Replication-Defective Bovine Adenovirus Type 3 as an Expression Vector†

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Although recombinant human adenovirus (HAV)-based vectors offer several advantages for somatic gene therapy and vaccination over other viral vectors, it would be desirable to develop alternative vectors with prolonged expression and decreased toxicity. Toward this objective, a replication-defective bovine adenovirus type 3 (BAV-3) was developed as an expression vector. Bovine cell lines designated VIDO R2 (HAV-5 E1A/B-transformed fetal bovine retina cell [FBRC] line) and 6.93.9 (Madin-Darby bovine kidney [MDBK] cell line expressing E1 proteins) were developed and found to complement the E1A deletion in BAV-3. Replication-defective BAV-3 with a 1.7-kb deletion removing most of the E1A and E3 regions was constructed. This virus could be grown in VIDO R2 or 6.93.9 cells but not in FBRC or MDBK cells. The results demonstrated that the E1 region of HAV-5 has the capacity to transform bovine retina cells and that the E1A region of HAV-5 can complement that of BAV-3. A replication-defective BAV-3 vector expressing bovine herpesvirus type 1 glycoprotein D from the E1A region was made. A similar replication-defective vector expressing the hemagglutinin-esterase gene of bovine coronavirus from the E3 region was isolated. Although these viruses grew less efficiently than the replication-competent recombinant BAV-3 (E3 deleted), they are suitable for detailed studies with animals to evaluate the safety, duration of foreign gene expression, and ability to induce immune responses. In addition, a replication-competent recombinant BAV-3 expressing green fluorescent protein was constructed and used to evaluate the host range of BAV-3 under cell culture conditions. The development of bovine E1A-complementing cell lines and the generation of replication-defective BAV-3 vectors is a major technical advancement for defining the use of BAV-3 as vector for vaccination against diseases of cattle and somatic gene therapy in humans.

Replication-defective, recombinant adenoviral vectors are widely used for in vivo gene transfer because of the ability of these vectors to enter many different target cells efficiently and to express the transgene (6). Although these vectors are effective with respect to the entry and expression processes, expression of the transgene is transient (8). The transient expression is explained at least in part by the host immune responses, both innate and acquired, to cells transduced with adenoviral vectors (37). To overcome the problem associated with the pre-existing immune responses, nonhuman adenoviruses have been proposed as vectors for gene therapy (21, 29, 30, 38, 39, 42). Animal adenoviruses are species specific, can enter human cells but do not replicate, and thus have great potential as gene transfer vectors. In addition, these vectors could be used as live viral vaccines in animals. Currently used recombinant adenovirus vectors are made replication defective by deletion of E1A and E1B sequences (4). The E1A region encodes the immediate-early proteins that transactivate all other viral early region genes. By deleting these sequences, the expression of all other early and the late region genes is reduced to a large extent, resulting in lower immune responses to adenoviral proteins and thus in long-term expression of a transgene. In addition, deletion of the E1 region removes the oncogenic potential of the viruses.

Bovine adenoviruses (BAVs) belong to the Mastadenovirus genus of Adenoviridae family. Currently, the accepted 10 serotypes of BAVs are divided into two subgroups on the basis of the differences in their biological and serological properties (3). Serotypes 1, 2, 3, and 9 belong to subgroup I and grow relatively well in established bovine cell lines. We have been studying BAV type 3 (BAV-3) with a goal of developing it as an expression vector. BAV-3 was chosen for this purpose based on the lack of virulence and the ability of the virus to grow to high titers in cell culture. In addition, experimental infection of calves with BAV-3 failed to produce either clinical signs or gross lesions, but all animals seroconverted (22). Molecular studies of the genome are essential for the development of BAV-3 as an expression vector. Determination of the complete nucleotide sequence and transcription map of BAV-3 was recently reported (30). Nucleotide sequence analysis of the E1 region of BAV-3 identified open reading frames for proteins that are homologous to the E1A and E1B proteins of human adenovirus type 5 (HAV-5) (11, 43). Transcription analysis of the E1 region in BAV-3 has indicated that the transcripts of the E1A, E1B, and pIX regions are 3′ coterminal (28). The proteins produced from the E1 and neighboring pIX region of BAV-3 were also identified and characterized by using specific antibodies raised in rabbits (28).

