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Evaluation of promoters for foreign gene expression in the E3 region of bovine adenovirus type-3☆

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Abstract

In order to optimize foreign gene expression in the E3 region of BAdV-3, we constructed full-length BAdV-3 genomic DNA clones containing a reporter gene (truncated glycoprotein gD of bovine herpesvirus 1, gDt), under the control of exogenous promoters inserted in either direction in the E3 region. Irrespective of exogenous transcriptional elements, viable recombinant BAdV-3 viruses could only be isolated when the gDt expression cassettes were inserted in the E3 region parallel to the direction of E3 transcription. Introduction of exogenous promoters altered the kinetics and amount of gDt expression in recombinant BAdV-3 infected cells. Interestingly, recombinant BAdV-3 containing gDt under the control of the mouse cytomegalovirus (MCMV) immediate early (IE) promoter expressed gDt more efficiently with noticeable differences in the amount and kinetics of expression. Moreover, animals immunized with recombinant BAdV-3 expressing gDt under the control of the MCMV IE promoter induced strong immune responses with reduced pathological lesions. These results suggest that BAdV vectors with the MCMV IE promoter may be useful for transgene expression and the development of vaccines.
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Keywords: BAdV-3; Promoters; MCMV; HCMV; IE; E3 region

1. Introduction

Genetically engineered virus genomes are being developed and used as vectors to express and deliver genes of other pathogens in vivo. One such recombinant viral system is based on human adenovirus (HAdV) (Graham and Prevec, 1992). Both replication-defective and replication-competent HAdV vectors have been engineered to express various foreign genes (Hitt and Graham, 2000). In addition to stable foreign expression, engineered adenoviruses have been shown to induce humoral, cellular and mucosal immune responses (Imler, 1995; Hitt and Graham, 2000). We have been characterizing bovine adenoviruses (BAdVs) with the aim of developing BAdV as a live viral vector for animal vaccination.

Based on the lack of virulence and the ability of the virus to grow to high titers in cell culture, we chose to develop BAdV-3 (Darbyshire et al., 1965) as a live viral vector. Moreover, as BAdV-3 is a natural pathogen of cattle, it has an inherent advantage over other available systems for gene delivery in cattle. Earlier, we determined the complete DNA sequence and transcriptional map of the BAdV-3 genome (Baxi et al., 1999; Idamakanti et al., 1999; Reddy et al., 1999b). In addition, we have constructed replication-competent (Baxi et al., 1999; Reddy et al., 2000; Zakhartchouk et al., 1998) or replication-defective (Reddy et al., 1999a) BAdV-3s, and have demonstrated the ability of the recombinant BAdV-3 vector to induce protective immune responses in calves (Zakhartchouk et al., 1999).

Deletion of the non-essential E3 region has facilitated the construction of replication-competent BAdV-3s expressing different foreign genes (Baxi et al., 2000; Reddy et al., 2000; Zakhartchouk et al., 1998). In previous studies, most of the foreign genes expressed in the E3 region have been under the control of either BAdV-3E3/MLP promoters (Baxi et al.,

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2000; Reddy et al., 2000; Zakhartchouk et al., 1998) or human cytomegalovirus (HCMV) immediate early (IE) promoter (Baxi et al., 2000; Reddy et al., 2000). Although work has been done to develop and characterize recombinant BAdV-3 vectors with inserts in E3, little has been done to study the influence of upstream and downstream flanking sequences on gene expression. Here, we introduced several cellular and viral promoter elements linked to a reporter gene (bovine herpes virus-1 [BHV-1] glycoprotein gDt) into the E3 region of BAdV-3 and compared the level of expression of gDt in vitro with the magnitude of protective immune responses induced in vivo.

2. Materials and methods

2.1. Cells and viruses

Madin–Darby bovine kidney (MDBK; American Type Culture Collection [ATCC] CCL-22) and VIDO-R2 (HAdV-5 E1-transformed fetal bovine retina cells; Reddy et al., 1999a) cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Wild-type (WBR-1 strain) BAdV-3 was cultivated in MDBK and VIDO-R2 cells. The viral DNA was extracted from virus-infected cell monolayers using the method developed by Hirt (1967).

2.2. Construction of recombinant plasmids

Plasmids constructed by standard protocols (Sambrook et al., 1989) were used to transform *Escherichia coli* DH5α by the $CaCl_2$ method. Plasmid DNA was prepared by the alkaline lysis method and purified by the Qiagene kit protocol. Different *gDt* gene expression cassettes were inserted in the E3 region of an E3-deleted BAdV-3 genome (Zakhartchouk et al., 1998).

