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Construction and Characterization of E3-Deleted Bovine Adenovirus Type 3 Expressing Full-Length and Truncated Form of Bovine Herpesvirus Type 1 Glycoprotein gD[1]

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Using the homologous recombination machinery of *E. coli,* a 1.245-kb deletion was introduced in the E3 region of bovine adenovirus 3 (BAV3) genomic DNA cloned in a plasmid. Transfection of the restriction enzyme-excised, linear E3-deleted BAV3 genomic DNA into primary fetal bovine retina cells produced infectious virus (BAV3.E3d), suggesting that all the E3-specific open reading frames are nonessential for virus replication *in vitro*. Using a similar approach, we constructed replication-competent (BAV3.E3gD and BAV3.E3gDt) BAV3 recombinant expressing full-length (gD) or truncated (gDt) glycoprotein of bovine herpes virus 1. Recombinant gD and gDt proteins expressed by BAV3.E3gD and BAV3.E3gDt, respectively, were recognized by gD-specific monoclonal antibodies directed against conformational epitopes, suggesting that antigenicity of recombinant gD and gDt was similar to that of the native gD expressed in bovine herpes virus 1-infected cells. Intranasal immunization of cotton rats induced strong gD- and BAV3-specific IgA and IgG immune responses. These results suggest that replication-competent bovine adenovirus 3-based vectors have potential for the delivery of vaccine antigens to the mucosal surfaces of animals.

INTRODUCTION

Vaccination has proved to be the most effective means of controlling respiratory and enteric viral diseases, especially when live attenuated viral vaccines have been used. These vaccines, when administered orally or intranasally, induce a strong mucosal immunity, which is required to block the initial infection and to reduce the development of disease caused by these viruses. This approach has been extended by using genetically engineered virus genomes (virulence gene deleted) as vectors to express and deliver genes of other pathogens *in vivo* (Ertl and Xiang, 1996). One such recombinant viral vector system recently developed is based on human adenoviruses (HAVs) (Graham and Prevec, 1992). Both replication-defective and replication-competent HAV vectors have been engineered to express various foreign antigens (for reviews, see Grunhaus and Horwitz, 1992; Imler, 1995). In addition to stable foreign gene expression, engineered adenoviruses have been shown to induce humoral, cellular, and mucosal immune responses (Buge *et al.,* 1997).

We have been carrying out the molecular characterization of bovine adenovirus 3 (BAV3) with the aim of developing BAV3 as a live viral vector for animal vaccines and human gene therapy. BAV3, a representative of subgroup I of BAVs (Bartha, 1969), has been shown to replicate in the respiratory tract of cattle, producing mild or no clinical symptoms (Darbyshire *et al.,* 1965). Like other adenoviruses, BAV3 is a nonenveloped icosahedral particle of 75-nm diameter (Niiyama *et al.,* 1975) containing a linear double-stranded DNA molecule. Recently, the complete DNA sequence and transcriptional map of BAV3 genome were reported (Baxi *et al.,* 1998; Lee *et al.,* 1998; Reddy *et al.,* 1998). Although the size (34,446 bp) and the overall organization of the BAV3 genome appear to be similar to those of HAVs, there are certain differences (Reddy *et al.,* 1998). One of the distinctive features of BAV3 genome is the relatively small (1517 bp) size of the E3 coding region (Mittal *et al.,* 1993; Reddy *et al.,* 1998). Analysis of the sequence of the E3 region genome and RNA transcripts (Idamakanti, 1998) suggests that BAV3 E3 region may code at least four proteins, one (121R) of which shows limited homology to 14.7-kDa protein of HAV5 (Mittal *et al.,* 1993).

In this report, we describe an easy and efficient method of constructing an E3-deleted recombinant BAV3. In addition, we describe the construction of recombinant BAV3 expressing different forms of bovine herpes virus 1

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(BHV-1) glycoprotein gD and show that intranasal immunization of cotton rats leads to the induction of a gD-specific mucosal and systemic immune response.

RESULTS

Construction of E3-deleted recombinant BAV3

Initially, it was assumed that the role of BAV3 E3 in virus replication would be similar to the that of E3 region of HAV (Wold and Gooding, 1991). Therefore, the E3-based vectors were constructed by making deletions of the E3 sequences. However, attempts to isolate an E3-deleted BAV3 recombinant in different bovine cell lines, including Madin–Darby bovine kidney (MDBK), were unsuccessful. A partially deleted BAV3 (BAV3-Luc) recombinant expressing luciferase gene could only be isolated when BAV3 E1-transformed MDBK cells were used for transfection (Mittal *et al.,* 1995). However, this recombinant BAV3 could be propagated on normal bovine cells, suggesting that E3 region of BAV3 was not essential for virus replication *in vitro* (Mittal *et al.,* 1995). To increase the efficiency of isolating a BAV3 recombinant, we used primary fetal bovine retina (PFBR) cells and a novel procedure (Degryse, 1996) for generating BAV3 recombinants. Using this method, targeted modifications of the viral genome were introduced into a plasmid using the highly efficient homologous recombination machinery of *Escherichia coli.* The infectious virions are isolated after transfection of the adenovirus genome, excised by *Pac*I digestion from the plasmid vector by restriction endonuclease digestion, into appropriate host cells (Chartier *et al.,* 1996).

