Development of a bovine adenovirus type 3-based expression vector

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We constructed a non-defective bovine adenovirus type 3 recombinant (BAd3-Luc) containing the firefly luciferase gene inserted in the early region 3 (E3) of the BAd3 genome. Deletion of a 696 bp XhoI–NcoI E3 segment and insertion of the luciferase gene in E3 was confirmed by Southern blot analyses. Luciferase was expressed in Madin-Darby bovine kidney cells infected with BAd3-Luc as measured by enzymic assays and Western blotting. Analyses of luciferase expression in the presence or absence of 1-β-D-arabinofuranosylcytosine indicated that approximately 70–75% of luciferase expression was dependent on viral DNA replication, suggesting that transcription of the gene was at least partially under the control of a late promoter.

Although yields of infectious virus for BAd3-Luc were approximately 10-fold lower than for wild-type virus, replication of the vector was still relatively efficient. In a Western blot experiment, anti-luciferase antibody reacted with a 62 kDa protein which is of the same molecular mass as the purified firefly luciferase polypeptide. Luciferase was also expressed in the 293 cell line infected with BAd3-Luc for at least 6 days post-infection as monitored by luciferase assays. Based on these observations we suggest that BAd-based expression vectors should have excellent potential for the development of recombinant vaccines for cattle and may also be suitable as vectors for gene transfer into human cells.

Introduction

Bovine adenoviruses (BAdVs) are usually involved in subclinical respiratory and enteric infections in cattle; however, occasionally they may lead to clinical symptoms (Darbyshire, 1968; Mattson, 1973; Stott et al., 1980). BAd type 3 (BAd3) is the best characterized of the BAd serotypes and contains a genome of approximately 35 kb (Elgadi & Haj-Ahmad, 1992; Mittal et al., 1992). The regions encoding hexon (Hu et al., 1984), proteinase (Cai et al., 1990), early region 1 (El; Elgadi et al., 1993; Zheng et al., 1994), E3 and fibre (Mittal et al., 1992, 1993a) proteins in the BAd3 genome have been mapped and sequenced.

Adenoviruses have been shown to be excellent mammalian cell expression vectors and good candidates as recombinant viral vaccines (Berkner, 1988; Graham, 1990; Graham & Prevec, 1992). Adenoviruses can package approximately 105% of the wild-type (wt) human adenovirus type 5 (HAd5) genome (Ghosh-Choudhury et al., 1987; Bett et al., 1993), so that up to 1.8 kb of foreign DNA can be inserted into the HAd5 genome without any compensating deletion. To increase the amount of foreign DNA that can be inserted into HAdVs, two deletable regions, E1 and E3, have been extensively exploited in the generation of recombinant adenoviruses (Berkner & Sharp, 1984; Haj-Ahmad & Graham, 1986). E1 proteins are essential for virus replication in tissue culture but conditional helper-independent adenovirus recombinants containing foreign DNA in the E1 region can be propagated in the 293 cell line which constitutively expresses E1 (Graham et al., 1977). In contrast, E3 gene products are not required for in vitro or in vivo infectious virion production (Ginsberg et al., 1989) and helper-independent recombinant adenoviruses with foreign genes in the E3 region replicate very well in permissive cell lines (Johnson et al., 1988; Dewar et al., 1989; Lubeck et al., 1989; Chanda et al., 1990; Prevec et al., 1990; Mittal et al., 1993b). However, E3 gene products are involved in host immune responses to virus infection (Andersson et al., 1985; Burgert & Kvist, 1985, 1987; Gooding et al., 1988; Carlin et al., 1989; Tollefson et al., 1991; Wold & Gooding, 1991).