2.2.1. Construction of plasmid pFBAV.EFgDt

A 1.1 kb *Xba*I–*BgL*II fragment of pRSV1.3 (Tikoo et al., 1993) containing the coding sequence for truncated glycoprotein D (gDt) was ligated to a *Hinc*II-digested plasmid, pEF/MYC/CYTO (invitrogen), creating plasmid pLEFgDt. A 2.8 kb *SSp*I–*Srf*I fragment isolated from plasmid pLEFgDt was ligated to *Srf*I-digested pBAV301 (E3 transfer vector; Reddy et al., 2000), creating plasmid pBAV301.EFgDt. The plasmids pFBAV.EFgDt (parallel to E3 transcription) and pFBAV.EFgDta (antiparallel to E3 transcription) were generated by homologous recombination in *E. coli* BJ5183 (Chartier et al., 1996) between *Srf*I-linearized pFBAV302 (Zakhartchouk et al., 1998) and a 8.7 kb *Eco*RV–*Spe*I fragment of pBAV.EFgDt.

2.2.2. Construction of pFBAV348

A 0.5 kb *Pst*I fragment containing mouse phosphoglycerate kinase promoter (mPGK) was excised from plasmid pTG4671 (pTG6559 (Imler et al., 1995) without the E1b^{large} mutation) and ligated to a *Pst*I-digested plasmid, pLEFgDt, to create plasmid pLPGK.gDt. A 1.9 kb *Pvu*II fragment (containing gDt under the control of the PGK promoter and bovine growth hormone [BGH] poly[A]) of plasmid pLPGK.gDt was ligated to the *Srf*I-digested plasmid pBAV301 (E3 transfer vector; Reddy et al., 2000) creating plasmid p301.PGKgDt. The recombinant plasmids pFBAV348 (parallel to E3 transcription) and pFBAV348a (antiparallel to E3 transcription) were generated by homologous recombination in *E. coli* BJ5183 (Chartier et al., 1996) between *Srf*I-digested pFBAV302 DNA and a 8.9 kb *Asc*I–*Swa*I fragment of p301.PGKgDt.

2.2.3. Construction of plasmid pFBAV349

A 550 bp fragment containing human cytomegalovirus immediate early gene promoter was amplified by PCR using primers LZP35 (5′-AACTGCAGCCGAATTCTAACTTACGG-3′) and LZP36 (5′-AACTGCAGCTTAAGCTTAGGCGATCT-3′) and plasmid QB125 DNA as a template. The PCR fragment was digested with *Pst*I and ligated to a *Pst*I-digested plasmid, LPGKgDt, creating plasmid pLHCMVgDt. A 2 kb *Pvu*II fragment containing the *gDt* gene under the control of the HCMV IE promoter and BGH poly(A) was excised from plasmid pLHCMVgDt and ligated to the *Srf*I-digested plasmid pBAV-301 (E3 transfer vector; Reddy et al., 2000) creating pBAV301.HCMVgDt. The plasmids pFBAV349 (parallel to E3 transcription) and pFBAV349a (antiparallel to E3 transcription) were generated by homologous recombination in *E. coli* BJ5183 (Chartier et al., 1996) between *Srf*I-linearized pFBAV302 (Zakhartchouk et al., 1998) and a 5.1 kb *Asc*I–*Eco*NI fragment of pBAV301.HCMCgDt.

2.2.4. Construction of plasmid pFBAV-350

A 585 bp PCR fragment containing the murine cytomegalovirus (MCMV) IE promoter was amplified by PCR plasmid pIECAS using primers LZP37 (5′-AACTGCAGCTGCAGCGAGGAGCTCTG-3′) and LZP38 (5′-AACTGCAGAGGTCAATGGGAGGTAAG-3′) and plasmid pIECAS DNA as a template. The PCR fragment was digested with *Pst*I and ligated to the *Pst*I-digested plasmid pLPGK.gDt creating pLMCMVgDt. A 2.03 kb *Pvu*II fragment containing the *gDt* gene under the control of the MCMV IE promoter and BGH poly(A) was excised from plasmid pLMCMVgDt and ligated to the *Srf*I-digested plasmid pBAV301 (E3 transfer vector; Reddy et al., 2000) creating plasmid pBAV301.MCMVgDt. The plasmids pFBAV350 (parallel to E3 transcription) and pFBAV350a (antiparallel to E3 transcription) were generated by homologous recombination in *E. coli* BJ5183 (Chartier et al., 1996) between *Srf*I-linearized plasmid pFBAV302 (Zakhartchouk et al., 1998) DNA and a 5.1 kb *ASc*I–*Eco*NI fragment of pBAV301.MCMVgDt.