Among current methods for generating adenovirus-based vectors, the Escherichia coli BJ5183 recombination system is the most simple and efficient (5, 29, 42). We applied this method to clone the full-length BAV-3 genome as a stable...
infectious bacterial plasmid and to generate the replication-
competent viral vector (42). In this paper, we describe gener-
ation of E1A-complementing cell lines, E1A deletion mutants,
and replication-defective BAV-3 vectors. This is the first re-
port, to our knowledge, of the development of E1A-comple-
menting cell lines and construction of replication-defective
BAV-3 expression vectors.

MATERIALS AND METHODS

Viruses and viral DNA. The WBR-1 strain of BAV-3 was cultivated in Madin-
Darby bovine kidney (MDBK) and VIDO R2 cells. VIDO R2 is a transformed
fetal bovine retina cell line (FBRC) line expressing the E1 proteins of HAV-5. The
cells were grown in Eagle’s minimum essential medium supplemented with 5%
fetal bovine serum. The viral DNA was extracted from virus-infected cell mono-
layers by the method of Hirt (17). Replication-defective HAV-5 expressing β-
galactosidase (Ad5/E1A/AlacZ) was propagated as described previously (43).

Construction of recombinant plasmids. The recombinant plasmid vectors
were constructed by standard procedures (31) using restriction enzymes and
other DNA-modifying enzymes as directed by the manufacturers.

(i) Construction of plasmid pBAV500. Plasmid pTG5345, containing the
full-length BAV-3 genome in pPOLY1HS1m, was digested with HindIII, and the
recombinant plasmid was ligated with the EcoRI fragment from pBAV-101.d. The
cells were transfected with plasmid pBAV-101.d in the same orientation as the E3 transcription unit to generate plasmid pBAV101.d. A full-length plasmid (E1A-E3 deletion) with the E3 site of pBAV500 was inserted into the
vector pHIV-3 between the HindIII and XbaI sites.

(ii) Construction of recombinant plasmid pBAV502. The genome of recom-
binant BAV502 (27) contains the hemagglutinin-esterase (HE) gene under the
control of the simian virus 40 (SV40) immediate-early promoter, a 137-bp length
chimeric intron, and SV40 poly(A) signal. This plasmid was used as a control of
SV40 early promoter, a 137-bp chimeric intron, and SV40 poly(A) signal.

(iii) Construction of recombinant plasmid pBAV503. The genome of recom-
binant BAV503 contains the hemagglutinin-esterase (HE) gene under the
control of the simian virus 40 (SV40) immediate-early promoter, a 137-bp length
chimeric intron, and SV40 poly(A) signal.

(iv) Construction of plasmid pBAV304. The green fluorescent protein (GFP)
genome under the control of the cytomegalovirus (CMV) immediate-early promoter
and bovine growth hormone poly(A) signal was obtained from pQBI 25 (Quan-
tern Biotechnologies, Irvine, Calif.) for 4 h. The cells were washed once with phosphate-buffered
saline, 1,000 Ci/mmol; ICN Radiochemicals Inc., Irvine, Calif.) for 4 h. The cells were washed once with phosphate-buffered
saline, harvested by scraping, and then lysed with ice-cold modified radioimmuno-
assay (RIA) buffer. The radiolabeled proteins were immunoprecipitated with
a pool of anti-BHV-1 gD rabbit antibodies (9, 10) and analyzed by SDS-PAGE. The
bands were dried, and protein bands were visualized by autoradiography.

