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Optimization of bovine coronavirus hemagglutinin-esterase glycoprotein expression in E3 deleted bovine adenovirus-3

P. Seshidhar Reddy ^{a,1}, Neeraja Idamakanti ^a, Lexander N. Zakhartchouk ^{a,2},
Lorne A. Babiuk ^a, Majid Mehtali ^b, Suresh K. Tikoo ^{a,*}

^a *Virology group, Veterinary Infectious Disease Organization, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5E3*

^b *Gene Therapy Department, Transgene S.A. 67000 Strasbourg, France*

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Abstract

Adenoviral vectors expressing foreign genes have many desirable properties in applications such as vaccination. Recently, we have generated replication-competent (E3 deleted) bovine adenovirus-3 (BAV-3) recombinants expressing significant amounts of glycoprotein D (gD) of bovine herpesvirus-1 (a DNA virus). However, attempts to express the RNA virus genes using the same strategy were not successful. In an effort to optimize the expression, we have constructed several BAV-3 recombinants carrying the hemagglutinin esterase (HE) gene of bovine coronavirus (BCV) in the E3 region with or without exogenous transcription control elements. The expression studies suggest that the introduction of a 137 bp chimeric intron upstream of the HE cDNA is able to increase the level of HE gene expression. The introduction of a SV40 early promoter or human cytomegalovirus (HCMV) immediate early (IE) promoter into the expression cassette changed the kinetics of the HE expression. However, the recombinant BAV-3 containing HE under the HCMV IE promoter replicated less efficiently than the wild-type BAV-3. These studies should prove useful in expression of other RNA viral genes in the E3 region of BAV-3 expression system. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Bovine adenovirus-3; Bovine coronavirus; Gene expression

1. Introduction

Bovine coronavirus (BCV) contains a positive single stranded RNA genome of about 30 kb in length. The BCV genome encodes three membrane glycoproteins, the integral membrane protein (M), the spike protein (S), and the hemagglutinin–esterase (HE) (King and Brian, 1982).

* Corresponding author. Tel.: +1-306-9667482; fax: +1-306-9667478.

E-mail address: tikoo@sask.usask.ca (S.K. Tikoo).

¹ Present address: Genetic Therapy, Inc. Gaithersburg, MD 20878 USA.

² Present address: Introgene, Wassenaarseweg 72, P.O. Box 2048, 2301 CA Leiden, The Netherlands.

The S and HE proteins are major membrane associated glycoproteins and induce virus neutralizing antibodies (Deregt et al., 1989). The monoclonal antibodies raised against the HE protein neutralize the infectivity of BCV under cell culture conditions and protected the intestinal epithelia of cattle from the virus infection (Deregt and Babiuk, 1987). In addition, the immunogenicity of a recombinant human adenovirus-5 (HAV-5) expressing the HE gene was tested in cotton rats (Baca-Estrada et al., 1995). Although HAV-5 based vectors (expressing vaccine antigens from different bovine pathogens) have been used to test the efficacy and immunogenicity of protective antigens in experimental animals (Baca-Estrada et al., 1995; Breker-Klassen et al., 1995; Papp et al., 1997), their use as recombinant vaccines in cattle is limited due to their broad host range, which creates regulatory concerns.

In recent years, the idea of using bovine adenovirus-3 (BAV-3) as a vector (Baxi et al., 1999; Reddy et al., 1999; Zakhartchouk et al., 1998) for delivery of protective antigen from pathogens of cattle has received considerable attention. Deletion of the non-essential E3 region has facilitated construction of replication-competent BAV-3 vectors expressing authentic and truncated forms of glycoprotein D (gD) of bovine herpes virus-1 (Zakhartchouk et al., 1998). During infection of bovine cell lines, such recombinant BAVs produced large amounts of glycoprotein gD (a DNA virus gene), which has been shown to undergo proper post-translational modifications (Zakhartchouk et al., 1998). In addition, intranasal inoculation of calves with these recombinants has been shown to be safe and efficacious, making these vectors particularly attractive for vaccination of cattle (Zakhartchouk et al., 1999). However, similar attempts to obtain high levels of expression of HE gene of bovine corona virus in a replication-competent BAV-3 vector (Zakhartchouk et al., 1998) without any flanking upstream or downstream sequences have been unsuccessful. Since the level of expression of an antigen may determine the quality of immune response in animals immunized orally or intranasally with recombinant adenoviruses, it is necessary to maximize the expression of a vaccine

antigen. In addition, as the development of a quality immune response would correlate with levels of antigen produced by the recombinant adenovirus vectors, we investigated ways of increasing HE gene expression in the BAV-3 expression system. The main reason to choose BCV HE gene was that it has been difficult to over express HE using a variety of heterologous expression systems (unpublished data). In addition, BCV causes neonatal diarrhea in calves resulting in significant economic losses due to mortality and decreased productivity of the survivors. Since currently available vaccines against BCV are not effective (Waltner-Toews et al., 1995), better vaccines are needed to reduce the economic losses.