2.3. Isolation of recombinant BAdV-3s

VIDO-R2 cell monolayers in 60 mm dishes were transfected with 5–10 μg of *Pac*I-digested pFBAV.EFgDt, pFBAV348, pFBAV349 and pFBAV350 DNA by lipofectin (Gibco/BRL). After 2 weeks, the transfected cells showing cytopathic effects were collected, freeze-thawed three times and the recombinant viruses were plaque-purified on and amplified on MDBK cells (Zakhartchouk et al., 1998).

2.4. Immunoprecipitation

Confluent monolayers of MDBK cells in six-well dishes were infected with the virus at a multiplicity of infection (MOI) of 10. The cells were pre-incubated for 2 h in methionine–cystine-free Dulbecco's modified Eagle's medium prior to labeling with 100 μCi of [^{35}S] methionine–cysteine. At different times post-infection the radiolabeled proteins were immunoprecipitated from the medium with a pool of anti-BHV-1 gD monoclonal antibodies (MAbs; Hughes et al., 1988) and analyzed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE). The gels were dried and protein bands were visualized by autoradiography.

2.5. Animal inoculation

A total of 60 cotton rats (4–6 weeks old) of either sex were divided into five groups (12 animals/group). After anesthetizing the animals with halothane, animals were inoculated intranasally at day 1 and at day 21 with 50 μl of inoculum containing 10^7 p.f.u. of individual recombinant virus. Blood samples were collected at days 0, 21, 35 and 39 after the primary inoculation to examine the development of BHV-1 gD-specific antibodies by enzyme-linked immunosorbent assays (ELISA) and virus neutralization (VN) assays. Three animals in each group were killed at 21 and 35 days after the primary inoculations by an overdose of halothane. The lung and nasal secretions were collected separately to monitor the development of BHV-1 gD-specific mucosal IgG and IgA antibody responses by ELISA (Zakhartchouk et al., 1998). At 36 days post-primary inoculation, animals were anesthetized with halothane and intranasally challenged with 10^7 p.f.u. of BHV-1 (Papp et al., 1997). At 39 days post-primary inoculation, the animals were killed by an overdose of halothane and the right lung was collected in MEM for virus isolation. The left lung was inflated with 10% neutral buffered formalin for histopathological examination.

2.6. ELISA

Antibodies specific for BHV-1 in sera, lung and nasal secretions were determined by ELISA as described earlier (Zakhartchouk et al., 1998). Briefly, 96-well immunol-2 microtiter plates were coated with purified truncated gD (0.01 μg/well) and incubated with different dilutions of each sample. Antigen-specific IgG was detected using biotinylated rabbit anti-rat IgG. Antigen-specific IgA was measured by rabbit anti-rat IgA and horseradish peroxidase-conjugated goat anti-rabbit IgG. The reactions were visualized using *p*-nitrophenyl phosphate di (tris) salt [PNPP].

The yield of gDt in the supernatants of recombinant BAdV-3s-infected cells was determined by indirect ELISA. Briefly, 96-well immunol-2 microtiter plates were coated either with purified gD in serial dilutions or with supernatants from control and recombinant BAdV-3-infected cells. A cocktail of gD-specific MAbs (Hughes et al., 1988) followed by alkaline phosphatase-conjugated goat anti-mouse antibody were used for detection. The reactions were visualized using *p*-nitrophenyl phosphate di (tris) salt.

2.7. Virus neutralization

Two-fold serial dilutions of heat-inactivated serum samples were incubated with 100 p.f.u. of BHV-1 for 1 h at 37 °C. The virus sample mixture was then plated onto confluent MDBK cells in 12-well tissue culture plates and incubated for 2 days in MEM containing 2% FBS and 0.7% low melting agarose. Titers were expressed as reciprocals of the highest dilution that caused 50% reduction in the number of plaques relative to the control.