It seems likely that BAd vectors would be better suited for use as live recombinant virus vaccines in domestic farm animals than would an adenovirus of human origin. Development of vectors based on a non-human adenovirus have not previously been reported. If the E3 region of BAd is non-essential for virus replication in
(a) pSM17 (7.7 kb) 

Luciferase gene from pSVOA/L

BamHI

pSM41-Luc (11.7 kb) 

Luciferase

Klenow fragment

BamHI fragment

pSM44del2 (5.0 kb) 

pUC18

BamHI

fragment

(b) pSM43 (10.7 kb)

pSM51 (29.3 kb)

XhoI-NcoI deletion

Klenow fragment

NruI-SalI linker insertion

Fig. 1. For legend see opposite.
cultured cells, it should be possible to generate helper-independent BAd recombinants. We have previously sequenced the E3 region of BAd3 and identified the flanking sequences encoding the fibre protein and the C terminus of protein VIII (pVIII; Mittal et al., 1992, 1993a). To determine whether BAd3 E3 gene products are dispensable for virus growth in tissue culture, we substituted a 696 bp E3 deletion with a 1.7 kb fragment containing the firefly luciferase gene. The luciferase gene serves as a highly sensitive reporter gene when introduced into the E3 region as was shown previously with HAd5-Luc recombinants (Mittal et al., 1993b; S. K. Mittal and others, unpublished results). Here we report the construction of a BAd3 recombinant containing the luciferase gene as an E3 substitution and demonstrate that the vector can express luciferase in infected cells.

**Methods**

**Cells and viruses.** Cell culture media and reagents were obtained from Gibco-BRL. Media were supplemented with 25 mm-HEPES and 50 μg/ml gentamicin. Madin-Darby bovine kidney (MDBK) cells and MDBC cells transformed with a plasmid containing BAd3 E1 sequences (S. K. Mittal and others, unpublished results) were grown in MEM supplemented with 10% fetal bovine serum. We BAd3 and BAd3-luciferase recombinants were grown and titrated in MDBK cells.

**Enzymes, bacteria and plasmids.** Restriction and other enzymes required for DNA manipulations were purchased from Pharmacia, Boehringer-Mannheim, New England Biolabs or Gibco-BRL and used according to the manufacturer's instructions. Restriction enzyme fragments of BAd3 DNA were inserted into pUC18 or pUC19 (Yanisch-Perron et al., 1985) following standard procedures (Sambrook et al., 1989). Escherichia coli strain DH5 was transformed with recombinant plasmids by electroporation (Dower et al., 1988). Plasmid DNA was prepared using the alkaline lysis procedure (Birnboim & Doly, 1978) and was purified by isopycnic centrifugation in caesium chloride-ethidium bromide gradients. The plasmid pSVO/L containing the entire cDNA encoding firefly luciferase (de Wet et al., 1987), was a gift from D. R. Helinski (University of California, San Diego, Calif., USA).

**Construction of recombinant BAd3.** In an attempt to establish a bovine cell line with properties analogous to human 293 cells, MDBK cells were transformed with a plasmid containing BAd3 E1 sequences and the neomycin resistance gene. A number of neomycin-resistant colonies were isolated in the presence of G418, expanded and tested for the expression of BAd3 E1 messages by Northern blot analyses using a 32P-labelled DNA probe containing only the BAd3 E1 sequences. A BAd3 E1-transformed bovine cell culture (MDBK3.3) monolayer in 60 mm dishes was cotransfected with 5–10 μg of PvuI-digested BAd3 DNA and 5–10 μg of the plasmid pSM31-Luc (Figs 1 and 2) per dish using a lipofection-mediated cotransfection protocol (Gibco-BRL). The virus plaques produced following cotransfection were isolated, plaque-purified and the presence of the luciferase gene in the BAd3 genome was confirmed by agarose gel electrophoresis of recombinant virus DNA digested with appropriate restriction enzymes.