RESULTS

Development of MBDK cell line 6.93.9. Our initial goal was to develop an
MBDK cell line expressing E1 proteins of BAV-3, as these cells are commonly used for the propagation
of BAV-3. MBDK cells are also permissive to wild-type HAV-5 infection, and the available E1 deletion mutants
of HAV-5 would allow us the selection of the clones that are expressing E1 proteins of BAV-3 by cross-complementation (43). MBDK cells were transfected with plasmid pBD-Neo,
containing a SalI fragment of BAV-3 covering 0 to 23.4 map units on the viral genome and a Neo′ marker under the control
of the long terminal repeat from Rous sarcoma virus. The SalI
fragment contains all of the E1 region, pIX, Iva2, and pol
region of the terminal protein (pTP) gene. The Neo′ system
permits the selection of cells that take up the plasmid
vector containing the Neo′ marker. Several G418-resistant
clones were isolated, expanded, and tested for the ability to
support the growth of Ad5/E1A/AlacZ (43). Five clones supported
the growth of an E1A deletion mutant, and a clone
(6.93.9) which supported the growth of the virus to the highest
titer was selected and subjected to single-cell cloning. How-
ever, even though these cells supported the growth of an E1A
deletion mutant, we could not detect E1 protein expression
in any of the clones including 6.93.9. Western blotting using
polyclonal antibodies specific to E1 proteins of BAV-3 (28).Clone
6.93.9 cells grew slowly, rapidly acidified the medium,
and never formed a confluent monolayer.

Development of FBRC line VIDO R2. Since MBDK cells were not useful for the generation of replication-defective
BAV-3 vectors, we attempted to develop an FBRC-derived cell
line. Early-passage secondary FBRCs were prepared by standard
techniques. Two attempts to transform FBRCs by the E1 region
of BAV-3 (pBD-Neo) with or without G418 selection were
not successful. As our experience with clone 6.93.9 indicated
that the E1A region of BAV-3 could complement the
E1A of HAV-5, we assumed that the FBRCs transformed with
the E1 region of BAV-3 also could complement the E1A
region of BAV-3. Human fetal retina cells are efficiently
transformed with the E1 region of HAV-5 (13). To develop a bovine
cell line expressing the E1 protein of HAV-5, subconfluent
monolayers of FBRCs were transfected with plasmid pTG6671
(pTG6559 [20] without the E1b-gD mutation) by the calcium
phosphate technique. Plasmid pTG6671 contains the entire
E1A and E1B coding sequences of HAV-5 (nt 505 to 4034). The
transcription of E1A is under the control of the constitutive
mouse phosophoglycerate kinase gene (PGK) promoter, and
transcription of the E1B region is under the control of its
natural promoter and a globin poly(A) signal. This plasmid
also has a selection marker, the puromycin acetyltransferase
gene, under the control of the SV40 early promoter and SV40
poly(A) signal. Several morphologically transformed colonies

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were observed 4 weeks after transfection without selection for puromycin resistance. The cells in transformed foci showed altered morphology, smaller and rounded, whereas the untransformed FBRCs were long and slender (Fig. 1). The transformed cells expressed Vimentin, not cytokeratin, indicating that they are mesenchymal in origin (data not shown). Transformed cell line VIDO R2 was established from a separated foci and then subjected to single-cell cloning. Initially, the expression of E1 in VIDO R2 cell line was analyzed by reverse transcription (RT)-PCR using primers specific to E1A and E1B regions of HAV-5. The PCR products generated using the E1 region DNA as templates were used as size controls (Fig. 1C, lanes 3 and 6). As seen in Fig. 1C, HAV-5 E1A (lane 2) and 19-kDa E1B (lane 5) genes of expected lengths were transcribed in VIDO R2 cells. The RNA samples without reverse transcriptase added did not show any bands (lanes 1 and 4), indicating that genes were amplified from E1 mRNAs and not from residual DNA.

For complementation of an essential viral protein by a cell line, the protein of interest must be synthesized in sufficient quantities by the host cells. To determine the level of E1A proteins produced by VIDO R2, Western blot analysis was carried out. The mouse monoclonal antibody M73, which recognizes all HAV-5 E1A proteins, and antibody 3D11 (Calbiochem), directed against the 19-kDa HAV-5 E1B protein, were used. In VIDO R2, the levels of E1A proteins produced were similar to or little higher than those produced in 293 cells (Fig. 2A). However, the amount of 19-kDa E1B protein produced was slightly less in VIDO R2 cells (Fig. 2B). Western blot analysis using the same antibodies did not recognize any proteins from extracts from FBRCs (Fig. 2, lanes 2). Attempts to detect the HAV-5 E1B 55-kDa protein in 293 and VIDO R2 cells in immunoprecipitation assays using a rat Mab (DP 08; Cedarlane) specific to the protein were not successful.