2.8. Histopathology

The formalin-fixed cotton rat lungs were processed routinely and embedded in paraffin wax. Sections were cut at 5 μm and stained with haematoxylin and eosin for histological evaluation. Lesions were graded separately for the bronchioles based on the development of intranuclear inclusion bodies, and degree and extent of epithelial necrosis and inflammation; and for the alveoli based on intranuclear inclusion bodies, necrosis, neutrophils and macrophages in alveolar septa and type II pneumocyte hyperplasia. A scoring system of 0 (normal; no visible lesions); 1+ (mild; few necrotic bronchiolar epithelial cells, few alveolar macrophages); 2+ (moderate; moderate type II cell proliferation, moderate alveolar macrophages, few necrotic bronchiolar epithelial cells); 3+ (moderate to severe; high number of alveolar macrophages, moderate to severe degree of type II cell proliferation with some necrotic bronchiolar epithelial cells) and 4+ (severe; severe diffuse type II cell proliferation, moderate to high number of alveolar macrophages, moderate number of necrotic bronchiolar epithelial cells with giant cells and intranuclear inclusion bodies), based on severity of the lesions in the lower respiratory tract, was used for histopathological evaluation of the lungs.

3. Results

3.1. Construction of recombinant BAdV-3

To evaluate promoter efficiencies in the E3 region of BAdV-3, we developed a number of replication-competent

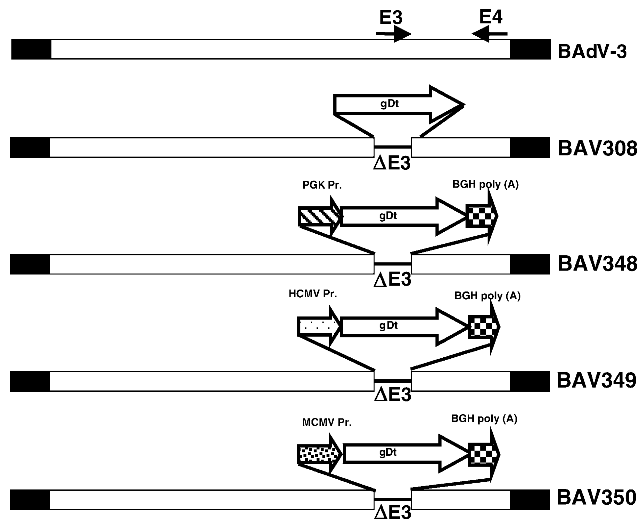

Fig. 1. Schematic representation of recombinant BAdV3s. Expression cassettes depicting gDt linked to various promoters are indicated. Mouse phosphoglycerate kinase promoter (PGK pr), Human cytomegalovirus immediate early gene promoter (HCMV pr) and mouse cytomegalovirus immediate gene early promoter (MCMV) promoter. Bovine growth hormone poly(A) [BGHpoly(A)]. BAdV-3 genome is depicted as a thick black line. Δ E3 (complete deletion of E3 region [open reading frames]).