In the present study, we examined the effect of exogenous transcription control elements on the expression levels of the HE gene in E3 deleted BAV-3 vector.

2. Materials and methods

2.1. Cells and viruses

Wild-type and recombinant BAV-3s were cultivated in Madin Darby bovine kidney (MDBK) and VIDO R2 cells (transformed fetal bovine retina cells; Reddy et al., 1999). The cells were grown in Eagle's minimum essential medium (MEM) supplemented with 5% fetal bovine serum. The viral DNA was extracted from virus infected cell monolayers by the method of Hirt (1967).

2.2. Construction of E3 transfer vectors

The original E3 transfer vector, pBAV-300 has the genomic DNA sequences between nts 24465 and 28593 (nt numbers are based on BAV-3 genome sequence; GenBank Accession No. AF030154) with a deletion of 1245 bp of the E3 region from nt 26458 to 27703, cloned into a bacterial plasmid (Zakhartchouk et al., 1998). This transfer vector has an overlap of 1992 bp on the left side and 889 bp on the right side of the E3 region for homologous recombination in *E. coli* BJ 5183 with the E3 deleted full-length clone

pFBAV-302 (Zakhartchouk et al., 1998). To increase the overlap, initially the KpnI-SspI fragment representing the right side of the BAV-3 genome between nt 24464 and 34060 was introduced into KpnI and blunt-ended NotI sites of pPOLYII sn 14 to generate a plasmid, pBAV-299. The region spanning the KpnI and XbaI sites of pBAV-299 was replaced with that of pBAV-300 to generate pBAV-301. The plasmid pBAV-301 can be digested with KpnI (nt 24464) and SpeI (nt 31570) enzymes and the gel purified fragment can be used for homologous recombination in *E. coli* BJ 5183. This new transfer vector has two unique restriction enzyme sites (SrfI and Sall) for cloning of foreign genes and an overlap of 1992 bp on the left side and 3866 bp on the right side of the E3 region for efficient homologous recombination with plasmid pFBAV-302 (Zakhartchouk et al., 1998), which dramatically increased the frequency of recombination in BJ 5183 cells.

Plasmid pBAV301b was constructed by cloning a 137 bp long chimeric intron amplified by PCR from pCI-neo (Promega) into the SrfI site of pBAV-301. The intron is composed of the 5'-donor site from the first intron of the human beta-globulin gene and the branch, and 3'-acceptor site from the intron of an immunoglobulin gene heavy chain variable region (Senapathy et al., 1990). The transgenes are introduced downstream of the intron in order to prevent utilization of possible cryptic 5'-donor splice sites within the BCV HE cDNA sequence.

2.3. Construction of recombinant plasmids

2.3.1. Construction of plasmid pFBAV303 and pFBAV332

The 1.3 kb BamHI fragment of a plasmid pCVE3 (Parker et al., 1989) containing the complete coding sequence of BCV HE gene was treated with T4 DNA polymerase and ligated to blunt end repaired SrfI digested plasmid pBAV301 to create plasmid pBAV301.HE and blunt end repaired Sall digested plasmid pBAV301b to create plasmid pBAV301b.HE. The recombinant BAV-3 genomes containing the gene encoding HE were generated by homologous recombination in *E. coli* BJ5183 between SrfI lin-

earized pFBAV302 and the 7.2 kb KpnI — SpeI fragment of pBAV301.HE creating plasmid pFBAV303, and between SrfI linearized pFBAV302 and the 7.3 kb KpnI — SpeI fragment of pBAV301b.HE creating plasmid pFBAV332.