Taking advantage of the homologous recombination machinery of *E. coli,* we constructed a plasmid (pFBAV302) that contained a 1.245-kb deletion (nucleotides 26,456–27,701) and an *Srf*I restriction enzyme site (Fig. 1). The *Pac*I digested pFBAV302 DNA when transfected into PFBR cells produced cytopathic effects in 14 days. The virus named BAV3.E3d was plaque purified and expanded in MDBK cells. The viral DNA was extracted and analyzed by agarose gel electrophoresis after digestion with *Bam*HI restriction enzyme. The wild-type BAV3 had a *Bam*HI fragment of 3.019 kb (Fig. 2, lane b) that was missing in the recombinant BAV3.E3d genome, which instead had a fragment of 1.774 kb (Fig. 2, lane a). This is consistent with the expected *Bam*HI fragment sizes for the wild-type and deletion mutants, thus confirming that we had isolated an E3-deleted recombinant BAV3. Comparison of the growth characteristics of recombinant virus with the wild-type BAV3 suggested no significant differences in the plaque size or replication in, as E3-deleted recombinant replicated with similar kinetics as wild-type BAV3 (data not shown).

Construction of recombinant BAV3 expressing glycoprotein gD

To determine the usefulness of E3-deleted (replication-competent) BAV3 recombinants as delivery vehicles for live recombinant vaccine antigens, we constructed recombinant BAV3 expressing different forms of BHV-1 glycoprotein gD (Tikoo *et al.,* 1993). The full-length and truncated form of gD genes (devoid of any exogenous promoter) were inserted individually into the E3 region of the BAV3.E3d genome in the same transcriptional orientation of E3, using the homologous recombination machinery of *E. coli* (Degryse, 1996). The *Pac*I-digested pFBAV302.gD or pFBAV302.gDt plasmid DNA was transfected into PFBR cells and produced cytopathic effects in 14 days. The infected cell monolayers showing 50% cytopathic effects were collected and freeze–thawed, and recombinant viruses were plaque purified and propagated in MDBK cells. The recombinant BAV3s were named BAV3.E3gD (full-length gD) and BAV3.E3gDt (truncated gD). The viral DNA was extracted and analyzed by agarose gel electrophoresis after digestion with different restriction enzymes. Because the gD gene contains a unique *Nde*I site (Tikoo *et al.,* 1990), the recombinant viral DNA was cut with *Nde*I. As seen in Fig. 2, compared with the BAV3.E3d (Fig. 2, lane e), the BAV3.E3gD (Fig. 2, lane f) or BAV3.E3gDt (Fig. 2, lane g) genomes contain an additional expected band of 4.6 kb, suggesting that recombinant BAV3.E3gD and BAV3.gDt contained gD or gDt genes in the E3 region. To differentiate between the gD and gDt gene, the recombinant viral DNAs were digested with *Nhe*I because the gDt, but not the gD, gene contains a unique *Nhe*I restriction enzyme site (Tikoo *et al.,* 1993). As expected, the 5.4-kb BAV3.E3gD DNA fragment (Fig. 2, lane c) was replaced with a 5.0-kb fragment in BAV3.E3gDt (Fig. 2, lane d). This suggested that recombinant BAV3.E3gD and BAV3.E3gDt contained gD and gDt genes, respectively. A comparison of the growth characteristics of these recombinants with wild-type or E3-deleted BAV3 showed no significant differences both in the kinetics of replication and in the titer of virus produced (data not shown).

Analysis of expression of gD by BAV3.E3gD and BAV3.E3gDt

To examine the product or products expressed by recombinant BAV3 containing the BHV-1 gD or gDt gene, MDBK cells were infected with recombinant BAV3.E3gD, BAV3.E3gDt, or BAV3.E3d and metabolically labeled with [^{35}S]methionine-cysteine for different time periods. For comparison with authentic gD, MDBK cells were infected with BHV-1 and labeled similarly with [^{35}S]methionine-cysteine. The radiolabeled proteins