**Luciferase assays.** Enzymatic assays for luciferase activity were carried out essentially as described previously (Mittal et al., 1993b). Briefly, MDBK cell or 293 cell monolayers in 25 mm multi-well dishes (Corning Glass) were infected with BAd3-luciferase recombinants at a m.o.i. of 50 p.f.u. per cell. At the indicated times post-infection, recombinant virus-infected cell monolayers were washed once with PBS (0.137 M-NaCl, 2.7 mM-KCl, 8 mM-Na₂HPO₄, 1.5 mM-KH₂PO₄), harvested in 1 ml luciferase extraction buffer (100 mM-potassium phosphate pH 7.8, 1 mM-DTT) and centrifuged in a microfuge at 13000 r.p.m. for 2 min to pellet cells. The virus-infected

![Fig. 1. Construction of BAd3 E3 transfer vector containing the firefly luciferase gene. (a) The 3.0 kb BamHI 'D' fragment of the BAd3 genome between m.u. 77.8 and 864, which contains almost the entire E3 region (Mittal et al., 1992, 1993a) was isolated and cloned into pUC18 at the BamHI site to obtain pSM14. Similarly, the 50 kb BamHI 'C' fragment of BAd3 DNA from m.u. 864 to 100 was isolated and inserted into pUC18 at the BamHI–HincII site to produce pSM17. To delete a 696 bp XhoI–NcoI fragment from E3 of the BAd3 genome, pSM14 was cleaved with XhoI and NcoI, the larger fragment was purified, the ends made blunt with the Klenow fragment of DNA polymerase I and a NruI–SalI linker inserted to generate pSM14del2. To obtain pSM41 the 2.3 kb BamHI fragment containing BAd3 sequences, an E3 deletion and NruI and SalI cloning sites from pSM14del2 was inserted into pSM17 at the BamHI site. A 1716 bp fragment containing the firefly luciferase gene (de Wet et al., 1987) was isolated by digesting pSV0A/L (provided by D. R. Helinski) with BamI and SphI as described elsewhere (Mittal et al., 1993b) and the ends were made blunt with the Klenow fragment. The luciferase gene was inserted into pSM41 at the SalI site by blunt-end ligation. The resultant plasmid was named pSM41-Luc and contained the luciferase gene in the same orientation as the E3 transcription unit. The plasmid pKN30, containing the kanamycin resistance gene (kan'), was digested with XbaI and inserted into pSM41-Luc (partially cleaved with XbaI) at a XbaI site present within the luciferase gene to obtain pSM41-Luc-Kan. (b) The 18.5 kb XhoI 'A' fragment of the BAd3 genome, which falls between m.u. 31.5 and 84.3, was cloned into pUC18 at the XbaI site to give pSM21. The 3 kb BamHI fragment from plasmid pSM14 was isolated and inserted into pSM17 at the BamHI site to generate pSM43. An 18.5 kb XhoI fragment was purified from pSM21 after cleavage with XbaI and inserted into pSM43 at the XbaI site to generate pSM51. A 7.7 kb BamHI fragment containing the luciferase gene and kan' gene was isolated after digesting pSM41-Luc-Kan with BamHI, and this fragment was ligated to the partially BamHI-digested pSM51 to isolate pSM51-Luc-Kan in the presence of ampicillin and kanamycin. Finally the kan' gene was deleted from pSM51-Luc-Kan by partial cleavage with XbaI and religation to obtain pSM51-Luc. Plasmids are not drawn to scale.]
cell pellets were resuspended in 200 µl of luciferase extraction buffer and lysed by three cycles of freezing and thawing. After centrifugation at 13000 r.p.m. in a microfuge for 10 min at 4°C, the supernatants were assayed for luciferase activity. For the luciferase assay, 20 µl of undiluted or serially diluted cell extract was mixed with 350 µl of luciferase assay buffer (25 mM-glycylglycine pH 7.8, 15 mM-MgCl₂, 5 mM-ATP) in a 3-5 ml tube (Sarstedt). The luminometer (PicoLite Luminometer; Packard) used in the present study was programmed to inject 100 µl of luciferin solution (1 mM-luciferin in 100 mM-potassium phosphate buffer pH 7.8) into the tube present in the luminometer chamber to start the enzyme reaction. In the absence of luciferase a background count of approximately 300 to 450 light units was produced in a 10 s reaction time. Known amounts of purified firefly luciferase were used in assays to calculate the amount of active luciferase present in each sample. One pg of luciferase produced approximately 40000 light units.

