Porcine adenovirus-3 as a helper-dependent expression vector

P. Seshidhar Reddy,† Neeraja Idamakanti, Lorne A. Babiuk, Majid Mehtali and Suresh K. Tikoo

1 Virology Group, Veterinary Infectious Disease Organization, 120 Veterinary Road, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5E3
2 Gene Therapy Department, Transgene SA, 67000 Strasbourg, France

Porcine adenovirus has been proposed as a potential vector for generating novel and effective vaccines for pigs. As a prerequisite for the generation of helper-dependent porcine adenovirus-3 (PAV-3) vectors, two E1-complementing porcine cell lines expressing E1 proteins of human adenovirus-5 (HAV-5) were made. These cell lines could be efficiently transfected with DNA and allowed the rescue and propagation of a PAV-3 recombinant, PAV201, containing a 0.597 kb E3 deletion and a 0.803 kb E1A deletion. Our data demonstrate that E1A proteins of HAV-5 have the capacity to transform foetal porcine retina cells and complement for the E1A proteins of PAV-3. The green fluorescent protein (GFP) gene placed under the control of a cytomegalovirus immediate early promoter was inserted into the E1A region of the PAV201 genome. Using these cell lines, a helper-dependent PAV-3 recombinant expressing GFP, PAV202, was constructed and characterized. The wild-type PAV-3 and the recombinant PAV202 expressing GFP were used to determine the ability of the virus to enter and replicate in cells of human and animal origin under cell culture conditions. Our results suggest that PAV-3 enters but does not replicate in dog, sheep, bovine and human cells.

Introduction

Adenoviruses have proven to be effective vectors for the delivery and expression of foreign genes in a number of specific applications (for reviews see Gerard & Meidell, 1993; Imler et al., 1995). The ability of these vectors to mediate the efficient expression of candidate therapeutic or vaccine genes in a variety of cell types, including post-mitotic cells is considered an advantage over other gene transfer vectors. Adenovirus vectors are divided into helper-independent and helper-dependent groups based on the region of the adenovirus genome used for the insertion of transgenes. Helper-dependent vectors are usually made by deletion of E1 sequences and substitution with foreign DNA. They are produced in complementing cell lines such as 293 (Graham et al., 1977), 911 and PER (Fallaux et al., 1998) that constitutively express E1 proteins. Though these viruses do not replicate in cells that do not express E1 proteins, they express foreign proteins, provided the genes are placed under the control of a constitutive promoter (Xiang et al., 1996). Vaccination of animals with recombinants containing genes in the E1 region induced a systemic immune response and provided protection against subsequent challenge (Imler et al., 1995). This type of expression vector provides a significant safety profile to the vaccine as it eliminates the potential for dissemination of the vector within the vaccinee and, therefore, the spread of the vector to non-vaccinated contacts or to the general environment. However, the currently used human adenovirus (HAV)-based vectors are endemic in most populations, which provides an opportunity for recombination between the helper-dependent virus vectors and wild-type viruses. To circumvent some of the problems associated with the use of HAVs, we (Mittal et al., 1995; Zakhartchouk et al., 1998, 1999; Reddy et al., 1999) and others (Xu et al., 1997; Khatri et al., 1997; Klonjkowski et al., 1997; Sheppard et al., 1998) have developed non-human adenoviruses as expression vectors. All these vectors, except one developed by Klonjkowski et al. (1997), have the E1 region intact in their genomes. Use of such vectors for gene therapy in humans and vaccination in animals

Author for correspondence: Suresh Tikoo.
Fax +1 306 966 7478. e-mail tikoo@sask.usask.ca

† Present address: Genetic Therapy Inc., 938 Clopper Road, Gairhesburg, MD 20878, USA.
is unsafe, because they have the ability to replicate in normal cells and retain the oncogenic potential of the E1 region.