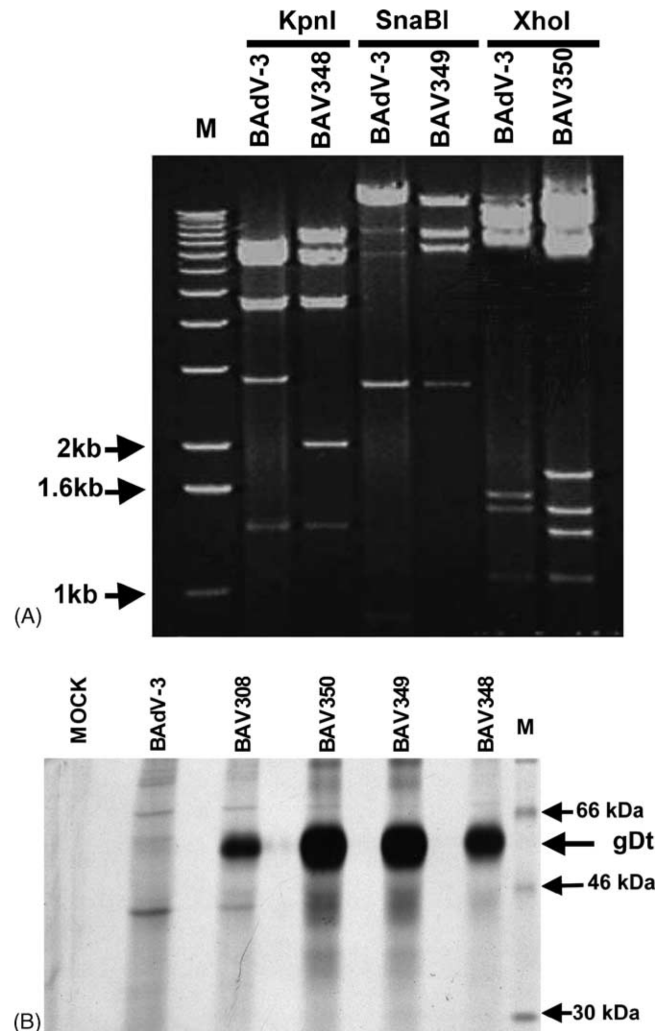

Fig. 2. Analysis of recombinant BAdV-3s. (A) Restriction enzyme analysis of recombinant BAdV-3 genome. The viral DNA was extracted from MDBK cells infected with BAdV3, BAV348, BAV349 or BAV350 by Hirt's method(1967) and digested with different restriction enzymes as shown. The 1 kb DNA ladder (*M*) from GIBCO/BRL was used for sizing the viral DNA fragments. (B) Expression of gDt in recombinant infected cells. Proteins from media of radiolabeled MDBK cells uninfected (mock) or infected with wild-type or recombinant BAdV-3s were immunoprecipitated with a pool of gD-specific MAbs and analyzed by SDS-PAGE under reducing conditions. Molecular size markers (*M*) are indicated in kDa.

BAdV-3 vectors in which gDt expression cassettes under the control of different promoters were introduced into the E3 region. By using the homologous recombination machinery of *E. coli*, the *gDt* gene under the control of different promoters, along with BGH poly(A), was inserted into the E3 region of BAdV-3 in both orientations of E3. *Pac*I-digested pFBAV348, pFBAV349 or pFBAV350 plasmid DNA was transfected into VIDO-R2 cells and produced cytopathic effects in 2 weeks. However, repeated transfection of VIDO-R2 cells with individual *Pac*I-digested pFBAV.EFgDt, pFBAV.EFgDta, pFBAV349a, pFBAV349a or pFBAV350a plasmid DNA did not produce any cytopathic effects. The transfected cell monolayers were collected, freeze-thawed and recombinant viruses were plaque-purified and propagated in MDBK cells. The recombinant BAdV-3s were named BAV348 (mPGK promoter), BAV349 (HCMV IE promoter) and BAV350 (MCMV IE promoter) (Fig. 1). The viral DNA was extracted from virus-infected cells by the Hirt extraction method, 1967 and analyzed by agarose gel electrophoresis after digestion with restriction enzyme. Since BAV348 contains an additional *Kpn*I site in the mPGK promoter sequence, the viral DNAs were digested with *Kpn*I. As seen in Fig. 2A, compared with wild-type BAdV-3, the BAV348 genome contains an additional expected band of 2 kb. As BAV349 contains an additional *Sna*BI site in the HCMV IE promoter sequence, the viral DNAs were digested with *Sna*BI. As seen in Fig. 2A, compared to wild-type BAdV-3, the BAV349 genome contains expected bands of 8.5 and 7.5 kb. As BAV350 contains an additional *Xho*I site in MCMV IE promoter sequence, the viral DNAs were digested with *Xho*I. As seen in Fig. 2A, compared to wild-type BAdV-3, the BAV350 genome contains additional expected bands of 1.6 and 1.3 kb bands. The construction and characterization of BAV308 has been described earlier (Zakhartchouk et al., 1998). The BAV308 contains the *gDt* gene under the control of the BAdV-3E3/major late promoter inserted in the E3 region (parallel to E3 transcription) of the E3-deleted BAdV-3 genome (Zakhartchouk et al., 1998).

3.2. Detection of truncated gD expression in MDBK cells

To examine gDt expression by recombinant BAdV-3s, MDBK cells were infected at an MOI of 10 p.f.u.