2.3.2. Construction of plasmid pFBAV333 and pFBAV334

Plasmid pSVPIA containing a unique Sall cloning site was constructed by ligating a 209 bp SV40 promoter (isolated from pCAT-Promoter plasmid; Promega), 137 bp chimeric intron and 240 bp SV40 late poly(A) signal (isolated from pCI-neo; Promega) to plasmid pPOLYIIsn. Plasmid pCMVPIA is similar to plasmid pSVPIA except that SV40 promoter is replaced by a 510 bp HCMV promoter (isolated from pCMV β ; clontech). The 1.3 kb blunt end repaired BamHI fragment containing the HE gene (Parker et al., 1989) was ligated, to blunt end repaired Sall digested plasmid pSVPIA to create plasmid pSVPIA.HE, and to blunt end repaired Sall digested plasmid pCMVPIA to create plasmid pCMVPIA.HE. A 1.9 kb fragment of plasmid pSVPIA.HE and a 2.0 kb fragment of plasmid pCMVPIA.HE containing the HE gene under appropriate transcriptional elements were isolated and ligated individually to blunt end repaired SrfI digested plasmid pBAV301 to create plasmids pBAV301.Hesv and pBAV301.HEcmv respectively. Finally, the recombinant BAV-3 genomes were isolated by homologous recombination in *E. coli* BJ5183 between SrfI linearized plasmid pFBAV302 and the 7.8 kb KpnI — SpeI fragment of pBAV301.Hesv creating plasmid pFBAV333, and between SrfI linearized plasmid pFBAV302 and the 8.1 kb KpnI — SpeI fragment of pBAV301.HEcmv creating plasmid pFBAV334.

2.4. Isolation of recombinant BAV-3

VIDO R2 cell monolayers in 60 mm dishes were transfected with 5–10 μ g of Pac-I digested pFBAV303, pFBAV332, pFBAV333 and pFBAV334 recombinant plasmid DNAs using lipofectin reagent. After incubation at 37°C, the transfected cells showing cytopathic effects were collected, freeze thawed two times and the recom-

binant viruses were plaque purified on MDBK cells.

2.5. Southern blot hybridization

DNA fragments obtained after restriction enzyme digestion of virion DNA were transferred from agarose gels to Nytran membranes (Schleicher and Schuell) as described (Reddy et al., 1993). The gene coding for HE of BCV was labeled with ^{32}P dCTP by the random primer labeling technique. Hybridizations were carried out at 42°C in the presence of 50% formamide. Prehybridization, hybridizations and washing of membranes were carried out as described elsewhere (Reddy et al., 1993).

2.6. Northern blot hybridizations

MDBK cells grown in petri dishes were infected with 5 pfu per cell of recombinant BAV-3s. Total RNA was extracted from mock infected or recombinant BAV-3 infected cells with acid guanidinium thiocyanate-phenol-chloroform mixture as described by Chomczynski and Sacchi (1987). RNA (10 µg) was separated on 1% agarose-formaldehyde gels and transferred to Nytran membranes (Sambrook et al., 1989). The blots were baked, prehybridized, hybridized and washed as described (Sambrook et al., 1989). The gene coding for HE of BCV, labeled with α - ^{32}P dCTP by the random primer labeling technique was used as a probe.

2.7. Immunoprecipitations

Confluent monolayers of MDBK cells in six well dishes were infected with the virus at a multiplicity of infection of five. The cells were preincubated for 2 h in MEM deficient of methionine and cysteine prior to labeling with 50 µCi of ^{35}S methionine (Trans (^{35}S) label [1000 Ci/mmol] ICN Radiochemicals Inc., Irvine, CA) for 4 h. The cells were washed once with PBS, harvested by scraping and then lysed with ice-cold modified radioimmunoprecipitation assay buffer. The radiolabeled proteins were immunoprecipitated with polyclonal anti-BCV rabbit antibodies

(Deregt and Babiuk, 1987) and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions. The gels were dried and protein bands were visualized by autoradiography.

3. Results

3.1. Generation of recombinant BAVs containing HE of BCV

Our initial attempts to insert the BCV HE gene in the E3 region of plasmid pFBAV302 (E3 deleted full length BAV-3 genomic clone; Zakhartchouk et al., 1998) by homologous recombination in *E. coli* BJ5183 between the KpnI — XbaI fragment (with an overlap of 1992 bp on left side and 889 bp on the right side of E3 region) of plasmid pBAV300 (Zakhartchouk et al., 1998) containing the BCV HE gene and SrfI linearized plasmid pFBAV302 were unsuccessful. In order to increase the efficiency of inserting foreign genes in the E3 region of plasmid pFBAV302 by homologous recombination in *E. coli* BJ5183, we first constructed a modified transfer plasmid pBAV301. This plasmid contains an overlap of 1992 bp on the left side and 3866 bp on the right side of the E3 region with plasmid pFBAV302. Use of this plasmid dramatically increased the frequency of recombination in *E. coli* BJ5183 and helped to successfully clone BCV HE.