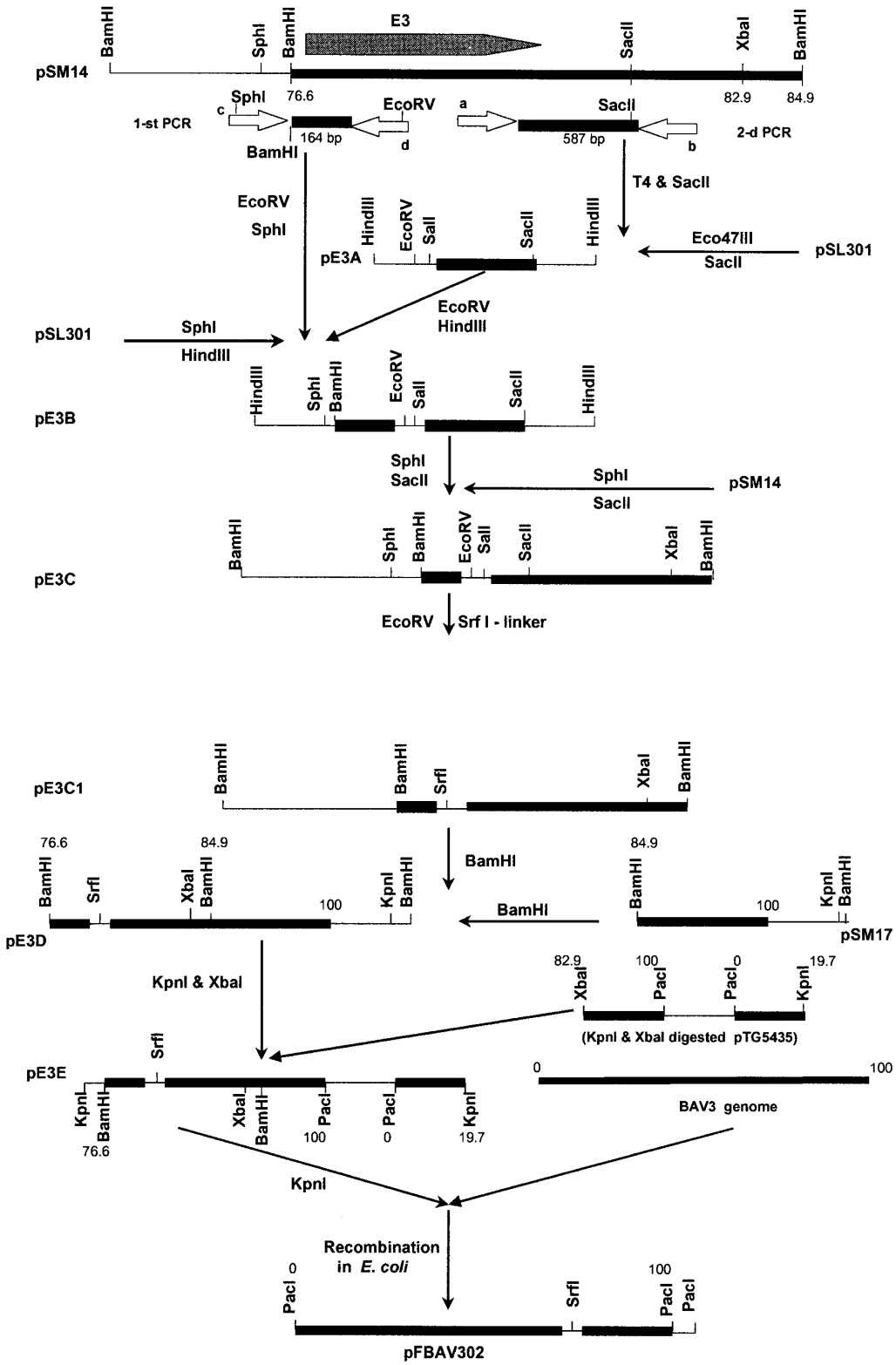

FIG. 1 Construction of a plasmid containing E3-deleted BAV3 genomic DNA. The plasmid pFBAV302 was constructed from different genomic clones as described in the text. The origin of the DNA sequences is as follows: plasmid DNA, thin line; BAV3 genomic DNA sequences, thick line. (Hollow arrows) PCR primers: a, ACGCGTCGACTCCTCCTCA; b, TTGACAGCTAGCTTGTTG; c, CCAAGCTTGCATGCCTG; and d, GGCGATATCTCAGCTATAAC-CGCTC. (The plasmid maps are not drawn to scale.)

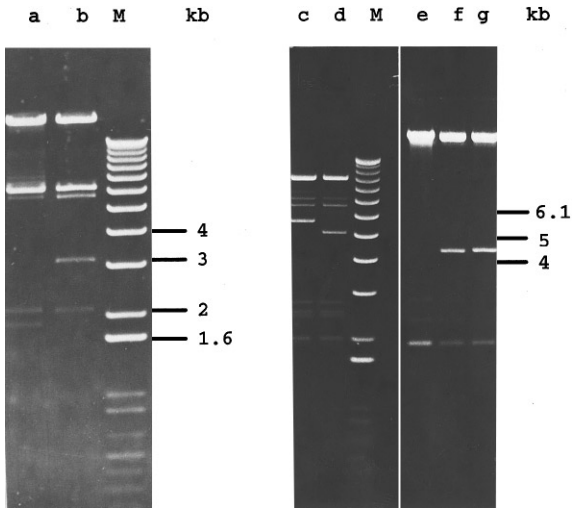

FIG. 2. Restriction enzyme analysis of recombinant BAV3 genomes. The DNAs were extracted from the BAV3- (lane b), BAV3.E3d- (lanes a and e), BAV3.E3gD- (lanes c and f), and BAV3.gDt- (lanes d and g) infected MDBK cells by Hirt's method (Hirt, 1967) and digested with *Bam*HI (lanes a and b), *Nhe*I (lanes c and d), and *Nde*I (lanes e–g). The 1-kb-plus DNA ladder (M) from GIBCO BRL was used for sizing the viral DNA fragments. The sizes of some marker bands are indicated by arrows.

were immunoprecipitated with a pool of gD-specific monoclonal antibodies (MAbs) (Hughes *et al.,* 1988) and analyzed by SDS–PAGE under reducing conditions. The immunoprecipitation of recombinant BAV3.E3gD-infected cells revealed a major band of ~71 kDa (Fig. 3A, lanes b–d), which comigrated with the gD protein produced in BHV-1-infected cells (Fig. 3A, lane a) suggesting that the recombinant gD contained posttranslational modifications similar to authentic gD. No similar band was observed in uninfected cells (Fig. 3A, lane h) or cells infected with recombinant BAV3.E3d (Fig. 3A, lanes e–g). Radioimmunoprecipitation of recombinant BAV3.E3gDt-infected cell supernatants revealed a major band of 61 kDa (Fig. 3B, lanes b–d). No similar band was observed in the supernatants of cells infected with BAV3.E3d (Fig. 3B, lanes e–g). Both recombinant proteins were expressed throughout the infection of MDBK cells (Fig. 3).

