155R is a novel structural protein of bovine adenovirus type 3, but it is not essential for virus replication

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Abstract

Bovine adenovirus (AdV) type 3 (BAdV-3) E1 region shares functional homology with E1 of human AdV type C5. Sequence analysis of the BAdV-3 E1 region revealed the presence of a novel 155R ORF that is not observed in other AdVs, on the lower strand antiparallel to a portion of the E1B region. The 155R gene products in BAdV-3-infected cells were identified by Northern blot, reverse transcriptase PCR followed by sequencing and Western blot analysis using the 155R-specific antibody. 155R seems to be a late protein and is present in purified BAdV-3 particles. Replication kinetics of BAdV mutants with either one (BAdV/155R/mt1) or two (BAdV/155R/mt2) stop codons in the 155R ORF were comparable to those of BAdV-3, indicating that 155R is not essential for virus replication in cell culture. These results suggest that 155R-deleted BAdV-3 vectors could be generated in a cell line that fully complements BAdV-3 E1 functions.

Adenoviruses (AdVs) are non-enveloped dsDNA viruses with icosahedral symmetry in the family Adenoviridae. In mammalian hosts, they are involved in inapparent or subclinical infections to respiratory illness, conjunctivitis, gastroenteritis or systemic infections. AdVs have demonstrated incredible potential as gene delivery vector systems for recombinant vaccines and gene therapy applications [1, 2].

Hexon, penton base and fibre constitute the major capsid proteins, whereas the minor capsid proteins (IIa, VI, VIII and IX) connect the major capsid proteins with each other and with the viral core [3, 4]. The viral core consists of the dsDNA genome and five proteins: V, VII, µ, terminal protein and the viral protease. In addition, IVa2 and E2 72K proteins are involved in genome packaging and are present in the purified infectious AdV particles [5].

Bovine AdVs (BAdVs) are represented by 10 types (BAdV-1–10) and BAdV-3 is the most studied among all BAdVs. BAdV-3 was first isolated in 1967 from a healthy cow, but it is considered as a pathogen that can cause inapparent to mild respiratory or enteric infections in cattle [6, 7]. It is classified under the genus Mastadenovirus and the type species Bovine mastadenovirus B [8]. The structural genes and their arrangements on the BAdV-3 genome are similar to those of human AdV type C5 (HAdV-C5) [9]. A noticeable difference is in the BAdV-3 fibre which is significantly longer than the HAdV-C5 fibre and is bent at several locations [10, 11]. However, the number of non-structural genes in the early 3 (E3) region of BAdV-3 is considerably lower than that in the HAdV-C5 E3 region [10]. BAdV-3 E1 region is similar to that of HAdV-C5 E1, and BAdV-3 E1 proteins (E1A, E1B-157R and E1B-420R) demonstrate amino acid sequence or functional homologies with the corresponding E1 proteins of HAdV-C5 [12]. Since E1A is essential for virus replication and BAdV-3 E1A can complement HAdV-C5 E1A functions [12], bovine origin cell lines expressing HAdV-C5 E1 were isolated to generate and grow E1A-deleted replication-defective BAdV-3 vectors [13, 14]. BAdV-3-based vectors appear to have immense potential to supplement or to serve as an alternate to HAdV vectors for a number of reasons. BAdV-3 vectors are capable of overcoming very high levels of pre-existing HAdV-specific neutralizing antibodies (‘vector immunity’) [15], since HAdV-specific humoral and cell-mediated immune
responses do not cross-react with BAdV-3 [16]. In vitro and in vivo tropism of BAdV-3 is distinct from that of HAdV-C5 [17, 18]. BAdV-3 internalization is not via HAdV-C5 receptors (coxackievirus and adenovirus receptor and αvβ3- or αvβ5-integrin) [19], but it uses α(2,3)-linked as well as α(2,6)-linked sialic acid as a major receptor for virus entry [20].