Five porcine adenovirus (PAV) serotypes have been identified to date (Derbyshire et al., 1975; Hirahara et al., 1990). Of the five serotypes, type 3 (PAV-3) replicates to high titres in cell culture (Hirahara et al., 1990). The prototype of this virus was first isolated from a rectal swab collected from a healthy piglet (Clarke et al., 1967), and experimental infections of piglets with PAV-3 have been subclinical or associated with transient diarrhoea (Derbyshire et al., 1975). The proposed use of this virus as a vector has stimulated interest in the molecular genetics of the virus. The complete nucleotide sequence of the PAV-3 genome was determined and a transcription map for the whole genome was established (Reddy et al., 1998a,b). Genomic and cDNA sequence analysis identified promoters, cap sites, intron–exon boundaries, poly(A) signals and poly(A) sites in the virus genome. Though the overall genome organization is similar to that of human adenovirus-2 (HAV-2), the prototype of HAVs, there were some distinct features of the PAV-3 genome. A relatively high G+C content, organization of the late region genes into six families, the absence of additional leader sequences in transcripts of the fibre, and the presence of a single small virus-associated RNA gene are some of the distinctive features of the PAV-3 genome. Availability of the complete sequence information and a transcription map for the whole genome facilitated the development of PAV-3 as a helper-independent expression vector (Reddy et al., 1999). Using the Escherichia coli BJ 5183 recombination system, an infectious full-length clone of the PAV-3 genome was recently constructed and non-essential sites suitable for the insertion of transgene expression cassettes were identified (Reddy et al., 1999). In the present study, we developed E1-complementing cell lines and isolated a helper-dependent PAV-3 vector. In addition, we constructed a helper-dependent PAV-3 expressing a green fluorescent protein (GFP), which was used to determine the host range of PAV-3.

**Methods**

**Cells and viruses.** The 6618 strain of PAV-3 was cultivated in a swine testis (ST) cell line obtained from the ATCC (CRL-1740). Replication-defective HAV-5 expressing β-Gal (Ad5dlE1AlacZ) was cultivated in 293 cells (Zheng et al., 1994). Foetal porcine retina cells (FPRC) were obtained from the eyeballs of piglets delivered by caesarean section of a pregnant sow, 2 weeks before the parturition date. Transformed FPRC (VIDO R1) and ST cells (STR) expressing HAV-5 E1 proteins were used for the generation and cultivation of helper-dependent recombinants of PAV-3. All cells were grown in Eagle’s minimum essential medium supplemented with 5% foetal bovine serum. Viral DNA was extracted from infected cell monolayers by a modified Hirt method (Hirt, 1967).

**Construction of recombinant plasmids.** The recombinant plasmid vectors were constructed by standard procedures using restriction enzymes and other DNA-modifying enzymes as directed by the manufacturers.

(a) Construction of plasmid pFPAV201. The left (nt 1–2130) and the right (nt 32659–34094) terminal restriction enzyme fragments generated by Ncol were subcloned from pFPAV200 (Reddy et al., 1999) to generate the plasmid pFPAV-101. Nucleotide numbers of the PAV-3 genome referred to in this paper are according to GenBank (accession no. AF083132; Reddy et al., 1998b). The E1A sequences between nts 407 and 1210 were deleted using Ncol and Asel digestions and a unique Srl site was engineered into the region to create plasmid pFPAV-102. The plasmid pFPAV201, which has the full-length PAV-3 genome minus E1A and part of the E3 sequences, was created by co-transforma-

(b) Construction of plasmid pFPAV202. A 2.3 kb fragment containing the cytomegalovirus (CMV) immediate early promoter, the GFP gene and the bovine growth hormone (BGH) poly(A) signal was isolated by digesting pQBI 25 (Quantum Biotechnology) with BglII and Dnoll followed by filling the ends with T4 DNA polymerase. This fragment was inserted into the Srl site of pFPAV-102 in both orientations to generate pFPAV-102GFP. This plasmid was digested with PacI and SmaI en-

Results

**Generation of E1-complementing cell lines**

Deletion of essential regions such as E1 restricts the growth of the resulting viruses to cells expressing these genes. Use of...
complementing cell lines is therefore critical for growing these viruses. For studies with HAVs, the 293 cell line is most extensively used for this purpose. HAVs do replicate in cells of porcine origin but PAVs do not replicate in human cells, so cells of human origin cannot be used for PAV. To develop an E1-complementing cell line for PAVs, ST cells were transfected with a plasmid pTG 4671 (Transgene) using Lipofectin (Gibco-BRL). The plasmid has the complete E1 region of HAV-5 (nt 505–4034) under the control of a constitutive promoter of the mouse phosphoglycerate kinase gene and the selective puromycin-N-acetyltransferase gene under the control of the constitutive simian virus 40 immediate early promoter. Forty-eight hours post-transfection, the cells were subcultured and grown in 7 μg/ml puromycin (Clontech). Two weeks after transfection, several puromycin-resistant clones were obtained. These clones were amplified and partially characterized. One clone (STR), which supported the growth of an E1A deletion mutant of HAV-5 (Ad5dlE1ALacZ; Zheng et al., 1994) to high titre, was selected and subjected to single cell cloning. STR cells expressed the HAV-5 E1A and E1B 19 kDa proteins constitutively. Expression levels of E1A proteins (Fig. 1a, lane 3) were slightly higher, and that of the E1B 19 kDa protein (Fig. 1c, lane 3) was lower, than the corresponding proteins in 293 cells (Fig. 1a, c, lane 1). No such proteins were detected in untransformed ST cells (Fig. 1a, c, lane 2). Attempts to detect E1B 55 kDa protein in 293 and STR cells in Western blot/immunoprecipitation assays using rat monoclonal antibodies (DP 08; Cedarlane Laboratories) specific to the protein were unsuccessful.

