vector (Zhou and Tikoo, 2001). The construction of PAV219 further demonstrated the feasibility of using this vector system for foreign gene expression. These viruses could be propagated in VR1BL cells with growth kinetics similar to those of wild type indicating that neither the extended deletion (complete E1, E3) nor the foreign gene insertion alters the viability of the viruses.

Recombinant PAV-3 also has potential as a vector for gene delivery in humans. In this respect recombinant PAV219 (expressing GFP) is a valuable tool as it provides a simple and reliable method for quantitative detection of transgene expression in different cells. As expected, transduction of different human cell lines with PAV219 was variable. Interestingly, both human 293 cells and SAOS-2 osteosarcoma cells were transduced efficiently by PAV219. In addition, human sera containing anti-HAV-5 antibodies does not neutralize PAV-3 (unpublished observations), suggesting that PAV-3 may be suitable as a gene delivery vector for the treatment of osteosarcoma in humans.

PAV-3 did not transduce A549 and Hep2 cells as efficiently as does HAV-5 (Horwitz, 1996). Although HAV-5 has been shown to bind to a number of receptor moieties (Dechecchi et al., 2000; Davison et al., 1999; Hong et al., 1997), the transduction efficiency of HAV-5 mainly depends on the amount of CAR (Bergelson et al., 1997) and integrin molecules present on the surface of the cells. In contrast to HAV, initial interactions between PAV-3 and host cells is not well understood. No primary receptor for fiber has been identified and because of the absence of RGD motif in penton base protein (Reddy et al., 1998), the requirement of cell surface integrins for PAV-3 internalization remains to be determined. The fact that PAV-3 transduces A549 and Hep2 cells poorly suggests that PAV-3 may utilize receptor(s) that is distinct from the CAR receptor. Similar observations have been reported for ovine adenovirus 287, human adenovirus 3 and CELO virus (Xu and Both, 1998; Stevenson et al., 1995; Tan et al., 2001).

Materials and methods

Cells and viruses

VIDO R1 (Reddy et al., 1999a) cells were grown and maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Swine testicular (ST), 293 (human embryo kidney), MRC-5 (human lung fibroblasts), A549 (human lung carcinoma), Hep2 (human larynx carcinoma), HeLa (human cervix carcinoma), U118-MG (human glioblastoma), SK-N-MC (human neuroblastoma), SAOS-2 (human osteosarcoma), and K562 (human myologenous leukemia) cells were cultivated according to ATCC recommendations. VR1BL cells were cultivated and maintained in MEM with 10% FBS and 100 μg/ml of hygromycin B. The mutant and wild-type PAV-3 (strain 6618) were propagated and titrated in VR1BL cells (this study).

Construction of plasmid pIRES hyE1BL

The construction of plasmid pIRES–Hyg will be described elsewhere (manuscript in preparation). Briefly, the hygromycin resistance gene, isolated as a 1.304-kb PCR fragment using sequence-specific primers and plasmid pDR2 (Clontech) as a template, was ligated to *Msc*I–

*Eco*RV-digested plasmid pCITE-1 (Clontech) to create plasmid pIRES–Hyg. A 1.6-kb *Pvu*II–*Eco*RV fragment containing the IRES–Hyg cassette was excised from plasmid pIRES–Hyg and ligated to *Eco*RV-digested pCDNA-3 to create plasmid pcIRES–Hyg. A 1612-bp *Hin*dIII fragment of the PAV-3 genome (nt 1767 to 3379) was blunt-end repaired with T4 polymerase and ligated to *Bam*HI-digested (T4 polymerase-treated) plasmid pcIRES–hyg to create plasmid pIRES hyE1BL.

Recombinant plasmids

The recombinant plasmid vectors were constructed by standard procedures using restriction enzymes and other DNA-modifying enzymes.

Construction of pFPAV507

Plasmid pFPAV507 (Fig. 4A) contains full-length PAV-3 genomic DNA with stop codons in all three reading frames inserted in $E1B^{large}$ (nt 2109) and deletion of the E3 region (nt 28112–28709)(Zhou and Tikoo, 2001).

Construction of pFPAV227

To delete the E1 region, the PAV-3 genome between nt 0 and 524 was amplified by using primers YZ-13 and YZ-14 and pPAVXhoIRL as a template as described previously (Zhou and Tikoo, 2001). The PAV-3 genomic DNA between nt 3274 and 4159 was amplified by using primers P3274 (5′-GACTAGTTCCT CT TGCAGGTACGTG-3′) and P6205C (5′-TGTCCATCACGGATCCTCAG-3′) and DNA of pPAVXhoIRL as a template. The PCR products were digested with *Bam*HI and *Spe*I and gel purified. The digested products were cloned in pPAVXhoIRL and digested with *Bam*HI in a three-way ligation. The resulting plasmid, pPAV227XhoIRL carried a 2750-bp (nt 524 to 3274) deletion of E1 and the inserted *Spe*I site. The plasmid containing recombinant PAV-3 genome with the deletions in the E1 and E3 regions (pFPAV227; Fig 4A) was generated by homologous DNA recombination in *E.coli* BJ5183 between *Xho*I-linearized pPAV227XhoIRL and PAV-3 genome excised by *Pac*I from pPAV-300 (Reddy et al., 1999b).