To investigate the complementing properties of the VIDO R2, the cells were infected with the E1A deletion mutant of HAV-5 (Ad5dlE1AlacZ [43]). This cell line supported the growth of the deletion mutant to 10^7 PFU/ml. To determine whether the VIDO R2 cell line could support plaque formation, cells cultured in 35-mm-diameter dishes were infected with BAV-3 or HAV-5 and incubated in a CO_2 incubator. Clear plaque formation was evident on days 5 and 7 postinfection with HAV-5 and BAV-3, respectively. We also observed a substantially more rapid onset of viral cytopathic effect in E1-expressing cell lines than in MDBK cells and FBRCs. In addition, VIDO R2 supported the formation of clear plaques by recombinant BAV-3.

Transfectability of E1-expressing cell lines. To test the ability of the cells to take up large DNA, MDBK, 6.93.9, and VIDO R2 cells in 35-mm-diameter dishes were transfected with 1 to 3 μg of PacI-restricted plasmid pFBAV304 DNA (described below) by using Lipofectin (GIBCO/BRL). This plasmid contains the entire BAV-3 genome with the E3 region replaced by a GFP gene under the control of a CMV immediate-early promoter. When observed under a fluorescence microscope 24 h posttransfection, more than 3% of VIDO R2 cells showed fluorescence, as opposed to less than 0.1% cells in MDBK and 6.93.9 cultures. Further incubation of the transfected VIDO R2 cells for 10 to 14 days resulted in the production of a recombinant virus (named BAV304) expressing GFP. These observation suggest that VIDO R2 is a better cell line for the generation of recombinant BAV-3 due to its greater transfection efficiency and/or the presence of HAV-5 E1A/B sequences.

Construction of E1A deletion mutant of BAV-3. To construct a replication-defective recombinant BAV-3, full-length BAV-3 genomic DNA containing deletions in the E1A (nt 536 to 1077) and E3 (nt 26456 to 27701) regions was cloned in a plasmid named pFBAV500 (Fig. 3a). Plasmid pFBAV500 DNA digested with PacI (to release the recombinant viral genome from the plasmid) was used to transfect VIDO R2 cells. A recombinant virus (named BAV500) with the E1A and E3 regions deleted was obtained 10 days following transfection.
The virus was amplified in VIDO R2 cells, and the viral DNA was extracted from infected cells. The DNA was analyzed after digestion with restriction enzyme ClaI. The wild-type BAV-3 had ClaI fragments of 3.1, 6, and 7.5 kb (Fig. 4, lane 1), which were missing in the recombinant BAV500 genome, which instead had fragments of 2.5 and 12.2 kb (lane 2). This is consistent with the expected ClaI fragments of the wild type and the deletion mutant.

**Construction of E1A deletion-based recombinants.** To determine the usefulness of E1A-deleted (replication-defective) BAV-3 recombinants as gene delivery vehicles, we constructed recombinant BAV-3 expressing BHV-1 gD (32) or BCV HE (26). The full-length gD gene [flanked by the SV40 early promoter, chimeric intron, and SV40 poly(A) signal] was inserted into the E1A region of the BAV500 genome in the same transcriptional orientation as E1 (using the homologous re-
combination machinery of E. coli), (5), creating plasmid pFBAV501 (Fig. 3b). Earlier, we constructed a replication-competent recombinant BAV-3 (BAV333) expressing BCV HE (27). To construct a replication-defective recombinant BAV-3 expressing BCV HE, the full-length HE gene [flanked by the SV40 promoter, chimeric intron, and SV40 poly(A) signal] was inserted into the E3 region of the BAV500 genome in the same transcriptional orientation as E3 (using the homologous recombination machinery of E. coli), (5), creating plasmid pFBAV502 (Fig. 3c). The PacI-digested pFBAV501 or pFBAV502 DNA was transfected into VIDO R2 cells to isolate a recombinant virus named BAV501 or BAV502, respectively. BAV501 and BAV502 were amplified in VIDO R2 cells, and viral DNAs were extracted from the infected cells. The presence of foreign genes in viral genomes (gD in BAV501 and HE in BAV502) was confirmed by ClaI restriction enzyme analysis. The BAV500 genome had a ClaI fragment of 2.5 kb (Fig. 4A, lane 2) that was missing in the recombinant BAV501 genome, which instead had a fragment of 4.4 kb (Fig. 4A, lane 3). This suggested that recombinant BAV501 contained a gD gene, a possibility confirmed by Southern blot analysis (Fig. 4B, lane 2). As expected, the 12.2-kb BAV500 DNA fragment (Fig. 4A, lane 2) was replaced with a 14.1-kb DNA fragment in BAV502 (Fig. 4A, lane 4). This suggested that recombinant BAV502 contains an HE gene in the E3 region, a possibility confirmed by Southern blot analysis (Fig. 4B, lane 4).