To test the antigenicity of recombinant gD proteins, radiolabeled proteins were immunoprecipitated from recombinant BAV3 infected cell lysate (BAV3.E3gD) or supernatant (BAV3.E3gDt) with gD-specific MAbs (Hughes *et al.,* 1988) and analyzed by SDS–PAGE under reducing conditions. As shown in Fig. 4, both gD (lanes 1–e) and gDt (lanes f–j) proteins were recognized by MAbs directed against discontinuous epitopes Ib (MAb 136; lanes a and f), II (MAb 3E7; lanes b and g), IIIb (MAb 4C1; lanes c and h) IIIC (MAb 2C8; lanes d and i), and IIId (MAb 3C1; lanes e and j). These results suggests that the antigenic structures of recombinant proteins gD and gDt are similar to that of authentic gD produced in MDBK cells (Tikoo *et al.,* 1993).

To determine whether gD expression occurred in the absence of DNA synthesis, we compared the amount of gD produced in BAV3.E3gD-infected MDBK

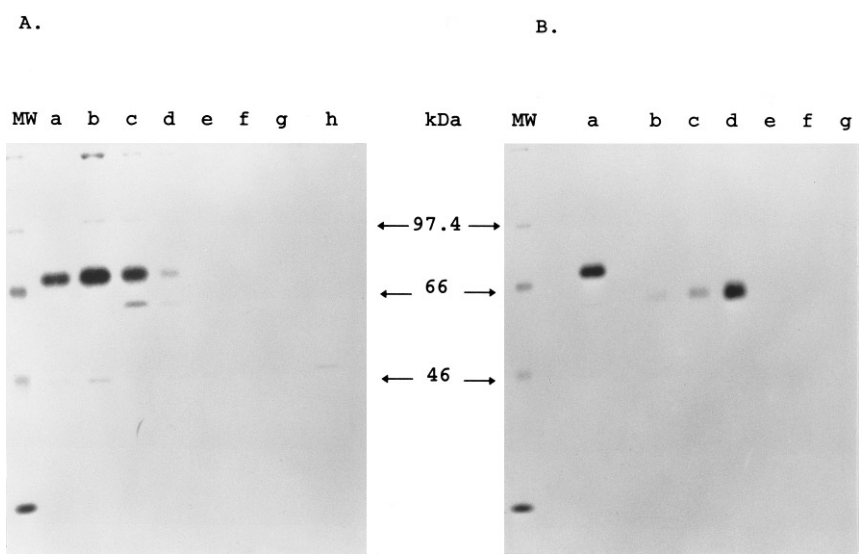

FIG. 3. Expression of gD proteins in MDBK cells infected with recombinant BAV3 viruses. (A) Proteins from lysates of radiolabelled MDBK cells uninfected (lane h) or infected with BHV-1 (lane a), BAV3.E3d (lanes e–g), or BAV3.E3gD (lanes b–d) were immunoprecipitated with a pool of gD-specific MAbs and analyzed by SDS–PAGE under reducing conditions. Proteins were labeled as 6–16 h (lanes a and h), 36–48 h (lanes b and e), 48–50 h (lanes c and f), and 60–62 h (lanes d and g) p.i. (B) Proteins from culture medium of radiolabeled MDBK cells infected with BHV-1 (lane a), BAV3.E3d (lanes e–g), or BAV3.E3gDt (lanes b–d) were immunoprecipitated with a pool of gD-specific MAbs and analyzed by SDS–PAGE under reducing conditions. Proteins were labeled as 6–16 h (lane a), 12–14 h (lanes b and e), 16–18 h (lanes c and f), and 22–26 h (lanes d and g) p.i. Molecular size markers (MW) are given in kDa.

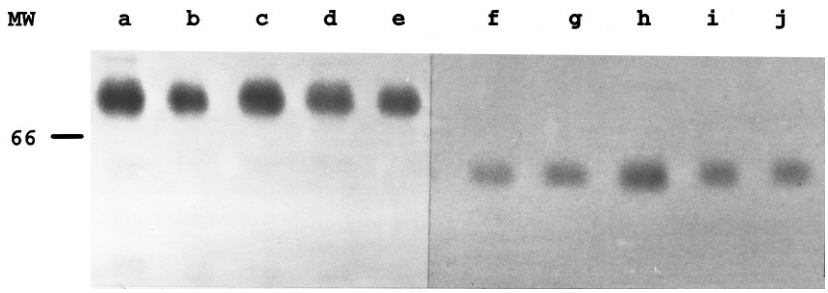

FIG. 4. Antigenic analysis of recombinant gD and gDt proteins. Proteins from lysates (lanes a–e) and culture medium (lanes f–j) of radiolabeled MDBK cells infected with BAV3.E3gD (lanes a–e) or BAV3.E3gDt (f–j) recombinant viruses were immunoprecipitated with MAb 136 (lanes a and f), MAb 3E7 (lanes b and g), MAb 4C1 (lanes c and h), MAb 2C8 (lanes d and i), and MAb 3C1 (lanes e and j) and analyzed by SDS–PAGE under reducing conditions. Molecular size marker (MW) is given in kDa.

cells in the presence (Fig. 5, lanes a and b) and absence (Fig. 5, lanes c and d) of an inhibitor of DNA synthesis, 1-β-D-arabinofuranosylcytosine (AraC). The results suggest that gD expression was reduced in the presence of AraC.