Recently, it was shown that human retina cell lines expressing E1 proteins of HAV-5 have very good transfection efficiency and support the growth of adenoviruses to high titres (Fallaux et al., 1998). To develop a similar cell line for PAVs, early passage secondary FPRC were established by standard tissue culture protocols. Subconfluent monolayers of FPRC were transfected with the plasmid pTG 4671 by means of the calcium phosphate technique. Several morphologically transformed colonies were noticed 4 weeks following transfection without the use of any selection pressure. Since the culture contained both transformed and untransformed cells, it was necessary to select the transformed cell phenotype. Untransformed cells were removed by passing the cells three times in medium containing the drug puromycin (7 μg/ml). A transformed cell line was established following single cell cloning and designated VIDO R1. The transformed cells were smaller and rounded, whereas the untransformed cells were long and slender (Fig. 2). The VIDO R1 cells express vimentin not cytokeratin as determined by immunochemical analysis, indicating that these cells are mesenchymal rather than epithelial in origin (data not shown). To investigate whether the established cell line has E1 sequences integrated in the genome, PCR analysis using two primers specific to the E1A and E1B regions of HAV-5 was carried out. From the PCR, a product of the expected size was obtained, the identity of which was further confirmed by Southern blot analysis using a 32P-labelled E1A region as a probe (data not shown). To determine the levels of E1A and E1B 19 kDa proteins produced by this cell line relative to those produced in 293 cells, Western blot analysis was carried out. The expression levels of E1A produced in VIDO R1 cells (Fig. 1b, lane 3) were higher, while the E1B 19 kDa protein level (Fig. 1d, lane 3) was lower than the corresponding proteins produced in 293 cells (Fig. 1b, d, lane 1). Western blot analysis using the same antibodies did not recognize the corresponding proteins from the extracts of untransformed FPRC (Fig. 1b, d, lane 2). To assess the stability of E1 expression, VIDO R1 cells were cultured more than 50 times, split one in three twice weekly and tested for their ability to support the replication of E1A-deleted HAV-5 (Zheng et al., 1994). Expression of the E1 proteins at regular intervals was also monitored by Western blot. The results indicated that the VIDO R1 cells retained the ability to support...

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**Fig. 1.** Western blot analyses of E1A (a, b) and E1B 19 kDa (c, d) proteins produced in STR (a, c) and VIDO R1 (b, d) cells. In (a) and (b), the blots were treated with MAb (3D11) specific to E1B 19 kDa protein. In (c) and (d), the blots were treated with MAb (M73) specific to E1A. In (c) and (d), lanes 1, 293 cell lysate; 2, ST cell lysate; 3, STR cell lysate. In (b) and (d), lanes 1, 293 cell lysate; 2, FPR cell lysate; 3, VIDO R1 cell lysate.

**Fig. 2.** Comparison of cell morphology. FPRC (a) and VIDO R1 (b) cells were stained with crystal violet and photographed at a magnification of x 100.
Fig. 3. Schematic representation of the strategy used for plasmid and recombinant virus construction. (a) Construction of plasmid pFPB201 and recombinant virus PAV201; (b) construction of plasmid pFPB202 and recombinant virus PAV202. Broken thin line, plasmid DNA; unfilled boxes, PAV-3 genomic DNA; filled boxes, PAV-3 genome inverted terminal repeats.

the growth of E1A-deleted HAV-5 and expressed similar levels of E1 proteins during more than 50 passages in culture (data not shown). Therefore, VIDO R1 can be considered to be an established cell line.