To demonstrate that infection with the E1 mutant viruses is abortive in noncomplementing cell lines, MDBK, FBRC, 6.93.9, and VIDO R2 cultures were used for infection studies. Cells were infected at an MOI of less than 1, grown for a week, subjected to two freeze-thaw cycles, and titrated on the VIDO R2 cells. Wild-type BAV-3 and BAV-3.E3d (E3 deletion [42]) grew to high titers (10^9 PFU/ml) in all cells tested, whereas the replication-defective recombinant viruses (E1A-E3 deletion) grew only in the VIDO R2 (Fig. 5) and 6.93.9 (10^6 to 10^7 PFU/ml) cells. This reduction in titer of replication-defective BAV-3s could be due to lower than optimum level of E1A expression in cell lines or differences in the transcriptional activation property of different E1As in different cell lines, which may affect the efficiency of virus production. Alternatively, it is possible that certain unspecified replication functions of bovine E1A cannot be efficiently replaced by HAV-5 E1A.

**Kinetics of expression of gD and HE.** The kinetics of gD expression by recombinant BAV501 was determined at three different time points postinfection in VIDO R2 and MDBK cells by immunoprecipitation assay. Electrophoretic analysis of metabolically radiolabeled immunoprecipitates from recombinant BAV501-infected VIDO R2 cells lysates revealed immunoreactive proteins with approximate molecular masses of 63 kDa (unglycosylated) and 71 kDa (glycosylated) (Fig. 6A, lanes 5 and 6). No corresponding proteins could be detected in mock (lane 1) or wild-type BAV-3 (lane 2)-infected cells. The molecular weight of the unglycosylated and glycosylated gD corresponds to that of the authentic gD immunoprecipitated from BHV-1-infected cell extracts (lane 3). In BAV501-infected cells, expression of gD was first detected at 24 h postinfection (lane 5) and continued to be produced up to 36 h postinfection (lane 6), the last time point used in the study. Similar kinetics of gD expression was observed in MDBK cells (Fig. 6B, lanes 5 and 6).

The kinetics of HE expression by recombinant BAV502 was...
determined in VIDO R2 cells. Anti-BCV polyclonal rabbit serum immunoprecipitated a 65-kDa polypeptide from the cells infected with BAV502 (Fig. 7, lanes 5 and 6). This polypeptide comigrated with the authentic HE protein produced from BCV-infected cells (lane 3). No such protein was immunoprecipitated from mock (lane 1)- or wild-type BAV-3 (lane 2)-infected cells. The kinetics of HE expression (lanes 5 and 6) was similar to that observed for gD in VIDO R2 cells (Fig. 6A, lanes 5 and 6). Glycosylation of the recombinant gD and HE proteins was examined by labeling the virus-infected cells with [3H]glucosamine and then performing immunoprecipitation assays. The results confirmed that the proteins produced by recombinant adenoviruses are glycosylated and are indistinguishable in migration rate from the authentic proteins produced from virus-infected cells (data not shown).

**Construction of replication-competent BAV-3 expressing GFP.** Plasmid pFBAV304 DNA (Fig. 3d) was digested with PaeRI and transfected into VIDO R2 cells to isolate recombinant virus BAV304. BAV304 was amplified in MDBK cells, and viral DNA was extracted from the infected cells. The viral DNA was analyzed by agarose gel electrophoresis after digestion with restriction enzyme BamHI. We observed a 2.3-kb DNA fragment in the BAV304 genome (Fig. 8A, lane 3) which was absent in the BAV3.E3d genome (Fig. 8B, lane 2). This suggested that the BAV304 genome contained a GFP gene, a possibility confirmed by Southern blot analysis (Fig. 8B, lane 3). A similar 2.3-kb DNA fragment was also observed in the pFBAV304 genome (Fig. 8A, lane 4) but not in pFBAV302 (lane 5). This suggests that pFBAV304 contained a GFP gene, a possibility confirmed by Southern blot analysis (Fig. 8B, lane 5).