Next, we constructed recombinant BAV-3s expressing the BCV glycoprotein HE. The full length HE gene alone or with different exogenous transcriptional elements was inserted individually into the E3 region of plasmid pFBAV302 in the same transcriptional orientation of E3 using homologous recombination in *E. coli* (Chariter et al., 1996). The Pac-I digested pFBAV303, pFBAV332, pFBAV333 or pFBAV334 plasmid DNA was transfected into VIDO R2 cells (transformed fetal bovine retina cells). The infected monolayers showing 50% cytopathic effects were collected, freeze thawed and recombinant viruses were plaque purified and propagated in MDBK cells. The recombinant viruses were named BAV303 (HE without exogenous elements),

BAV332 (HE with chimeric intron), BAV333 (HE with SV40 promoter, chimeric intron and SV40 polyA) and BAV334 (HE with CMV promoter, chimeric intron, SV40 polyA) (Fig. 1). The viral DNA was extracted from infected cells by the Hirt method (Hirt, 1967) and analysed by agarose gel electrophoresis after digestion with BamHI restriction enzyme. The digestion of wild type BAV-3 viral DNA with BamHI yields five fragments and the fragment D (3.019 kb) contains the E3 region (Fig. 2A, lane 1). The difference in the size of modified BamHI 'D' fragment of BAV3.E3d (Fig. 2A, lane 2) BAV303 (Fig. 2A, lane 3), BAV332 (Fig. 2A, lane 4), BAV333 (Fig. 2A, lane 5) and BAV334 (Fig. 2A, lane 6) was as expected. This was confirmed by Southern blot analysis of BamHI digested genomic DNA of wild type and recombinant BAV3s. As seen in the Fig. 2B, the same modified BamHI 'D' fragment (s) from the recombinant viruses hybridized to α - 32 P dCTP labeled HE gene in the Southern blot hybridization (Fig. 2B, lanes 3,4,5,6). This suggested that recombinants BAV303, BAV332, BAV333 and BAV334 contained the HE gene.

3.2. Northern analysis of HE transcripts

To analyze transcription of the HE gene, RNA was prepared from mock infected or recombinant BAV-3 infected cells at 18 and 28 h postinfection. The RNA was separated on agarose–formaldehyde

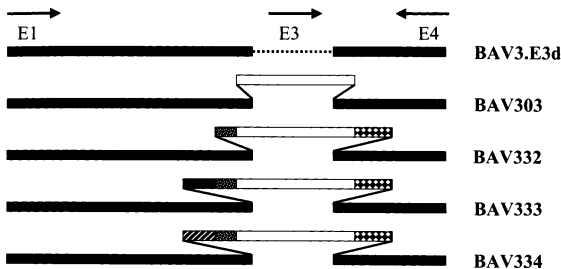


Fig. 1. Schematic representation of recombinant E3 deleted full length BAV-3 genomic clones. BAV-3 genome (■), HE gene (□), SV40 early promoter (○), chimeric intron (◐), SV40 poly(A) (△), HCMV IE promoter (◑). The locations of early (E) region E1, E3 and E4 are depicted. The arrow represents the direction of transcription. The name given to each recombinant virus is depicted on the right.