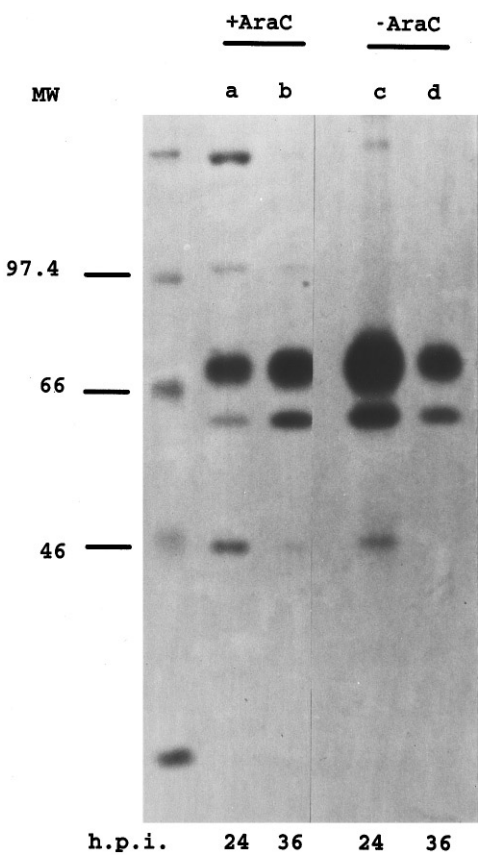

FIG. 5. Effect of AraC on gD expression in MDBK cells. Proteins from lysates of MDBK cells infected with BAV3.E3gD (lanes a–d) in the presence (lanes a and b) or absence (lanes c and d) of 100 μl/ml AraC and radiolabeled for 2 h at 22 h (lanes a and c) or 34 h (lanes b and d) p.i. were immunoprecipitated with a pool of gD MAbs and analyzed by SDS–PAGE. Molecular size markers (MW) in kDa.

Antibody responses in animals

To determine the ability of BAV3 recombinants to induce gD-specific immune responses, cotton rats were inoculated twice intranasally, 3 weeks apart, with 10^7 PFU of BAV3.E3gD, BAV3.E3gDt, or BAV3.E3d recombinants. Serum, lung washes, and nasal washes were collected for the analysis of IgG and IgA antibodies, whereas lungs were collected for analyzing the number of IgA antibody-secreting cells (ASC). Both BAV3.E3gD and BAV3.E3gDt induced gD-specific IgG antibody response (Fig. 6B) in the serum and lung washes, which was significantly higher ($P < 0.05$) than the response induced in BAV3.E3d-immunized animals (control). However, gD-specific IgG response in the serum induced by BAV3.E3gDt was higher than the response induced by BAV3.E3gD ($P < 0.05$). No gD-specific IgG was detected in the nasal washes (Fig. 6B).

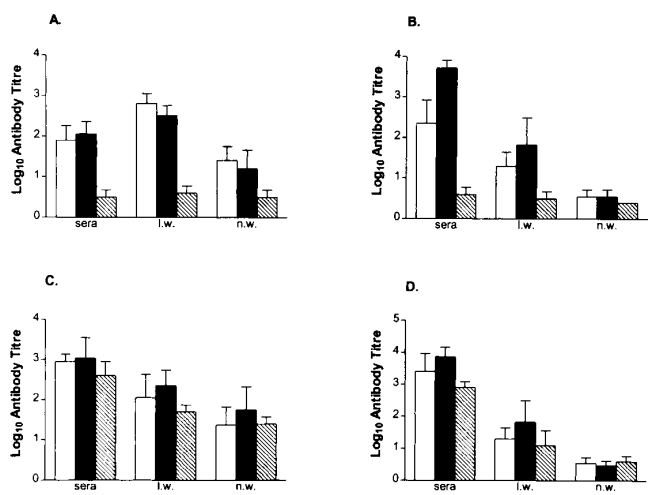

FIG. 6. Antibody responses in cotton rats. Glycoprotein gD- (A and B) or BAV3- (C and D) specific IgA (A and C) or IgG (B and D) ELISA titers in sera, lung washes (l.w), and nasal washes (n.w) 12 days after secondary immunization with recombinant BAV3.E3d (stipled bar), BAV3.E3gD (open bar), and BAV3.E3gDt (filled bar). Error bars represent the standard error of the mean of four animals per group.

TABLE 1

Frequency of gD- and BAV3-Specific Antibody-Secreting Cells in the Lungs of Cotton Rats Immunized Intranasally with Recombinant Adenoviruses

	IgA	
Immunization	gD-specific ASC/10^6 cells	BAV3-specific ASC/10^6 cells
After first immunization		
BAV3.E3gD	12	4
BAV3.E3gDt	4	4
After second immunization		
BAV3.E3d	6	50
BAV3.E3gD	30	58
BAV3.E3gDt	22	40

Immunization with BAV3.E3gD and BAV3.E3gDt induced significantly higher ($P < 0.05$) IgA antibody responses to gD in the serum and lung washes than immunization with BAV3.E3d (Fig. 6A). However, there was no significant difference in the IgA antibody response between BAV3.E3gD- and the BAV3.E3gDt-immunized groups. These recombinants also induced a BAV3-specific IgG antibody response (Fig. 6D) in the serum and lung washes and IgA antibody response (Fig. 6C) in serum, lung washes, and nasal washes, which was not significant different among the groups.