Western blotting. Mock or virus-infected MDBK cells were lysed in 1:2 diluted 2 x loading buffer (80 mM-Tris–HCl pH 6.8, 0.97 M-urea, 25% glycerol, 2.5% SDS, 1 mM-mercaptoethanol, 0.001% bromophenol blue), boiled for 3 min and then centrifuged to pellet cell debris. Proteins were separated by SDS–PAGE on 0.1% SDS 10% polyacrylamide gels (Laemmli, 1970). After the end of the run, polypeptide bands in the gel were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad). The membrane was incubated at room temperature for 2 h with 1:4000-diluted rabbit anti-luciferase antibody (Mittal et al., 1993b). The binding of anti-luciferase antibody to the specific protein band(s) on the membrane was detected with 1:5000-diluted horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) and with an ECL Western blotting detection system (Amersham).

**Results**

We assumed that BAd3 would be similar to HAd5s in terms of packaging capacity and the role of E3 in virus replication. Thus we began the development of BAd3-based vectors by making a deletion of E3 sequences and substituting them with a cloning linker for introduction of foreign DNA inserts. Sequencing across the E3 region of BAd3 revealed that it was approximately 1.5 kb, about half the size of E3 of HAd2 or HAd5 (Mittal et al., 1992, 1993a). Therefore, the maximum possible size of E3 deletion in the BAd3 genome was expected to be correspondingly smaller compared to HAd5 E3 deletion vectors. Because we selected a small E3 deletion for our first series of vectors we needed to insert a relatively small reporter gene so that its size remained within the expected insertion capacity of the vector. The firefly luciferase gene was chosen for the following reasons: (i) highly sensitive assays exist to measure luciferase expression; (ii) the relatively small size of the luciferase gene, approximately 1.7 kb, should be within the packaging capacity of the BAd3 genome and (iii) inserts without an exogenous promoter have been shown to be expressed at high levels when inserted in E3 of HAd5 (S. K. Mittal and others, unpublished results).

Attempts to isolate a BAd3 recombinant in two different bovine cell lines, which support the formation of infectious virus progeny following BAd3 DNA-
mediated transfection, were unsuccessful. The 293 cell line, human fetal kidney cells transformed with HAd5 DNA and which express E1, is an excellent host for HAd5 DNA transfection but 293 cells are non-permissive for BAδ3 replication (S. K. Mittal and others, unpublished results). Therefore, we attempted to construct a comparable bovine cell line. A number of bovine cell lines expressing BAδ3 E1 were isolated following transformation of MDBK cells with the BAδ3 E1 sequences and one of these cell lines, MDBK3.3, was used to generate BAδ3-luciferase recombinants.

Construction of BAδ3-luciferase recombinants

The construction of a BAδ3 E3 transfer vector containing the luciferase gene in place of a 696 bp *XhoI*-NcoI deletion (between m.u. 78.8 and 80.8) in the E3 coding region is described in detail in Fig. 1 (a, b). The firefly luciferase gene without any flanking regulatory sequences was inserted into the E3 locus of BAδ3 in the same orientation as the E3 transcription unit. The final E3 transfer vector, pSM51-Luc contained BAδ3 sequences between m.u. 31.5 and 100 with a 696 bp E3 deletion substituted by the 1.7 kb luciferase gene.

BAδ3 DNA was digested with *PvuI*, which makes two cuts within the BAδ3 genome at m.u. 65.7 and 71.7, and cotransfected with the plasmid pSM51-Luc into MDBK3.3 cells (Fig. 2a). Recombination between these DNA molecules will produce a BAδ3 recombinant virus having the luciferase gene in the E3 region. The digestion of the wt BAδ3 DNA with *PvuI* minimized the generation of wt virus plaques following cotransfection.