To investigate the complementing properties of VIDO R1, the cells in one well of 96-well plates were infected with $10^5$ p.f.u./ml of an E1A-deletion mutant of HAV-5 (Ad5dl-E1AlacZ; Zheng et al., 1994) and incubated for 5–7 days. This cell line supported the growth of the deletion mutant to $10^5$ p.f.u./ml. To determine whether VIDO R1 could support plaque formation, cells cultured in 35 mm diameter dishes were infected with PAV-3 or HAV-5 and incubated in medium containing 5% foetal bovine serum and 0–75% low melting agarose. Clear plaque formation could be observed 7–9 days post-infection.

**Generation of E1A deletion mutants of PAV-3**

To test whether these cell lines can be used for the generation and propagation of PAV-3 mutants with deletions in E1A and E3, the full-length PAV-3 genomic DNA containing deletions in E1A (nt 407–1210) and E3 (nt 28112–28709) regions was cloned in a plasmid named pFPB201 (Fig. 3a). This plasmid was digested with AseI and analysed by agarose gel electrophoresis. pFPB200 (which contains full-length genomic DNA of PAV-3; Reddy et al., 1999) had an AseI fragment of 2–11 kb (Fig. 4, lane 1), which is missing in pFPB201 (Fig. 4, lane 2), confirming that the expected deletion has been created. A viable virus (named PAV201) was obtained following the transfection of PacI-restricted pFPB201 into VIDO R1 cells. Virus was harvested, amplified and viral DNA was prepared by Hirt extraction (Hirt, 1967). The structure of the viral DNA was established by cleavage with AseI (Fig. 4). The wild-type PAV-3 had an AseI fragment of 1-280 kb (Fig. 4, lane 4), which was missing in the recombinant PAV201 genome (Fig. 4, lane 5). This is consistent with the expected AseI restriction pattern. The deletion mutant could only be grown in VIDO R1 and STR cells but not in ST and FPR cells.

**Construction of replication-defective PAV-3 expressing GFP**

To determine if replication-defective vector could be used for the expression of foreign genes, the plasmid pFPB202 was constructed by inserting a 2-3 kb DNA fragment [con-
taining the GFP gene flanked by the CMV promoter and BGH poly(A) in plasmid pFP201 was confirmed by homologous recombination in E. coli (Fig. 3b). Since the CMV promoter contains an AseI site, the presence of the GFP gene in pFP202 was confirmed by AseI restriction enzyme analysis. As expected, pFP202 contains an additional band of 3.3 kb (Fig. 4, lane 3), which is missing in pFP201 (Fig. 4, lane 2). The recombinant PAV-3 virus (named PAV202) expressing GFP was generated following transfection of VIDO R1 cells with PstI-restricted pFP202 that had the GFP transcription unit in the opposite orientation to the E1. A recombinant virus with the GFP in the same orientation as the E1 could not be rescued from transfected cells. Since the CMV promoter contains an AseI site, presence of the GFP gene in the PAV202 virus genome was confirmed by AseI restriction enzyme analysis (Fig. 4). As expected, compared to PAV201 (Fig. 4, lane 5), the genome of PAV202 contains an additional AseI band of 2.3 kb (Fig. 4, lane 6). The recombinant virus replicated in VIDO R1 cells 2 logs less efficiently than the wild-type PAV-3. This is not due to expression of GFP, since recombinant PAV202 also replicates with similar efficiency in VIDO R1 cells. At present, we do not know the reason for the low titre.

In order to determine the expression of GFP protein, recombinant PAV202 virus-infected cell lysates were analysed by Western blotting using GFP-specific polyclonal antibodies (Clontech). As seen in Fig. 5, anti-GFP serum identified a band by Western blotting using GFP-specific polyclonal antibodies recombinant PAV202 virus-infected cell lysates were analysed we do not know the reason for the low titre.

**Discussion**

Adenoviruses have a great potential as vectors for immunization against human and veterinary diseases and somatic gene therapy in humans. At present adenovirus vectors are constructed by replacing the essential E1 region with a foreign gene of choice. This replacement makes them helper-dependent for E1 functions and therefore they must be propagated in a cell line that expresses E1 proteins. Currently, 293 (Graham et al., 1977), 911 (Fallaux et al., 1996) and PER (Fallaux et al., 1998) cells are used for the production of E1-deleted recombinant HAVs. These cell lines were generated by transformation of human embryonic cells with the E1 region of HAV-5. They are not suitable for the generation of helper-dependent PAVs as these viruses do not replicate in human cells. To generate the E1-region-deleted recombinant PAVs, we developed two porcine cell lines that constitutively express the E1 proteins of HAV-5.