Interestingly, nasal washes contained only IgA antibodies specific for gD (Figs. 6A and 6B) or BAV3 (Figs. 6C and 6D). In addition, IgA ASC specific for both gD and BAV3 could be detected in the lung of immunized animals, the number of which increased significantly after booster immunization (Table 1).

To measure the biological activity of the gD specific serum antibody, anti-BHV-1 titers were determined. Immunization with BAV3.E3gDt induced a BHV-1 $\log_2$ titer of 4.3 ± 0.5, which was significantly higher ($P < 0.05$) than the titer of 3.0 ± 0.6 and 0.8 ± 0.3 induced by BAV3.E3gD and BAV3.E3d, respectively.

DISCUSSION

Currently, recombinant human adenoviruses represent one of the most efficient vector systems for delivery of vaccine antigens to the mucosal surfaces. However, use of human adenoviruses as a vaccine delivery system in domestic animals is limited. Because nonhuman adenoviruses are species specific, the development of animal specific adenovirus as a vaccine delivery system would be a logical choice. In this report, we describe the development of a replication-competent (E3-deleted) recombinant BAV3 for use in the delivery of vaccine antigens to the mucosal surfaces of animals. In addition, we constructed replication competent BAV3-expressing BHV-1 gD or gDt glycoprotein and tested their ability to induce mucosal and systemic immune responses in cotton rats.

Initial attempts to isolate an E3-deleted BAV3 recombinant in different bovine cell lines, which support the formation of infectious progeny after wild-type BAV3 DNA-mediated transfection, were unsuccessful. However, an E3 (partial)-deleted BAV3 recombinant-expressing luciferase gene was isolated when a BAV3 E1-transformed MDBK cell line was used for transfection (Mittal *et al.,* 1995). This suggested that E1 region proteins may be required for the isolation and propagation of E3-deleted BAV3. However, recombinant BAV3-expressing luciferase gene in E3 region replicated efficiently in normal MDBK cells, suggesting that BAV3 E1 proteins are not required for the replication of E3-deleted BAV3 (Mittal *et al.,* 1995). Alternatively, the normal MDBK cells may not be efficient for generating recombinant BAV3. To develop a reliable and efficient method of isolating replication-competent BAV3 recombinants without using BAV3 E1-transformed MDBK cells, we first used the homologous recombination machinery of *E. coli* (Chartier *et al.,* 1996; Degryse, 1996) to introduce a 1.245-kb deletion into the E3 region of full-length BAV3 genome cloned in a plasmid. Second, for isolating the recombinant virus, we used PFBR cells instead of MDBK cells for transfection of the modified BAV3 genome, excised from the plasmid.

It has been reported that up to 105% of the wild-type genome can be packaged into the HAV5 virion without causing any rearrangements or deletion of the foreign genes in subsequent rounds of replication (Bett *et al.,* 1993). Recently, it was reported that the insertion capacity of the ovine adenovirus (OAV) vector is 114% of the wild-type genome (Xu *et al.,* 1997). Because the size of BAV3 genome is similar to that of the HAV5 genome, we believe that the insertion capacity of BAV3 genome should be similar to that of HAV5. Therefore, because the BAV3.E3D vector has a 1.245-kb E3 deletion, the insertion capacity of our present BAV3 vector should be ~3.0 kb.

Earlier, it was reported that recombinant BAV3.luc containing a 0.696-kb deletion in the E3 region replicated less efficiently than the wild type in cell culture (Mittal *et al.,* 1995). In contrast, the recombinant BAV3.E3d containing a 1.245-kb deletion in the E3 region replicated as efficiently as the wild-type BAV3 in cell culture. This difference may be due to the insertion of the luciferase gene in recombinant BAV3.luc (Mittal *et al.,* 1995), which may affect the expression of genes downstream of the E3 region (Mittal *et al.,* 1995). However, the recombinant BAV3.E3gD or BAV3.E3gDt also replicated as efficiently as wild-type BAV3, suggesting that a foreign gene product rather than the E3 insertion itself may be affecting the replication of different recombinant BAV3 in cell culture.

To confirm the validity of the BAV3 vector system for

the expression of potential vaccine antigens, we constructed recombinant BAV3.E3gD- and BAV3.E3gDt-expressing BHV-1 gD or gDt glycoprotein, respectively. As expected, gD expressed by BAV3.E3gD was of the expected molecular weight and comigrated with the authentic gD expressed in BHV-1-infected cells. Similarly, the gDt expressed by BAV3.E3gDt was of the expected molecular weight (Tikoo *et al.,* 1993). In addition, gD and gDt expressed by BAV3 recombinants were recognized by MAbs directed particularly against conformational epitopes (Hughes *et al.,* 1988). These results suggest that recombinant gD and gDt had the posttranslational modification and antigenic profiles indistinguishable from those of the authentic gD synthesized after viral infection (Hughes *et al.,* 1988).