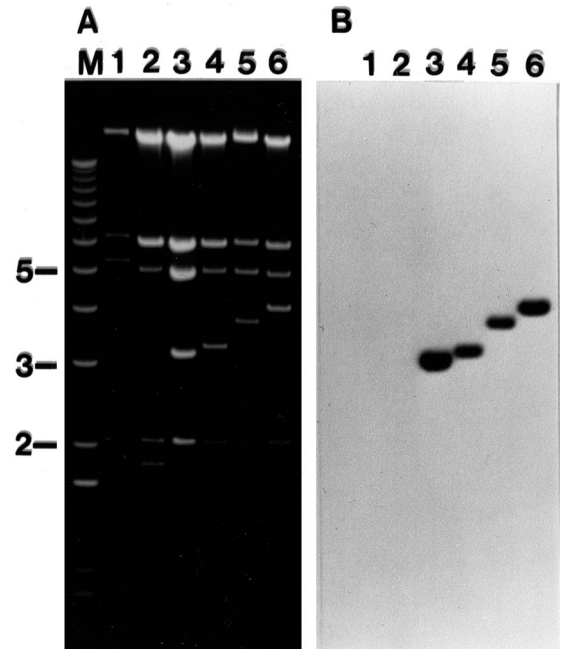


Fig. 2. Restriction enzyme analysis of recombinant BAV-3 genomes. (A) The DNAs were extracted from BAV-3 (lane1), BAV3.E3d (lane 2), BAV303 (lane 3), BAV332 (lane 4), BAV333 (lane 5) and BAV334 (lane 6) infected MDBK cells by Hirt method (Hirt, 1967) and digested with BamHI. (B) The fragments shown in panel A were transferred to Nytran membranes and probed with α - 32 P labeled HE probe. Lane M, 1 Kb Plus DNA ladder (Gibco/BRL) used for sizing the viral DNA fragments. Sizes in kb is shown on left.

hyde gels, transferred to Nytran membrane and probed with the α - 32 P labeled HE gene. The probe was expected to detect four L6 mRNAs (100 K, 33 K, 23 K and pVIII) and the mRNAs of HE transcribed from the E3 promoter and MLP. Unlike in HAV-2 (Ziff and Fraser, 1978), the transcripts from the L6 region in BAV-3 are polyadenylated at the poly(A) site of the E3 region (Reddy et al., 1998). Thus, all the transcripts of L6 region originating from the major late promoter (MLP) form a nested set of overlapping molecules with common 3' ends. Each mRNA contains all the sequences in the next smaller mRNA plus one additional ORF at the 5' end. Only the ORF at the 5' end of the mRNA is translated. When RNA was analyzed, several abundant mRNAs were identified that had HE sequences in them particularly in RNA that was

extracted at 28 h postinfection (Fig. 3). At 28 h postinfection, the larger transcripts were the dominant species of RNA extracted from BAV303 (lane 2) and BAV333 (lane 6) infected cells. During early stages of HAV-5 infection, the E3 promoter is used to express mRNAs from the E3 region and during late stages, transcription from the E3 promoter is reduced and some mRNAs are made from the MLP (Tollefson et al., 1992). The major late E3 mRNAs containing tripartite leader sequences are also produced in BAV-3 (Idamakanti et al., 1999). Since the sizes of the transcripts were considerably larger than the genomic distance between the E3 promoter and poly(A) site of the E3 region, they must have been generated by splicing of the primary transcripts produced from the MLP.

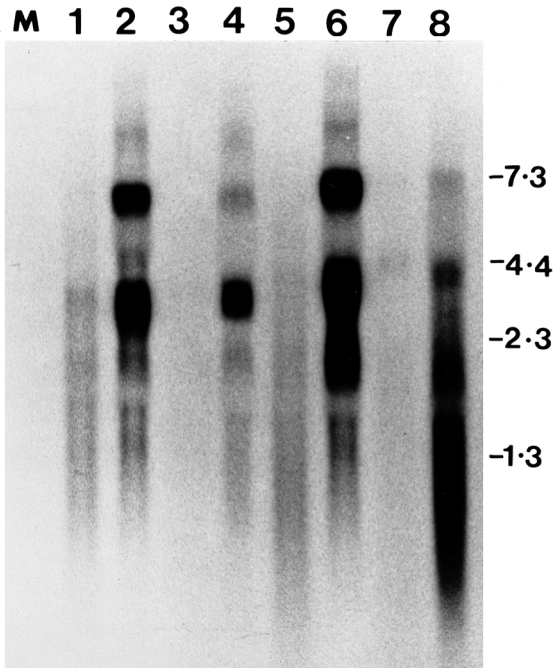


Fig. 3. Northern blot analysis of HE transcription. Total RNA was isolated from mock (M), BAV303 (lane 1, 2), BAV332 (lane 3, 4), BAV333 (lane 5, 6) and BAV334 (lane 7, 8) infected MDBK cells after 18 (lane 1, 3, 5, 7) or 28 (lane 2, 4, 6, 8) h post infection and analyzed by Northern blot analysis as described under 'Materials and Methods' using 1.3 Kb BamHI fragment (containing HE coding sequence) of pCVE3 plasmid (Parker et al., 1989) as a probe. Numbers on the right denote the estimated sizes of RNAs in kilobases.