ST cells are commonly used for the propagation of PAV-3, since the virus grows well in this cell line. These cells were chosen first for the stable transfection with the E1 expression plasmid pTG 4671. Two weeks following transfection, several puromycin-resistant clones were obtained. The puromycin-resistant clones supported the growth of an E1A-deleted HAV-5 (Zheng et al., 1994) although not as efficiently as did 293 cells. One of the clones, STR, was subjected to single cell cloning and the expression of E1 proteins was confirmed by Western blotting.

Although stable cell lines expressing E1 proteins could be established, stable and sustained expression of E1 proteins has been difficult, as these cell lines tend to gradually lose the transfected DNA (Imler et al., 1996). This was mainly attributed to E1-mediated cytotoxicity (Graham et al., 1977; Rao et al., 1992). This transient expression of E1 proteins through the established cell lines is dependent on the selection markers, such as hygromycin, G418 and puromycin, which are very expensive. Moreover, even under selection pressure, the expression of E1 proteins is often lost when these cells are cultured for a long time (Imler et al., 1996). To overcome these problems, we transformed FPRC with the E1 region of HAV-5. Transformation of cultured rodent cells by adenoviruses or adenoviral DNA has been well documented (Fisher et al., 1982). Transformation of porcine cells either with a PAV or a HAV has not been reported due to the fact that exposure of permissive or semi-permissive cells to adenovirus normally leads to lysis of infected cells (Graham et al., 1977). However, it was possible to transform human cells with the DNA

**Fig. 5.** Western blot analysis of GFP expression. Proteins from mock- (lane 1), PAV-3- (lane 3), purified GFP- (lane 2) or PAV202-infected cells harvested at 12 h (lane 4) and 24 h (lane 5) post-infection were separated on 10% SDS–PAGE under reducing conditions and transferred to nitrocellulose. The separated proteins were probed in Western blots using GFP-specific polyclonal antiserum.

**Infection of human and animal cells with PAV-3**

To initially characterize the host species restriction of PAV in vitro, we infected monolayers of 11 cell types from six different mammalian species with 10⁶ p.f.u. per 10⁶ cells of wild-type PAV-3. A tenfold increase in the virus titres in Vero and Cos cells and a 100-fold increase in those in cotton rat lung fibroblasts and VIDO R2 cells (E1-transformed FBRC) was noticed. No increase in the virus titres was noticed with 293, A549, MDBK, sheep skin fibroblasts, dog kidney and C3HA cells. However, all the cell types infected with PAV202 showed green fluorescence (data not shown). This suggests that PAV-3 infects but does not replicate in 293, A549, MDBK, sheep skin fibroblast, dog kidney and C3HA cells.

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encoding E1 proteins of HAVs. Using this approach several human cell lines, such as 293 (Graham et al., 1977), 911 (Fallaux et al., 1996) and PER (Fallaux et al., 1998), have been developed and used to generate helper-dependent HAV vectors. The approach used in the present study to create an E1-complementing cell line employing the E1 region of HAV-5 for PAVs is novel as E1A proteins of HAV-5 were shown for the first time to complement for those of PAV-3. There are several reasons for using the E1 region of HAV-5 for transformation of FPRC. The E1 region of HAV-5 was shown to transform human retina cells very efficiently (Fallaux et al., 1998). In contrast to the E1 region of PAV-3, the E1 region of HAV-5 has been thoroughly characterized and monoclonal antibodies against E1 proteins are readily available from commercial sources. In addition, the E1A region of HAV-5 was shown to complement the E1A functions of several non-human adenoviruses (Ball et al., 1988; Zheng et al., 1994).