To increase the foreign gene insertion capacity of BAdV-3, we tried to extend the E1A deletion to the E1B region. Repeated attempts were unsuccessful in generating infectious virus particles in bovine cell lines expressing HAdV-C5 E1, suggesting that either the HAdV-C5 E1B region does not support BAdV-3 E1B functions or another essential gene is present in this region of the BAdV-3 genome. Sequence analysis of the BAdV-3 E1 region demonstrated the presence of a potential 155R novel ORF on the lower strand antiparallel to the E1B region partially overlapping E1B-157R and E1B-420R ORFs (Fig. 1a). The 155R ORF on the lower strand has a start codon at nt 2146 and has a stop codon at 1679 nt. The Kozak consensus sequence (AXXATGG) that was found to be necessary for the efficient translation in eukaryotes [21] was also noticed in the 155R sequence around nt 2146 

420R ORFs (Fig. 1a). The 155R ORF on the lower strand has a start codon at nt 2146 and has a stop codon at 1679 nt. The Kozak consensus sequence (AXXATGG) that was found to be necessary for the efficient translation in eukaryotes [21] was also noticed in the 155R sequence around nt 2146 (AXXATGg) as well as around nt 2056 (AXXATGG). However, the linear discriminant function score for the prediction of translation initiation codons in cDNA sequences is low for nt 2056 (16%) compared to nt 2146 (67%) [22], indicating the higher probability of the use of 2146 position for initiation of translation. There is a presence of a potential TATA box (TTATGTT) at nts 2204-2197 and a potential polyadenylation signal (TTAAAAAA) at nts 1162-1155. The 155R gene product is predicted to be a 155 amino acid protein. A disulfide bond is predicted to be formed between two cysteine residues which are located at positions 82 and 143 [23]. No potential signal peptide was identified when the sequence was examined with SignalP [24], suggesting that 155R is not a transmembrane protein. The 155R sequence was also examined using InterProScan [25] for specific patterns and domains that might imply other molecular characteristics. No specific domains or sequence patterns were detected in the 155R sequence. There are two potential N-glycosylation sites predicted to be at Asp 127 and 149 using NetNGlyc 1.0 [26], whereas, 18 potential O-glycosylation sites are predicted using NetOGlyc 4.0 [27].

To determine whether the 155R ORF is expressed in BAdV-3-infected cells, virus-infected Madin–Darby bovine kidney (MDBK) cells were harvested at 12 and 24 h post-infection (p.i.), the total cellular RNA was extracted and treated with RNase-free DNase to get rid of the viral DNA. The presence

![Fig. 1. Identification of 155R as a novel structural protein of BAdV-3. (a) Diagrammatic representation of 155R and known ORFs in and near the E1 region of BAdV-3. The nucleotide position is shown at the top. The arrow denotes the direction of the ORF. ITR, inverted terminal repeat. The size and location of E1A deletion in BAdV-ΔE1AE3 is also indicated. (b) 155R expression kinetics in BAdV-3 infected cells. MDBK cells were either mock infected or infected with WT BAdV-3 at an m.o. of 5 p.f.u. and at 24 and 48 h p.i., the infected cells were harvested. Cell extracts were prepared and run on SDS-PAGE under reducing (upper blot) or non-reducing (lower blot) conditions and analysed for 155R expression by Western blot using the 155R-specific antibody. The molecular weight markers are shown on the left. (c) Presence of 155R in purified WT BAdV-3 or BAdV-ΔE1AE3 particles. A total of 1 or 10 µg of purified WT BAdV-3 or BAdV-ΔE1AE3 was processed for the Western blot analysis with the 155R-specific antibody. The molecular weight markers are shown on the left. (d) Locations of stop codons that were introduced in 155R to generate BAdV-3 mutants having one or two stop codons in the ORF of 155R. Amino acid residues of 155R are shown. The locations of stop codons are depicted by underlines, where Ala and Thr codons at positions nt 2096 and 2018 were replaced by stop codons. A stippled underline represents the amino acid residues of the immunogenic epitope that was used for raising the 155R-specific antibody.
of 155R-specific transcript was identified by the Northern blot analysis using a 155R-specific single-stranded synthetic probe (data not shown). DNA-free total cellular RNA samples from virus-infected cells were amplified by reverse transcriptase (RT)-PCR using a 155R-specific primer set with the following sequence: forward primer (nts 2113–2095): AAGGCAAAGCCTCAGTCGTC, and reverse primer (nts 1712–1731): TGAGCTGACATCTGGACACT. The amplified DNA bands were observed only in RNA samples from virus-infected cells but not in mock-infected cells (data not shown). There was no amplification when the same RNA samples and the 155R-specific primer set were used in PCR without prior reverse transcription, suggesting that the amplification by RT-PCR was not due to viral DNA contamination. The RT-PCR-amplified DNA fragment was sequenced and the nt sequence matched with the expected area of 155R ORF (data not shown). These results at least confirm the transcription of 155R ORF.