Construction of pFPAV219

DNA of pPAV227XhoIRL was digested with *Spe*I, blunt-end repaired with T4 polymerase, and ligated to a 2320-bp T4 polymerase-treated *Bgl*II–*Dra*III fragment of plasmid pQBI25 (QBIOgene), containing the human cytomegalovirus immediate-early promoter, gene for GFP, and bovine growth hormone (BGH) poly(A) signal. The final plasmid was named pPAV219XhoIRL. The plasmid containing recombinant PAV-3 genome with GFP expression cassette insertion in the E1 region of E3 and E1 deleted regions (pFPAV219; Fig. 4A) was generated by homologous DNA recombination in *E.coli* BJ5183 between *Eco*47III–*Tth*111I fragment of pPAV227XhoIRL and PAV-3 genome excised by *Pac*I from pPAV-300 (Reddy et al., 1999b).

Isolation of recombinant PAV-3

VR1BL cell monolayers seeded in 60-mm dishes were transfected with 8 μg of *Pac*I-digested pFPAV507, pFPAV219, and pFPAV227 recombinant plasmid DNAs using the lipofectin method (Invitrogen). After 14 days of incubation at 37°C, the transfected cells showing cytopathic effects were collected and freeze–thawed twice, and recombinant viruses were plaque purified and expanded on VR1BL cells.

RT-PCR analysis

For RT-PCR analysis, total RNA was extracted from 10^6 cells using the TRIzol reagent (Invitrogen). The RNA preparations were treated with 2 U of RNase-free DNase (Ambion) at 37°C for 30 min. Then, DNase inactivation reagent (Ambion) was added. Two micrograms of total RNA was subjected to analysis using QIAGEN one-step RT-PCR kit and the specific primers (PBL1, 5′-CCCTGCTGGAGGC-GCGAA-3′; PBL2, 5′-CACACCT GCTGATAGCTAAC) for the $E1B^{large}$ gene of PAV-3.

Southern blot analysis

Five micrograms of the DNA extracted from the cells using DNeasy Tissue Kit (QIAGEN) was digested with *Hin*dIII and loaded on 0.8% TAE agarose gel. After electrophoresis, the DNA was transferred to Hybond-N nylon membrane (Amersham) as described by Sambrook and Russell (2001). The membranes were then probed by Southern blot hybridization analysis using random primed [^{32}P]dCTP-labeled PAV-3 $E1B^{large}$ gene as a probe.

Western blot analysis

Five micrograms of cell protein extract was separated on sodium dodecyl sulfate (SDS)–polyacrilamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane. Nonspecific binding sites on the membrane were blocked with 1% BSA fraction V. $E1B^{large}$ protein of PAV-3 was detected by exposing the membrane to rabbit polyclonal anti-sera against $E1B^{large}$ of PAV-3 (Zhou and Tikoo, 2001) followed by anti-rabbit IgG conjugated to alkaline phosphatase. The blot was developed using AP conjugate substrate kit (Bio-Rad).

Indirect immunofluorescence microscopy

VIDO-R1 or VR1BL cells were plated on Lab-Tek chamber slides (Nalge Nunc) and grown to confluency. The cells were fixed with absolute methanol for 10 min at −20°C. Cells were washed with PBS and incubated with

rabbit polyclonal anti-E1B^{large} serum followed by anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC). The cells were examined using an Axiovert 200M inverted microscope (Zeiss) equipped with a cooled CCD camera and AxioVision 3.0 software.

Virus growth curve

Cells were infected with mutant or wild-type PAV-3 at an m.o.i. of 5. The infected cells, harvested at the indicated times postinfection, were lysed in the infection medium by three rounds of freeze–thawing. Virus titers were determined by serial dilution infections of VR1BL cells followed by immunohistochemical detection of DNA binding protein (Zhou et al., 2001). Titers were expressed as infectious units (IU) in which 1 IU was defined as one positive focus at 3 days postinfection.

Detection of GFP-positive cells by flow cytometry

The 1×10^5 cells were infected with PAV219 at the desired m.o.i. The cells were harvested at 24 hpi, washed in PBS three times, and analyzed on a FACS Calibur flow cytometer (Becton Dickinson). Acquired data were analyzed using Cell Quest software. The efficiency of infection of human cell lines was calculated as the percentage of GFP-positive cells in a human cell line divided by the percentage of GFP-positive cells found after infection of ST cells.

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