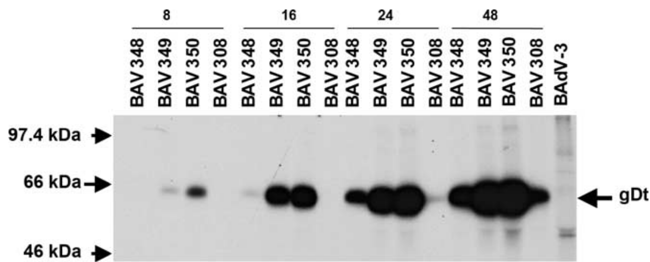

Fig. 3. Kinetics of gDt expression. Proteins from media of radiolabeled MDBK cells infected with recombinant or wild-type BAdV-3 collected at different times post-infection were immunoprecipitated with a pool of gD-specific MAbs and analyzed by SDS-PAGE under reducing conditions. Size of the marker proteins is depicted to the left of the panel.

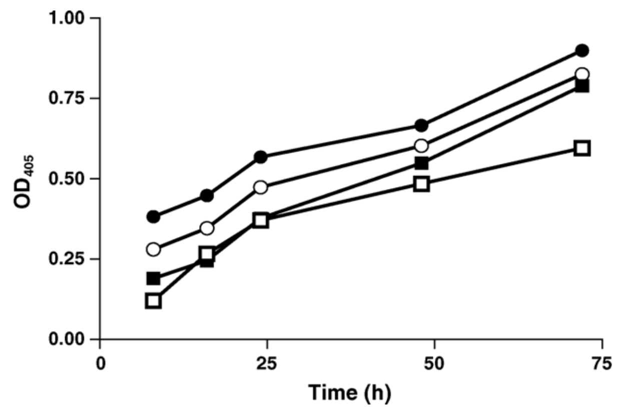

Fig. 4. Analysis of gDt expression by ELISA. Media from MDBK cells infected with wild-type or recombinant BAdV-3s were collected at different times post-infection and analyzed by ELISA as described in the text. BAV308 (□), BAV348 (■), BAV349 (○), BAV350 (●). The media from wild-type MDBK cells showed an OD_{405} of 0.05–0.08.

with recombinant BAV308, BAV348, BAV349, BAV350 or wild-type BAdV-3 and metabolically labeled with [^{35}S] methionine–cysteine. The medium of the infected cells was harvested and the radiolabeled proteins were immunoprecipitated with a pool of gD-specific monoclonal antibodies and analyzed by SDS-PAGE under reducing conditions. As seen in Fig. 2B, the immunoprecipitation revealed a major band of 61 kDa in supernatants of cells infected with BAV348, BAV349 or BAV-350, which co-migrated with gDt produced in BAV308-infected cell supernatants. No corresponding protein could be detected in supernatants of mock or wild-type BAdV-3-infected cells.

To determine the kinetics of gDt expression, the cells infected with recombinant BAdV-3s were labeled with [^{35}S] methionine–cysteine. At different times post-infection, the cell supernatants were collected and the radiolabeled proteins were immunoprecipitated using gD-specific MAbs. As seen in Fig. 3, expression from the MCMV IE promoter in BAV350 was detected as early as 8 h and increased steadily until 48 h post-infection. Expression from the HCMV IE promoter in BAV349 was barely detectable at 8 h post-infection; however, efficient expression could be detected at 16, 24 and 48 h post-infection. Expression from the mPGK promoter in BAV348 was barely detectable at 16 h post-infection; however, reasonable expression could be detected at 24 and 48 h post-infection. Expression from the E3/MLP promoter in BAV308 was barely detectable at 24 h post-infection and was reasonably low at 48 h post-infection.

To determine the level of gDt expression at different times post-infection, quantitative ELISA was performed. The amount of gDt produced was extrapolated from a standard curve developed using known quantities of purified gDt. As seen in Fig. 4, the MCMV IE promoter in BAV350 consistently produced higher levels of gDt in BAV350-infected cells than the HCMV IE promoter in BAV349 or the mPGK promoter in BAV348-infected cells. However, there was a significant difference in the amount of gDt produced in cells infected with recombinant BAdV-3 expressing gDt under E3/MLP versus exogenous promoters.

3.3. Antibody response after immunization

To determine if recombinant BAdV-3 expressing gDt could produce an immune response in animals, cotton rats were immunized intranasally twice, 3 weeks apart. Serum samples collected at days 0, 21, 35 and 39 were tested for gD-specific IgG antibodies and BHV-1 neutralizing antibodies. As seen in Fig. 5A, animals immunized with BAV308, BAV348, BAV349 or BAV350 showed significant levels ($P < 0.05$) of gD-specific IgG titers after primary immunization versus the response induced in animals immunized with BAV302 (control). After the second immunization, all vaccinated cotton rats showed increases in gD-specific titers, whereas control animals did not. Similarly, animals immunized with BAV308, BAV348, BAV349 or BAV350 developed significantly higher ($P < 0.05$) BHV-1 neutralizing antibody titers after primary immunization than control animals immunized with BAV302 (Fig. 5C). After the second immunization, all vaccinated animals showed an increase in BHV-1 neutralizing antibody titers versus control animals. However, there was no significant difference in neutralizing antibody titers observed in animals immunized with BAV308, BAV348, BAV349 or BAV350.