Foreign genes without any flanking regulatory sequences, inserted in the same transcriptional orientation as E3, in the E3 region of human adenovirus are expressed efficiently, either from the upstream MLP or E3 promoter of the viral genome (Dronin *et al.,* 1993; Morin *et al.,* 1987; Xu *et al.,* 1995). Glycoprotein gD expression was partially reduced in the presence of AraC, suggesting that foreign gene expression was driven by both the MLP and E3 promoter of BAV3. Similar results have been reported for the luciferase gene expressed from the partially deleted E3 region of BAV3 (Mittal *et al.,* 1995). In addition, it appears that gD-specific mRNAs may be using BAV3 E3-specific poly(A)$^+$ signals (Idamakanti, 1998). This suggestion is based on the following observations: (1) an E3 deletion vector still retains the E3-specific polyA signal sequence, (2) the other probable poly(A)$^+$ signal sequence is $\sim$3.3 kb from the E3-specific poly(A)$^+$ signal (Reddy *et al.,* 1998), and (3) the gD genes are not flanked by exogenous poly(A)$^+$ signals.

Intranasal immunization of cotton rats with BAV3.E3gD or BAV3.E3gDt induced gD-specific mucosal and systemic immune responses. The gDt induced a higher IgG antibody response than to gD. In contrast, intradermal immunization of cotton rats with recombinant HAV5 induced a lower immune response to gDt than to gD (Mittal *et al.,* 1996). A purified preparation of recombinant gDt incorporated with an adjuvant when injected into mice (Baca-Estrada *et al.,* 1997) produced a secondary immune response similar to that of authentic gD. This strongly suggests that the route of immunization and vaccine formulation may effect the ability of the immunogen to induce an effective immune response and that whether the immunogen is anchored in the membrane (gD) or secreted as a soluble protein (gDt) may not in itself influence the immunogenicity of the protein.

Surprisingly, gD and gDt elicited similar IgA but significantly different IgG responses in the serum. Mucosal and systemic immune responses have been shown to be elicited and regulated with a considerable degree of independence (Alley and Mestecky, 1988; Conley and Delacroix, 1987). Induction of an immune response in one of these systems does not necessarily lead to a response in the other. Thus it is possible that the two different forms of gD are recognized differently in mucosal and systemic compartment.

Induction of mucosal immunity is thought to be crucial in protecting the host from a respiratory or enteric infection. Moreover, secretory IgA has usually been found to correlate with resistance to such infections (Murphy, 1994). Interestingly, intranasal immunization induced gD-specific IgA antibody response, not only in the serum and lung but also in nasal washes of cotton rats. A high level of IgA and the presence of ASC in the lung suggest that the antibody was produced locally.

In conclusion, we have described an efficient and reliable system for the production of BAV3 recombinants. In addition, we have shown that intranasal immunization of cotton rats with replication competent recombinant BAV3 induces vaccine antigen-specific mucosal and systemic immune responses.

MATERIALS AND METHODS

Cells and viruses

MDBK cells and PFBR cells were grown in Eagle's minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS). The wild-type (WBR-1 strain) and recombinant BAV3s were propagated in MDBK cells as described previously (Mittal *et al.,* 1995). The P8-2 strain of BHV-1 was propagated and quantified as described earlier (Rouse and Babiuk, 1974).

Animals

An inbred colony of cotton rats (*Sigmodon hispidus*) maintained at the Veterinary Infectious Disease Organization Saskatoon was the source of animals for this study.

Construction of recombinant plasmids

Construction of plasmid pFBAV302. A T4 polymerase-treated 587-bp fragment isolated by PCR amplification [using oligonucleotides (1) ACGCGTCGACTCCTCCTCA and (2) TTGACAGCTAGCTTGTTG; and plasmid pSM14 (Mittal *et al.,* 1995) as a template] was digested with *Sac*II and ligated to *Eco*47III–*Sac*II-digested plasmid pSL301 creating plasmid pE3A. A 164-bp fragment isolated by PCR amplification [using oligonucleotides (1) CCAAGCTTGCATGCCTG and (2) GGCGATATCTCAGCTATAACCGCTC and plasmid pSM14 (Mittal *et al.,* 1995)] was digested with *Sph*I–*Eco*RV and ligated to 531-bp *Eco*RV–*Hin*dIII fragment of pE3A and *Sph*I–*Hin*dIII-digested plasmid pSL301 creating plasmid pE3B. The 614-bp *Sph*I–*Sac*II fragment was isolated from plasmid pE3B and ligated to *Sph*I–*Sac*II-digested pSM14 to cre-

ate plasmid pE3C. The plasmid pE3C was digested with *Eco*RV and ligated to *Srf*I linker (TTGCCCGGGCTT) creating plasmid pE3C1. A 1755-kb *Bam*HI fragment of pE3C1 was isolated and ligated to *Bam*HI-digested pSM17 (Mittal *et al.,* 1995) creating plasmid pE3D. Finally, a 8783-bp *Kpn*I–*Xba*I fragment of plasmid pE3D was isolated and ligated to *Kpn*I–*Xba*I-digested plasmid pTG5435 (which contains full-length BAV3 genomic DNA) to create plasmid pE3E. The plasmid pE3E contained end fragments of the BAV3 genome (0–19.7 m.u. and 76.6–100 m. u.) with a 1.245-kb deletion in the E3 region and a unique *Kpn*I site.