Eight virus plaques were obtained in four independent cotransfection experiments. These virus isolates were expanded in MDBK cells. The viral DNA from isolated viruses was extracted and analysed by agarose gel electrophoresis after digestion with either *BamHI*, *EcoRI* or *XbaI* to identify recombinant viruses containing the luciferase gene in the viral genome (data not shown). Two virus isolates obtained in two independent cotransfection experiments, which were found to contain the luciferase gene, were selected for further investigation. These recombinant viruses were plaque-purified and named BAδ3-Luc (3.1) and BAδ3-Luc (3.2) to represent plaques obtained from two independent experiments. Since both recombinant virus isolates were identical they are referred to as BAδ3-Luc. The luciferase gene, without flanking control sequences, was inserted into BAδ3 E3 following blunt-end ligation of an intermediate E3 transfer plasmid, pSM41, cleaved with *SalI*, and a 1716 bp *BsmI*-SspI fragment containing the luciferase gene. We were not sure, therefore, whether luciferase was expressed as a fusion protein with part of an E3 protein. To explore this possibility we sequenced the DNA at the junction of the luciferase gene and the BAδ3 E3 sequences in the plasmid pSM51-Luc using a synthetic primer that annealed to luciferase coding sequences near the initiation codon (data not shown). The luciferase coding region was present in the BAδ3 E3 open reading frame (ORF), F2 (Fig. 2b) and the luciferase initiation codon was the first start codon in this ORF. The presence of the luciferase gene in both BAδ3-Luc isolates was further confirmed by Southern blot analyses and luciferase assays using extracts from recombinant virus-infected cells.

Characterization of BAδ3 recombinants

Southern blot analysis of the genomic DNA of wt BAδ3 and recombinants, digested either with *BamHI*, *EcoRI* or *XbaI*, was carried out using sequences containing the luciferase gene as a probe to confirm the presence and orientation of the luciferase gene in the E3 locus (Fig. 3). There were strong hybridization signals with the DNA fragments from the recombinant viruses [Fig. 3; 4.0 kb *BamHI* fragment (lane 4 and 7); 6.0 and 3.2 kb *EcoRI* fragment (lane 6 and 9)].
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Fig. 4. (a) Single-step growth curve for wt BAd3 and BAd3-Luc. Confluent monolayers of MDBK cells in 25 mm multi-well culture plates were inoculated with the wt BAd3 (○) BAd3-Luc (3.1) (●) or BAd3-Luc (3.2) (□) at a m.o.i. of 10 p.f.u. per cell. The virus was allowed to adsorb for 1 h at 37 °C, cell monolayers were washed three times with PBS (0.137 M-NaCl, 2.7 mM-KCl, 8 mM-Na2HPO4, 1.5 mM-KH2PO4, 0.01% CaCl2 . 2H2O and 0.01% MgCl2 . 6H2O) and incubated at 37 °C in 1 ml of maintenance medium containing 2% horse serum. At various times post-infection, cells were harvested along with the supernatant, frozen and thawed three times and titrated on MDBK cells by plaque assay. Results are the means of duplicate samples. (b) Kinetics of luciferase expression in BAd3-Luc-infected MDBK cells. Confluent MDBK cell monolayers in 25 mm multi-well culture plates were infected with BAd3-Luc (3.1) (●) or BAd3-Luc (3.2) (□) at a m.o.i. of 50 p.f.u. per cell. At indicated time post-infection, virus-infected cells were harvested and assayed in duplicate for luciferase activity.

fragments (lanes 5 and 8); 16.7 and 2.9 kb XbaI fragments (lanes 6 and 9). When the blot was probed with the 696 bp XhoI–NcoI fragment of E3 of the BAd3 genome, the expected bands (3.0 kb BamHI, 8.1 kb EcoRI and 18.5 kb XbaI fragments) of the wt BAd3 DNA showed hybridization. However, no signal was detected with the DNA fragments from the recombinant viruses, confirming that the 696 bp XhoI–NcoI fragment of the E3 region was indeed deleted in BAd3-Luc genomic DNA (data not shown). These results confirm that the BAd3-Luc contained the luciferase gene in the E3-parallel orientation, with a 696 bp XhoI–NcoI E3 deletion.