The construction of PAV201 is the first step towards the development of replication-defective PAV-3 containing deletions in E1 and E3 regions. The construction of PAV202 further demonstrated the feasibility of using this vector system for foreign gene expression. In addition, deletion of 1-4 kb (E1A + E3) should also increase the capacity of the resulting vector, PAV201, to accommodate 3-2 kb of foreign DNA. The presence of low levels of helper-independent vectors in the batches of helper-dependent adenoviruses that are grown in 293 or 911 cells have been reported (Fallaux et al., 1998). This occurs as a result of recombination events between the viral DNA and the integrated adenoviral sequences present in the complementing cell line (Hehir et al., 1996). This type of contamination constitutes a safety risk, which could result in the replication and spread of the virus. Complete elimination of helper-dependent adenoviruses in the batches of helper-dependent vectors can be achieved using two approaches. First, by developing new helper cell lines and matched vectors that do not share any common sequences (Fallaux et al., 1998). Second, by using cross-complementation that exists between two distantly related adenoviruses such as HAV-5 and PAV-3. VIDO R1 cells contain the E1 coding sequences of HAV-5. Although there is no significant identity between the E1 regions of HAV-5 and PAV-3 at the nucleotide sequence level, the proteins produced from the region can complement the function of each other. Thus, the problem of helper-independent vector generation by homologous recombination should be rare when VIDO R1 cells are used for the propagation of recombinant PAV-3.

HAV serotypes belonging to all subgroups can transform rodent cells in tissue culture (Tooze, 1981). The transforming region is comprised of E1A and E1B and both are necessary for complete transformation (Van den Elsen et al., 1983). The E1A proteins of HAV-2 have been extensively characterized. Three conserved regions (CR1 to CR3) of the E1A proteins have been identified that are required for the interaction of the protein with cellular proteins. CR1 and CR2 are required for transformation and serve as binding sites for the product of the retinoblastoma susceptibility gene (Rb) and p300 protein respectively. This activity is responsible for freeing of E2F transcription factor from the clutches of Rb and p300, which is responsible for induction of S phase events (Bagchi et al., 1990). Sequences within CR3 are critical for the transcriptional activation capacity of the E1A protein. The expression of E1A stimulates apoptosis, an important cellular defence against virus infection and perturbation of cell growth control. Both proteins encoded by the E1B region are involved in preventing the E1A-mediated apoptosis (Rao et al., 1992; Debbas & White, 1993). Thus, the expression of E1B protein makes cells tolerant of levels of E1A that are required for cell transformation (Lowe & Ruley, 1993). The ability of E1 proteins of HAV-5 to transform FPRC into a stable cell line suggests that similar molecular mechanisms of transformation are used in foetal porcine cells.

The E1 region in PAV-3 is located within the least 12% of the genome and encodes proteins that are analogous to those found in HAV-5 (Reddy et al., 1998a). There is a high degree of conservation in the CR2 and CR3 between the E1A proteins of PAV-3 and HAVs. In this study, we have demonstrated that the E1A proteins of HAV-5 could complement for the E1A of PAV-3. Earlier, functional similarities between the E1A proteins of HAV-5 and simian adenovirus-7 (Kimelman et al., 1985); between HAV-5 and mouse adenovirus-1 (Ball et al., 1988); and also between HAV-5 and bovine adenovirus-3 (Zheng et al., 1994) were demonstrated in transient transfection assays or using co-infections.

The use of the GFP as a marker for recombinant virus allows the visualization of infected cells by fluorescence microscopy. Since the recombinant GFP gene, isolated from the jellyfish Aequorea victoria was first used as a genetic marker in Caenorhabditis elegans, it has been used in a variety of organisms (Cubitt et al., 1995). GFP is a valuable biological marker, which allows a non-evasive detection that requires only illumination by UV light to yield green fluorescence. Future investigations on the virus tropism will likely involve the study of cell populations in which only a subset of cells may be infected by PAV-3. In such cell populations, the GFP can allow the isolation of virus-infected cells for subsequent experiments regarding the study of virus persistence.

The finding that PAV-3 was effective in entering human, canine, sheep and bovine cells in which it does not replicate or replicates poorly is an important observation. This may have implications in designing a PAV-3 vector for vaccination in human and animal species. Recombinant HAVs expressing virus antigens have been shown to induce protective immune responses in animals such as mice and dogs, in which HAV replicates poorly or not at all (Prevec et al., 1989).

We thank Dr F. L. Graham, McMaster University for Ad8dlE1AlacZ virus and Caron Pyne for helping with Western blot analysis of GFP. This work was supported by grants from National Science and Engineering
Research Council of Canada, Saskatchewan Agriculture Development Fund, Western Economic Diversification, and Alberta Agriculture Research Institute. P.S.R. was a recipient of a post-doctoral research fellowship from Health Services Utilization and Research Commission, Saskatoon, Saskatchewan, Canada.

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Received 27 April 1999; Accepted 2 July 1999