To determine 155R protein expression in BAdV-3-infected cells, there was a need to develop the 155R-specific antibody. A 155R peptide representing amino acid residues 101–115 (GTQRSTTRKPEKPE) predicted to be immunogenic was synthesized and linked to keyhole limpet haemocyanin (KLH). The KLH-155R immunogenic peptide complex was used for immunizing a rabbit (New Zealand White). The hyperimmune serum was then purified on a protein A sepharose column to obtain the rabbit anti-155R-specific IgG antibody fraction. The 155R-specific rabbit antibody reacted well with the 155R immunogenic peptide and extracts from BAdV-3-infected cells in a Western blot, and this reactivity can be adsorbed with the peptide treatment (data not shown). To ascertain expression of 155R in BAdV-3-infected cells, MDBK cells were mock infected or infected at an m.o.i. of 5 p.f.u. per cell with WT BAdV-3, and at 24 and 48 h p.i. cells were harvested. Cell extracts were analysed by Western blot using the 155R-specific antibody under reducing (5% β-mercaptoethanol in Laemmli buffer) or non-reducing (Laemmli buffer without β-mercaptoethanol) conditions. Under reducing conditions, a dominant band at approximately 17 kDa was observed both at 24 and 48 h p.i. in WT BAdV-3-infected MDBK cell extracts (Fig. 1b; upper panel). In addition to a 17 kDa band, another prominent band at approximately 35 kDa also appeared in the 48 h sample. The 17 and 35 kDa bands may represent the monomer and dimer forms of 155R protein, respectively. As anticipated, mainly a 35 kDa band was observed with BAdV-3-infected MDBK cell extracts under non-reducing conditions (Fig. 1b; lower panel) with a faint band at 17 kDa in overexposed gels (data not shown). These observations confirmed that 155R protein is expressed in BAdV-3-infected MDBK cells and it is a late protein.

AdV late proteins represent the viral structural components. To determine whether 155R is a structural protein for BAdV-3, BAdV-3 or BAdV-E1AE3 were purified by caesium chloride density-gradient centrifugation and extracts prepared from purified virus particles were analysed by Western blot using the 155R-specific antibody. 155R-specific bands were observed both with extracts of BAdV-3 or BAdV-E1AE3-purified preparations (Fig. 1c), indicating that 155R is a structural protein. Two closely migrating bands that reacted with the 155R-specific antibody may represent 155R with variable secondary modifications. Additional experiments will be required to examine secondary modifications of 155R and its location on the virion.