We also compared the growth characteristics of the recombinant viruses with the wt BAd3 in a single-step growth curve (Fig. 4a). Titres of infectious virus in MDBK cells infected with the wt BAd3 started increasing at 12 h post-infection, reached a maximum at 36–48 h post-infection and then declined slightly. The recombinant viruses replicated with similar kinetics but reached maximum titres that were approximately 10-fold lower than wt virus. The plaque size of the recombinant viruses was also smaller than with wt virus (data not shown).

**Kinetics of luciferase expression by BAd3-Luc**

Luciferase activity in BAd3-Luc-infected MDBK cells was monitored at various times post-infection by luciferase assays as described in Methods (Fig. 4b). A low level of luciferase activity was first observed at 12 h post-infection, reached a maximum at 30 h post-infection and subsequently decreased. A maximum of approximately 1000 pg luciferase was detected in 10⁶ BAd3-Luc (3.1)-infected MDBK cells. In MDBK cells infected with the wt BAd3, luciferase expression was not detected (data not shown). The kinetics of luciferase expression by BAd3-Luc (3.1) and BAd3-Luc (3.2) were essentially identical (Fig. 4b), with the bulk of enzyme expression occurring late in infection when compared with the wt BAd3 and BAd3-Luc growth curves (Fig. 4a). To determine whether luciferase expression occurred in the absence of viral DNA replication, BAd3-Luc-infected MDBK cells were incubated in the presence of an inhibitor of DNA synthesis, 1-β-D-arabinofuranosylcytosine (AraC). Luciferase activity was then measured in virus-infected cell extracts at various times post-infection and compared to luciferase expression obtained in the absence of AraC (Fig. 5). In the presence of AraC, viral DNA synthesis was approximately 1% of the viral DNA synthesis in its absence (data not shown). When the recombinant virus-infected cells were incubated in the presence of AraC, luciferase expression at 18, 24 and 30 h post-infection was approximately 20–30% of the value obtained in the absence of AraC. These results
indicated that approximately 70–75% of luciferase expression in MDBK cells infected with BAd3-Luc was dependent on viral DNA synthesis.

The levels of luciferase expression in 293 cells infected with BAd3-Luc was detectable at 12 h post-infection, reached a maximum level at 3 days post-infection and then slowly declined. However, even at 6 days post-infection the level of luciferase expression was approximately 60% of the maximum level (Fig. 6). A peak value of approximately 115 pg luciferase per 10^6 cells was reached at 3 days post-infection, which was approximately 10% of the maximum enzyme expression obtained in MDBK cells. Since 293 cells constitutively express HA5 E1 it can be argued that HA5 E1 may be trans-activating the BAd3 E3 promoter and/or the major late promoter (MLP) to yield luciferase expression in 293 cells. However, the kinetics of luciferase expression by BAd3-Luc in 293 cells shows that BAd3 recombinants do infect human cells in culture resulting in foreign gene expression. Presently we are monitoring luciferase expression by BAd3-Luc in a number of BAd3-nondemissive cell lines of human, monkey and mouse origin and are also examining the role of constitutively expressed BAd3 E1 in augmenting enzyme expression by BAd3-Luc in BAd3-permissive cell lines.