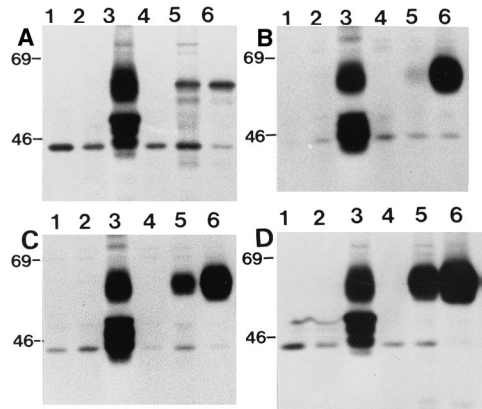


Fig. 4. Expression of HE protein in MDBK cells infected with recombinant BAV3 viruses. Proteins from lysates of radiolabeled mock infected (lane 1), BAV-3 infected (lane 2), BCV infected (lane 3), BAV303 infected (panel A, lanes 4–6), BAV332 infected (panel B, lanes 4–6), BAV333 infected (panel C, lanes 4–6) or BAV334 infected (panel D, lanes 4–6) MDBK cells were immunoprecipitated with polyclonal anti-BCV serum and analyzed by SDS-PAGE under reducing conditions. The cells were harvested at 12 (lane 4), 24 (lane 5) and 36 h (lane 6) postinfection. The positions of the size markers (in kilodaltons) are shown to the left of each panel.

3.3. Kinetics of HE expression in MDBK cells

Proteins from cell lysates, collected at different times post infection of MDBK cells with recombinant BAV-3, were analyzed by immunoprecipitation assays using BCV specific polyclonal antiserum. Electrophoretic analysis of metabolically radiolabeled immunoprecipitates from BCV (Fig. 4ABCD, lane 3) infected cell lysates detected a protein of 65 kDa. No such protein was detected from mock (Fig. 4ABCD, lane 1) or BAV-3 (Fig. 4ABCD, lane 2) infected cell lysates. The recombinant BAV303 contains the HE cDNA sequence substituting for BAV-3 E3 in the parallel orientation so as to allow expression from endogenous promoters. Immunoprecipitation analysis of BAV303 infected cell lysates showed little or no HE expression (Fig. 4A, lanes 4, 5, 6). The recombinant BAV332 has the HE sequence in the E3, downstream of an exogenous chimeric intron and upstream of SV40 late poly(A) signal. Immunoprecipitation analysis of BAV332 infected cell lysates detected a specific band of 65 kDa at

36 h post infection (Fig. 4B, lane 6). The recombinant BAV333 and recombinant BAV334 are similar to BAV332 except that they have either SV40 or CMV immediate early promoters upstream of chimeric introns, respectively. Immunoprecipitation analysis of BAV333 infected cells detected a specific band of 65 kDa at 24 (Fig. 4C, lane 5) and 36 h (Fig. 4C, lane 6) post infection. Similarly, immunoprecipitation analysis of BAV334 infected cells also detected a specific band of 65 kDa at 24 (Fig. 4D, lane 5) and 36 h (Fig. 4D, lane 6) post infection.

3.4. Growth of recombinant viruses

To determine whether insertion of exogenous transcription control elements into the E3 had any noticeable effect on the ability of these recombinant to replicate in MDBK cells, virus titer were determined. Deletion of the E3 region had no detectable effect on the virus yield. BAV303, BAV332, and BAV333 grew to similar titers as BAV3.E3d (E3 deleted) virus. However, BAV334 grew to a final titer that was $1.0 \log_{10}$ lower than the BAV3.E3d (E3 region deleted virus; Zakhartchouk et al., 1998).