To examine the expression of GFP, recombinant BAV304-infected cell lysates were analyzed by Western blotting using GFP-specific polyclonal antibodies (Clontech). The anti-GFP serum identified a band of 28 kDa in recombinant BAV304-infected cells (Fig. 9, lanes 1 to 3). No such band was observed in mock (lane 4)- or wild-type BAV-3 (lane 5)-infected cells. In BAV304-infected cells, GFP was detected from 12 to 36 h postinfection (lanes 1 to 3).

**Replication of BAV-3 and expression of GFP in different cell types.** To determine the host species restriction of BAV-3, 13 different cell types obtained from six different mammalian species were infected with wild-type BAV-3. BAV-3 replicated in cells of bovine origin and cotton rat lung fibroblasts. In contrast to the earlier observations (23), BAV-3 was able to replicate in cotton rat lung cells as well as or better than in bovine cells. No virus replication was apparent in cells derived from all other species, since titers were lower than those of input virus. To further characterize the host species restriction of BAV-3, we examined GFP expression in cells infected with BAV304. The greatest number of GFP-expressing cells were obtained in MDBK, VIDO R2, STR, and cotton rat lung fibroblast cultures, where as very few sheep skin fibroblasts and VIDO R1 cells showed fluorescence. Human cell lines 293 and HeLa infected with BAV304 showed mild fluorescence, while A549 and HepG2 cells showed no fluorescence (data not shown). All other virus-infected cells had low-level or no fluorescence (data not shown).

**DISCUSSION**

Human 293 (15), 911 (12), HAV-5 E1-expressing A549 (20), and PER (13) cells have been used to generate replication-defective HAVs. These cell lines are not suitable for BAV-3 infection, as they are nonpermissive for BAVs. To develop a bovine cell line for the generation of replication-defective...
BAV-3 vectors, initially MDBK cells were transfected with a plasmid, pVD-Neo, containing E1 sequences of BAV-3. A number of G418-resistant clones which supported the growth of an E1A deletion mutant of HAV-5 were selected. However, attempts to detect the E1 proteins of BAV-3 by Western blot analysis were unsuccessful. We have also not been able to generate E1 deletion mutants of BAV-3 by using this cell line. We hypothesized that this failure is due to the low transfection efficiency of MDBK cells. Consequently, we developed a second series of E1-complementing cell lines derived from FBRCs. Retina cells were chosen for the purpose because they are considered easy to transfect and transform with the E1 region of adenoviruses (13, 14). Two attempts to transform FBRCs with plasmid pVD-Neo containing the left end of the BAV-3 genome were not successful. Reasons for our failure to transform FBRCs with the E1 region of BAV-3 are unknown. However, several transformed foci were evident following the transfection of FBRCs with a plasmid, pTG4671, containing the E1 region of HAV-5 under the control of the mouse PGK promoter. The resulting transformed cells contained the integrated E1 sequences of HAV-5 in their genomic DNA as determined by PCR. These cells also produced E1A and at least one of the E1B proteins in amounts roughly as high as in 293 cells. The results of this study indicate that the diploid cultures of FBRCs could be immortalized with the E1 region of HAV-5. The resultant cell line, called VIDO R2, has been passed more than 50 times and shown to be stable with respect to retention of the E1 region, morphology, and the ability to support the replication of E1A deletion viruses.