To establish whether 155R is essential for virus replication, there was a need to generate BAdV-3 mutants affecting 155R expression. First, we needed to generate a cell line that constitutively expressed 155R. The 155R gene was synthesized commercially (Genscript) and was cloned into pcDNA3.1/Hygro (+) (Thermo Fisher Scientific) to generate pCDNA3.1/Hygro/155R. BHH3 (bovine–human hybrid clone 3) [28] cells were transfected with pCDNA3.1/Hygro/155R and a number of stable cell clones were isolated in the presence of hygromycin using cloning cylinders as described earlier [29]. Six independent clones were grown and cell extracts were examined for 155R expression by Western blot using the 155R-specific antibody. All six clones were found to express 155R (data not shown) and clone 5 (BHH3-155R) was used in subsequent studies. Since the 155R ORF overlaps with ORFs of E1B-157R and E1B-420R, we decided to mutate the 155R gene to add either one stop codon (at nt 2096) [155R/mt1] or two stop codons (at nts 2096 and 2018) [155R/mt2] to ensure that these nt changes did not alter the amino acid residue in the ORFs of E1B-157R and E1B-420R that are anti-parallel to the 155R ORF. The mutated 155R gene fragments were synthesized commercially and cloned into pBAdVE1 shuttle vector to generate pBAdVE1/155R/mt1 (stop codon at nt 2096) and pBAdVE1/155R/mt2 (stop codons at nts 2096 and 2018) (Fig. 1d). The full-length infectious clones, pBAdV/155R/mt1 and pBAdV/155R/mt2, were generated by bacterial recombination [30] by transforming BJ5183 Escherichia coli with WT BAdV-3 DNA and pBAdVE1/155R/mt1 or pBAdVE1/155R/mt2. To release BAdV/155R/mt1 or BAdV/155R/mt2 sequences from the plasmid, pBAdV/155R/mt1 and pBAdV/155R/mt2 were digested with PucI, and PacI-digested DNA samples were used to transfect BHH3-155R cells. Viral cytopathic effect was observed in 7–10 days post-transfection. BAdV/155R/mt1 or BAdV/155R/mt2 were grown in BHH3-155R cells for virus purification by caesium chloride density-gradient centrifugation and purified virus preparations were titrated in BHH3-155R cells by plaque assay. Purified BAdV/155R/mt1 or BAdV/155R/mt2 was used to extract genomic DNA and both mutations were confirmed by sequencing (data not shown).

Purified preparations of BAdV/155R/mt1, BAdV/155R/mt2 or WT BAdV-3 were used to infect BHH3 cells and at 48 h p.i., the cells were harvested. Mock or virus-infected cell extracts were analysed for expression of 155R by Western blot using the 155R-specific antibody. The full or truncated forms of 155R were not observed in BAdV/155R/mt1 or BAdV/155R/mt2-infected cell extracts (Fig. 2a).
To further elucidate the role of 155R expression in BAdV-3 replication, purified preparations of WT BAdV-3, BAdV-ΔE1AE3, BAdV/155R/mt1 or BAdV/155R/mt2 were used to infect MDBK, BHH3 or BHH3-155R cell lines. All three viruses, WT BAdV-3, BAdV/155R/mt1 and BAdV/155R/mt2, replicated equally well in all three cell lines (Fig. 2b), suggesting that 155R is not essential for virus replication in cell culture. Both BAdV/155R/mt1 and BAdV/155R/mt2 reached maximum titres at 36 h p.i., whereas the maximum titre with WT BAdV-3 was observed at 48 h p.i. Additional studies will be needed to confirm this information and its implication.

The above results confirm that 155R is a novel structural protein of BAdV-3 and it is not essential for virus replication in cell culture. It has been demonstrated at least in HAdV-C5 that short regions (60–72 or 110–114 amino acid residues) of protein IX [31], the entire fibre [32] or the fibre knob [33, 34] could be deleted without affecting virus replication in cell culture. Since cotton rats (Sigmodon hispidus) support the replication of BAdV-3 and thus serve as a replication-competent small-animal model for evaluating the efficacy of BAdV-3 vector-based vaccines as well as vector pathogenesis [35, 36]. Therefore, comparative in vivo assessment of BAdV/155R/mt1, BAdV/155R/mt2 and WT BAdV-3 in the cotton rat model will be helpful for deciphering the role of 155R in viral pathogenesis.

The above findings also suggest that the failure to generate BAdV-3 E1B deleted vectors in a cell line expressing HAdV-C5 E1A and E1B proteins was not due to the absence of BAdV-3 155R expression. Indirectly, it implies that the failure to rescue BAdV-3 E1B deleted vectors was most likely due to the lack of HAdV-C5 E1B to effectively complement BAdV-3 E1B functions. Further studies will be needed to determine the role of 155R in the BAdV-3 structure and/or viral pathogenesis.

Funding information
This work was supported by the Public Health Service grants – AI059374 from the National Institute of Allergy and Infectious Diseases and CA110176 from the National Cancer Institute, and the Hatch funds.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
No animals were used for the work presented in this manuscript.

References
4. Reddy VS, Nemerow GR. Structures and organization of adenovirus cement proteins provide insights into the role of