4. Discussion

The E3 region of BAV-3 located between nts 26185 and 27776 is 1591 bp in length. The promoter of the E3 is located within the coding sequence of the pVIII and shares its poly(A) site with the L6 (Idamakanti et al., 1999). Recently, we have developed a replication-competent BAV-3 vector with a 1.245 kb deletion in the E3 region, removing all E3 open reading frames, leaving the E3 promoter, 5' initiation site, and the poly(A) site intact (Zakhartchouk et al., 1998). Recombinant BAV-3 expressed high levels of full length or truncated forms of glycoprotein D (gD) of BHV-1 when the genes coding for these proteins were introduced into the E3 region in the same orientation as the E3 transcription unit without any exogenous transcription control elements (Zakhartchouk et al., 1998). However, when the cDNAs representing the protective antigen from

RNA viruses including BCV were inserted into the E3 region of BAV-3, no expression of the proteins was obtained (unpublished observation). In an attempt to optimize the expression of RNA virus genes in the BAV-3 expression system, we have constructed and characterized four recombinant viruses carrying the HE gene of BCV (with or without exogenous transcription control elements).

Adenoviruses replicate in the nucleus, whereas all except one RNA virus replicate in the cytoplasm. If a RNA virus gene is to be expressed through a recombinant adenoviral vector system, its mRNA must be free of any cryptic splice sites or else the message could be broken into pieces by the splicing machinery present in the cell nucleus. This can be prevented either by swapping codons to remove internal splice sites (expensive) or by inserting an intron upstream of the cDNA. Since transfection studies have demonstrated that an intron flanking the cDNA insert frequently increases the level of cDNA expression (Huang and Gorman, 1990), it was hypothesized that introduction of an intron upstream of a cDNA could prevent utilization of possible cryptic 5'-donor splice sites within the cDNA sequence (Huang and Gorman, 1990). Addition of poly(A)⁺ [e.g. SV40 late poly(A) signal] has also been shown to enhance RNA stability and translation (Carswell and Alwine, 1989; Jackson and Standart, 1990). However, the size of the poly(A) and the intron should be minimal as its inclusion will reduce the ultimate capacity of the adenovirus vector. The intron A, which is most widely used to express cDNAs is about 800 bp in length and will reduce the size limit of foreign DNA that can be put into BAV-3 vectors.

Keeping this in mind, the HE gene was flanked with a chimeric intron of 147 bp in length (Senapathy et al., 1990) and the SV40 late poly(A)⁺ signal at the 5' end and 3' end respectively. Addition of a chimeric intron and SV40 poly(A)⁺ signal dramatically increased the levels of HE gene expression. From the size of majority of mRNAs, it appears that the HE transcripts must have initiated from the major late promoter (MLP). It seems likely that the chimeric intron provided the strong splice acceptor site. Consis-

tent with this speculation is the fact that synthesis of HE was observed late (36 h) after infection, when much of the transcription occurs from MLP.

Introduction of a SV40 (BAV333) or HCMV IE (BAV334) promoter into the expression cassette modified the kinetics of HE expression as HE glycoprotein could be detected as early as 24 h post infection. It seems likely that early HE transcripts must have been initiated from the BAV-3 E3 promoter or SV40/HCMV promoter. Support for this hypothesis comes from the fact that (a) HE specific mRNAs of smaller size are abundant in BAV333 and BAV334 infected cells; (b) BAV-3 E3 284R protein expressed from the E3 promoter can be detected at 24 h but not at 12 h post infection (Idamakanti et al., 1999). However, it is not clear how many of these transcripts arise from either the E3 promoter or SV40 promoter, and are translated to give rise to the HE polypeptide expressed early in recombinant infected cells.

The BCV HE gene under the control of a SV40 promoter was cloned and expressed using HAV-5 (Yoo et al., 1992). Expression of the HE was seen as early as 6 h postinfection and produced throughout the infection. However, BAV-3 differs from HAV-5 with respect to replication kinetics. In BAV-3 infected cells, viral DNA replication begins at about 24 h postinfection and reaches a peak after 40 h, whereas viral DNA replication in HAV-5 infected cells occurs as early as 12 h postinfection (Niiyama et al., 1975). The green fluorescent protein (GFP) expression was noticed at 12 h postinfection when the gene for GFP was placed under the control of CMV immediate early promoter in the E3 region of BAV-3 (Reddy et al., 1999). This suggests that kinetics of foreign gene expression from the E3 region of BAV-3 may be influenced not only by exogenous transcriptional elements but also by the nature of the foreign gene.

In conclusion, we have shown that addition of intron 5' to the transgene increases the level of cDNA expression in E3 deleted BAV-3. Moreover, addition of heterologous promoter and intron 5' to transgene alters the kinetics of foreign gene expression from E3 region of BAV-3.

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