Functional homologies between the E1A proteins of the mouse adenovirus type 1 and HAV-5 were demonstrated in transient transfection assays (2). Similarly, Zheng et al. (43) showed transactivation activity of BAV-3 E1A proteins on the E2 and E3 promoters of HAV-5 in MDBK cells coinfected with E1A-deleted HAV-5 and BAV-3. In the present study, we demonstrated that the E1A deletion mutants of HAV-5 could be grown on 6.939 cells, suggesting that E1A proteins of BAV-3 could complement those of HAV-5. Using the VIDO R2 cell line and E1A deletion mutants of BAV-3, we further demonstrated that the E1A proteins of HAV-5 could complement those of BAV-3 during viral replication. This may be explained by strong homologies noticed among the E1A proteins of adenoviruses, especially in conserved region 3 (2, 28, 43). The amino-terminal part of conserved region 3 contains a zinc finger motif required for the transactivation function, and the carboxy terminus contains a domain required for association with various transcription factors (7). Once it was established that the E1A proteins of HAV-5 could complement for those of BAV-3, two BAV-3 recombinants expressing gD of BHV-1 and HE of BCV were generated from the same cell line. These viruses grow exclusively in bovine cell lines expressing E1 proteins of either BAV-3 (6,939) or HAV-5 (VIDO R2) but not in MDBK cells and FBRCs.

The presence of replication-competent adenoviruses (RCAs) in batches of replication-defective adenoviruses is a major problem for the use of these vectors in gene therapy and is potentially a safety risk. RCAs are generated by recombination between sequences in the adenovirus vector and the matching sequences in complementing cell lines such as 293 and 911, resulting in acquisition of the E1 region by the vector (16). The RCAs, in addition to providing helper function to replication-defective vectors, will aggravate the host immune response and enhance tissue damage (19). Although problems with RCAs can be overcome by carefully designing the vectors, an advantage of the VIDO R2 cells is that they contain the E1 region of HAV-5 and do not contain sequences that are present in replication-defective BAV-based vectors. Thus, the absence of homologous sequences should completely eliminate the problem of RCA generation by homologous recombination and allow the production of safe recombinant BAV-3 vectors.

The demonstration of a mucosal immune response following mucosal delivery of recombinant HAV-5 expressing BHV-1 gD or BCV HE in cotton rats (1, 24, 25) has stimulated interest in the development of BAV-3, vector-based vaccines in cattle. Recently, we constructed replication-competent BAV-3 recombinants expressing BHV-1 gD (42) and demonstrated that intranasal immunization of calves with these recombinants induced protective immune responses against BHV-1 (41). However, these vectors have the potential to shed recombinant virus into the environment. Ideally, a recombinant vaccine antigen should be produced in the target animal without virus replication, thus making the live vectors safe by eliminating transmission of the virus to other farm animals and humans. Cell lines complementing E1A function allowed us to construct replication-defective BAV-3 recombinants that may serve as safe expression systems or nonreplicating vaccine vectors for cattle. For our initial experiments, we chose genes coding for protective antigens from BHV-1 (a virus involved in the development of a complex respiratory disease syndrome called shipping fever [33–35, 40]) and BCV (a virus involved in causing neonatal diarrhea [9, 10, 26]). The availability of these recombinant viruses expressing vaccine antigens should help in determining the potential of the replication-defective recombinant BAV-3 for delivery of vaccine antigens to mucosal surfaces of cattle.

Recombinant BAV-3 also has potential as a viral vector for somatic gene therapy in humans. In this respect, a recombinant BAV-3 expressing GFP represents an important tool for characterizing and potentially improving its properties as a vector. The GFP gene was chosen as a marker because it provides a simple and reliable method for the detection of transgene expression in cells. The observation of low levels of GFP expression in human cells even though the gene was placed under the control of a strong CMV immediate-early promoter indicates that the entry of BAV-3 to human cells is restricted. For successful somatic gene therapy, we need to develop gene transfer vectors with very high transduction efficiency. There are several ways to improve the transduction efficiency of BAV-3 into human cells, by utilizing high doses of the virus or by combining the virus with cationic liposomes. An alternative strategy is to replace the knob region of the fiber of BAV-3 with that of an HAV. This seems to be possible, as the entry of an ovine adenovirus into human cells was enhanced when the knob region of the fiber was replaced with that of HAV-5 (38). The RGD motif, which interacts with surface integrins (36) and facilitates the entry of virus into cells, is present in the penton base protein of BAV-3 but absent in HAV-5 (30). Introduction of such a motif into BAV-3 may facilitate the entry of BAV-3 into human cells.

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