![Fig. 5. Luciferase expression in the presence of AraC in MDBK cells infected with BAd3-Luc. Confluent MDBK cell monolayers in 25 mm multi-well culture plates were infected with BAd3-Luc (3.1) at a m.o.i. of 50 p.f.u. per cell and incubated in the absence (□) or presence (□) of 50 μg arabinofuranosylcytosine/ml of maintenance medium. At indicated times post-infection, virus-infected cells were harvested and assayed in duplicate for luciferase activity. Similar results were obtained with the BAd3-Luc (3.2) recombinant (data not shown).](image1)

![Fig. 6. Kinetics of luciferase expression in the 293 cell line infected with BAd3-Luc. Confluent 293 cells monolayers in 25 mm multi-well culture plates were infected with CsCl density gradient-purified BAd3-Luc (3.1) at a m.o.i. of 50 p.f.u. per cell. At indicated times post-infection, virus-infected cells were harvested and assayed in duplicate for luciferase activity.](image2)

Western blot analysis of BAd3-Luc-infected cells

The above experiments showed that luciferase was expressed as an active enzyme in MDBK cells infected with BAd3-Luc. To confirm that luciferase protein produced by BAd3-Luc was of the same molecular mass...
as purified firefly luciferase, BAd3-Luc-infected, wt BAd3-infected or mock-infected MDBK cell extracts were reacted with an anti-luciferase antibody in a Western blot (Fig. 7). A band was visible in the BAd3-Luc-infected cell extracts (lanes 3 and 4) at approximately the same molecular mass (62 kDa) as pure firefly luciferase (lane 5).

Discussion

Adenovirus-based mammalian cell expression vectors have gained considerable importance in the last few years as possible vehicles for recombinant vaccine delivery and also for gene therapy. All such vectors currently under study are based on HAds, mainly HAd5. We are interested in extending the range of available vectors to other animal adenoviruses. BAd3-based expression vectors, in particular, may have potential for developing novel recombinant vaccines for veterinary use. The main objective of this study was to show that BAd3 E3 gene products are not essential for virus growth in cultured cells and that this locus could be used to insert foreign DNA sequences. As a first step in the development of BAd3-based vectors we developed a BAd3 E1-transformed bovine cell line suitable for transfection with BAd3 DNA and then constructed the vector BAd3-Luc which contains the firefly luciferase gene in the E3 region of the BAd3 genome in the E3-parallel orientation.

Luciferase was expressed as an active enzyme in BAd3-Luc-infected cultured cells. In the presence of AraC, luciferase activity was approximately 25–30% of the value obtained in the absence of AraC at 18, 24 or 30 h post-infection, indicating that the majority of luciferase expression was dependent on viral DNA synthesis. This suggested that the majority of luciferase expression is driven from the MLP, resulting in expression kinetics that parallel viral late gene expression. The reduced but significant levels of expression seen in the presence of AraC may be due to transcripts originating from the E3 promoter. In HAd5 vectors, the luciferase gene in E3 without any exogenous regulatory sequences displayed late kinetics and was strongly inhibited by AraC (S. K. Mittal and others, unpublished results). The bacterial β-galactosidase gene, when inserted into E3 of HAd5, exhibited both early and late expression kinetics comparable to the present study (S. K. Mittal and others, unpublished results).

The BAd3 recombinant virus replicated less efficiently than the wt BAd3 in cell culture, resulting in titres of infectious virus progeny approximately 10-fold lower than the wt BAd3. Infectious virus titres of some HAd5 recombinants were slightly lower than the wt HAd5 (Bett et al., 1993; S. K. Mittal and others, unpublished results) but reductions were not as low as observed with BAd3-Luc. This difference may be due to reduced expression of fibre protein in recombinant adenoviruses with inserts in the E3 region, compared to the wt virus (Bett et al., 1993; Mittal et al., 1993b). At present we are not sure whether the insertion of foreign genes in BAd3 E3 affects fibre production. It has been shown that up to 105% of the wt genome can be packaged into the HAd5 virion and such viruses do not rearrange or delete the foreign inserts at high frequency in successive rounds of replication (Ghosh-Choudhury et al., 1987; Bett et al., 1993). Our present BAd3-based vector has a 0.7 kb E3 deletion which theoretically could permit an insert up to approximately 2.5 kb in size if the BAd3 virion is also able to package up to 105% of the wt genome. The BAd3 E3 deletion could be extended up to probably 1.4 kb, which would increase the insertion capacity of this system by a further 0.7 kb.