The lung and nasal washes collected at days 0, 21, 35 and 39 were analyzed for gD-specific IgA antibodies. After primary immunization, animals immunized with BAV348, BAV349 or BAV350 showed gD-specific IgA titers in lung (Fig. 5B) and nasal washes (Fig. 5D). After the second immunization, all vaccinated animals had significantly ($P < 0.05$) higher levels of gD-specific IgA than control animals. Although there was no significant difference in the level of IgA response induced in animals immunized with different recombinant BAdV-3s expressing gDt, the animals immunized with BAV350 consistently showed higher IgA responses.

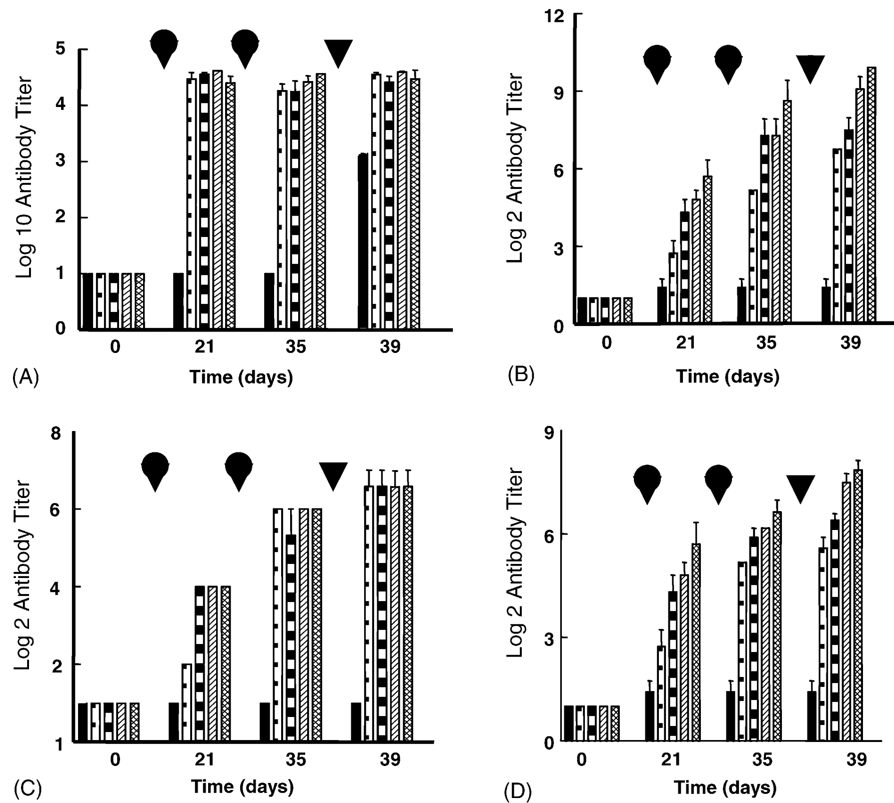

Fig. 5. Antibody response in serum and nasal secretions of calves. Cotton rats were immunized intranasally with BAdV-3 (■), BAV308 (⚃), BAV348 (▤), BAV349 (▨) or BAV350 (⊠). Arrows indicate the time points of immunization on days 0 and 21 and subsequent challenge with BHV-1 at day 35 post-primary immunization. (A) BHV-1 gD-specific IgG ELISA titers in sera. (B) BHV-1 gD-specific IgA ELISA titers in the lung secretions. (C) Virus neutralization antibody titers in sera (SN), expressed as 50% endpoint using 100 p.f.u. BHV-1. (D) BHV-1 gD-specific IgA ELISA titers in nasal secretions.