The plasmid (pFBAV302) containing the BAV3 genome with a 1.245-kb deletion in the E3 region was generated by homologous DNA recombination between *Kpn*I-digested pE3E and deproteinized BAV3 genomic DNA in *E. coli* BJ5183 (Degryse *et al.,* 1996).

Construction of plasmid pFBAV302.gD and pFBAV302.gDt. The transfer plasmid for generation of recombinant BAV3-expressing foreign genes in the E3 region was constructed by ligating a 8783-bp *Kpn*I–*Xba*I fragment of pFBAV302 to a *Kpn*I–*Xba*I-digested plasmid pGEM3zf(−), creating plasmid pBAV300. A 13-kb *Bgl*II fragment of a plasmid pRSV13 (Tikoo *et al.,* 1993) containing a BHV-1 full-length gD gene was treated with T4 DNA polymerase and ligated to *Srf*I-digested pBAV300, creating plasmid pBAV300.gD. Similarly, a 13-kb *Bg*1II fragment of plasmid pRSV13XN (Tikoo *et al.,* 1993) containing a BHV-1 truncated gD gene was treated with T4 DNA polymerase and ligated to *Srf*I-digested pBAV300, creating plasmid pBAV300.gDt.

The recombinant BAV3 genome containing a gene encoding full-length (pFABV302.gD) or truncated (pFBAV302.gDt) gD protein was generated by homologous DNA recombination in *E. coli* BJ5183 between *Srf*I linearized pFBAV302 and a 10-kb *Kpn*I–*Xba*I fragment of pBAV300.gD or between *Srf*I linearized pFBAV302 and a 10-kb *Kpn*I–*Xba*I fragment of pBAV300gDt, respectively.

Construction of recombinant BAV3

PFBR cell monolayers in 60-mm dishes were transfected with 10 μg of *Pac*I-digested pFBAV302, pFBAV302.gD, or pFDBAV302.gDt recombinant plasmid DNAs using the Ca^{2+}-phosphate method (Graham and van der Eb, 1973). After 15–20 days of incubation at 37°C, the transfected cells showing 50% cytopathic effects were collected and freeze-thawed two times, and the recombinant virus was plaque purified on MDBK cells (Mittal *et al.,* 1995).

Radiolabeling and immunoprecipitation on proteins

About 70–80% confluent MDBK cell monolayers in 28-cm^2 wells were infected with 10 pfu of recombinant or wild-type BAV3 per cell. After virus absorption for 60 min, the cells were incubated in MEM containing 5% FBS. At different times p.i., the cells were incubated in methionine–cysteine-free Dulbucco's modified Eagle's medium (DMEM) for 60 min before labeling with [^{35}S]methionine-cysteine (100 μCi/well). After 2 or 12 h of labeling, the cells or medium was harvested. Proteins were immunoprecipitated from the medium or the cells lysed with modified radioimmunoprecipitation (RIPA) buffer and analyzed by SDS–PAGE as described previously (Tikoo *et al.,* 1993).

Animal inoculations

A total of 25 cotton rats (4 to 6 weeks old) of either sex were divided into three groups (nine animals/group). After inducing anesthesia in the animals with halothane, animals were inoculated twice at day 1 and day 21 by the intranasal route with 100 μl of inoculum containing 10^7 pfu of individual recombinant virus. Blood samples were collected at days 0, 21, and 28 after the primary inoculation to examine the development of BHV-1 gD-specific and BAV3-specific antibodies by enzyme-linked immunosorbent assays (ELISA) and virus neutralization (VN) assays. Four animals in each group were killed at 21 and 28 days after the primary inoculation by an overdose of halothane. The lung and nasal secretions were collected separately to monitor the development of BHV-1 gD-specific and BAV3-specific mucosal IgG and IgA antibody responses by ELISA (Papp *et al.,* 1997). In addition, lungs were collected to determine the frequency of BHV-1 gD- and BAV3-specific IgA antibody secreting cells by enzyme-linked immunospot (ELISPOT) (Papp *et al.,* 1997).

Preparation of lymphocytes from the lungs

Aseptically removed lung tissue was cut into small pieces and incubated in complete medium: MEM supplemented with 10% FBS, 2 mM L-arginine, 1 mM sodium pyruvate, 100 μM nonessential amino acids, and 10 mM HEPES buffer plus 100 U/ml penicillin G, 100 μg/ml streptomycin solution, 150 U/ml collagenase A, and 50 U/ml Dnase I for 1 h and then pushed through a plastic mesh. The lung cell suspension was centrifuged through a discontinuous Percoll gradient and washed with MEM. The cells were resuspended in complete medium and incubated for 1 h in a flask to attach adherent cells. The nonadherent cell population was then resuspended and used in the antigen-specific ELISPOT assay as described earlier (Papp *et al.,* 1997).

ELISA

Antibodies specific for BHV-1 and BAV3 in sera in lung and nasal secretions were determined by ELISA as described earlier (Papp *et al.,* 1997). Briefly, 96-well Immunol-2 microtiter plates were coated with either purified truncated gD (0.01 μg/well) or BAV3 (0.5 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.

Virus neutralization

Two-fold serial dilutions of heat-inactivated serum samples were incubated with 100 pfu and 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. Titers were expressed as reciprocals of the highest antibody dilution that caused 50% reduction in the number of plaques relative to the control.

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