Luciferase expression by BAd3-Luc was approximately 1000-fold lower than a similar construct in a HAd5 vector (S. K. Mittal and others, unpublished results). This may be partially due to differences in the growth characteristics between the recombinant and the wt virus. Additionally, a downstream non-canonical poly(A) signal (ATAAA) of E3 transcripts (Mittal et al., 1992) may fail to provide a suitable poly(A) signal for the luciferase transcript. In this case the expression of a foreign gene inserted into E3 of BAd3 may be improved by introduction of a poly(A) signal such as that of simian virus 40. Presently we are continuing research into answering these questions and establishing conditions for optimum expression by the BAd3-based expression system.

The E3 region of BAd3 is approximately half the size of the E3 region of HAd2 or HAd5 and may encode only half the number of proteins compared to E3 of HAd2 or HAd5 (Hérissé et al., 1980; Hérissé & Galibert, 1981; Cladaras & Wold, 1985; Mittal et al., 1992, 1993a). One of the BAd3 E3 ORFs has been shown to have amino acid homology with the 14.7 kDa E3 protein of HAds (Mittal et al., 1992, 1993a) which prevents lysis of virus-infected mouse cells by tumour necrosis factor (Goding et al., 1988; Horton et al., 1990). The E3 19 kDa glycoprotein (gp19) of HAd reduces expression of class 1 major histocompatibility complex antigens on the surface of infected cells (Andersson et al., 1985; Burgert & Kvist, 1985, 1987) and mutants having deletions in the gp19 ORF produced significantly greater pathogenic effects than wt virus in experimental animals (Ginsberg et al., 1989). The E3 10.4 kDa and E3 14.5 kDa proteins of HAd have been shown to be involved in down-regulation of the epidermal growth factor receptor in virus-infected cells (Carlín et al., 1989; Tollefson et al., 1991). We have shown that BAd3 E3 gene products are not essential for
virus growth in tissue culture but it is not yet known whether BAd3 E3 gene products also act to allow virus to evade immune surveillance in vivo. The study of pathogenesis and immune responses of a series of BAd3 E3-deletion mutants in cattle should provide useful information regarding the role of E3 gene products in modulating these events in their natural host.

The generation of BAd-based expression vectors for the production of recombinant vaccines for farm animals is very promising. Since BAds infect the respiratory and gastrointestinal tracts of cattle, recombiant BAd-based vaccines might have considerable potential to provide a protective mucosal immune response, in addition to humoral and cellular immune responses, against a wide variety of pathogens of cattle.

HAd-based expression vectors are presently being studied for their potential use in human gene therapy (Stratford-Perricaudet et al., 1990; Rosenfeld et al., 1991, 1992; Ragot et al., 1993). A preferable adenovirus vector for gene therapy would be one which maintains expression of the required gene for indefinite or extended periods in the target organ without lytic infection. This may best be achieved by a virus which does not grow in a variety of pathogens of cattle.

Adenovirus genomes have a tendency to form circles in non-permissive cells (Ruben et al., 1981) and, if BAd3 does the same in human cells, it is possible that recombinant BAd3 genomes may persist and provide expression of inserted genes for extended periods of time. As adenovirus E1 has oncogenic potential, we are in the process of testing the state of BAd3 recombinant DNA in human cells with the anticipation of using BAd3 E1-deletion vectors in human gene therapy. Expressions of luciferase for at least 6 days post-infection in 293 cells infected with BAd3-Luc indicates that BAd3-based vectors have potential in this field.

References


(Received 1 June 1994; Accepted 25 August 1994)