3.4. Protection from challenge with BHV-1

After immunization of cotton rats with various BAdV-3 recombinants expressing gDt-induced gD-specific immune responses, the animals were challenged with BHV-1 to evaluate the effectiveness of the immune response in protection from infection. Fourteen days after the second immunization, animals were challenged with 10^7 p.f.u. of BHV-1. The animals immunized with BAV308, BAV348, BAV349 or BAV350 showed 1–2.6 log and 0.7–2.3 log-reductions in the virus titers recovered from the lungs compared to PBS and BAV302-immunized animals, respectively (Table 1). Lungs of cotton rats immunized with BAdV302 or PBS had severe lesions of interstitial pneumonia with type II pneumocyte proliferation and infiltration of macrophages. In contrast, lungs of cotton rats immunized with BAV308, BAV348, BAV349 or BAV350 did not have severe lesions of interstitial pneumonia (Table 1).

Table 1
Effect of immunization with recombinant BAdV-3s on protection of cotton rats against intranasal BHV-1challenge

Immunization	Virus isolation (log p.f.u./g lung tissue)	Interstitial pneumonia	Pathological score
BAV308	5.5 ± 0.3	No	3.0
BAV348	4.9 ± 0.4	No	2.5
BAV349	4.7 ± 0.5	No	2.0
BAV350	3.9 ± 0.2	No	1.0
BAV302	6.2 ± 0.3	Yes	4.0
PBS	6.5 ± 0.2	Yes	4.0

4. Discussion

Recombinant adenoviruses represent one of the most efficient vector systems for delivery of vaccine antigens to mucosal surfaces. As such, we have been developing bovine adenovirus-3 as a vaccine delivery vector for cattle. Earlier, we developed an E3-deleted BAdV-3 vector expressing vaccine antigen(s) (Zakhartchouk et al., 1998) and demonstrated the potential of using BAdV-3 vector(s) in inducing protective immune responses in cattle (Zakhartchouk et al., 1999). Although work has been done to characterize recombinant BAdV-3 vectors with inserts in E3, little has been done to study the importance of upstream flanking sequences on gene expression. In this report, we examined the effect of exogenous transcriptional control elements on the level of expression of the BHV-1 *gDt* gene in E3-deleted BAdV-3 vectors in vitro and the magnitude of antigen-specific immune responses induced in vivo.

Earlier, viable recombinant HAdV-5s expressing foreign gene(s) inserted in the E3 region (either orientation relative to E3 transcription) have been isolated (Both et al., 1993; Schneider et al., 1989). Moreover, expression of the transgene has been shown to be affected by orientation of the inserted expression cassette relative to the E3 transcription (Both et al., 1993; Schneider et al., 1989). However, irrespective of the type of exogenous transcriptional elements used, we could not isolate a recombinant BAdV-3 expressing the *gDt* gene inserted in the E3 region anti-parallel to the direction of E3 transcription. This may be due, in part, to the differences in the E3 transcription pattern of HAdV-5 and BAdV-3 (Idamakanti et al., 1999; Reddy et al., 1998). Since E3 and L6 region transcripts are 3′ coterminal (Idamakanti et al., 1999; Reddy et al., 1998), it is conceivable that insertion of the foreign gene cassette in the E3 region antiparallel to E3 transcription may adversely affect the transcription of *L6* genes.

Using BHV-1 gDt as a reporter, we have assessed the promoter activity of mouse PGK, HCMV, MCMV and E3/MLP in mDBK cells which are permissive for BAdV-3 replication. One of the most frequently used promoters for transgene expression is that of human cytomegalovirus immediate early gene, which directs high levels of transgene expression in a wide variety of cells (Addison et al., 1997; Ambriovic et al., 1997; Schmidt et al., 1990). Interestingly, our data suggest that BAdV-3 vector containing the MCMV promoter express gDt more efficiently, with noticeable difference in the kinetics of expression versus BAdV-3-specific or other exogenous promoters. Earlier, comparison of HCMV and MCMV IE promoters for transgene expression in the E1 region of HAdV-5 also suggested that the MCMV IE promoter has a much wider host range and drives high levels of gene expression in vitro and in vivo (Addison et al., 1997; Appleby et al., 2003).

Although BAdV-3s differing only in promoters used to drive the transcription of gDt provided protection against development of interstitial pneumonia, they differed strongly in the induction of immune responses, virus shedding and the severity of the pathological lesions. The BAV350 in which gDt transcription was driven by the MCMV IE promoter was consistently most effective in the induction of a strong mucosal antibody response, which resulted in reduced virus shedding and development of pathological lesions.

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