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A 72 bp Internal Deletion in the Left Inverted Terminal Repeat of the Bovine Adenovirus Type 3 Genome Does Not Affect Virus Replication

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Abstract

The genome of bovine adenovirus type 3 (BAV3) is flanked by 195 base pair inverted terminal repeats (ITR). We isolated a BAV3 mutant (BAV3c29) having an internal deletion within the left ITR. The deletion eliminated 72 bp between nucleotides nt 89 and 162, including most of the GC-rich sequences located close to the end of the ITR sequences. This deletion did not seem to have any affect on the virus plaque size or morphology and the kinetics of viral replication compared to wild-type (wt) BAV3. The nt sequence of the right ITR of BAV3c29 remained identical to the right or left ITR of wt BAV3. These results indicate that the *cis*-acting sequences present within the 72 bp between nt 89 and 162 of the left ITR are not essential for BAV3 DNA replication in cultured cells.

Keywords

Bovine adenovirus type 3 inverted terminal repeat deletion; adenovirus inverted terminal repeat; adenovirus recombinant

The bovine adenovirus type 3 (BAV3) genome is a linear double-stranded DNA molecule of 34,446 base pairs (bp) [1], flanked by 195 bp inverted terminal repeats (ITR) [2]. The presence of these terminal redundancies has been described for all known adenoviruses [2-7]. The left and the right ITRs are of the abc-c'b'a' conformation, with lengths ranging from 52 bp in the avian egg drop syndrome -76 adenovirus [2] to 238 bp in the porcine adenovirus type 4 [4].

The terminal protein (TP) is attached to the 5' -OH terminus of the genome by a phosphodiester bond [8]. The precursor of the terminal protein covalently binds to the first deoxycytidylyl nucleotide residue (dCMP) of the newly synthesized DNA chain [8]. The protein-bound dCMP then functions as a primer for DNA synthesis.

The ITR nucleotide (nt) sequences that are important in human adenovirus replication include the origin of replication (*ori*) represented by the first 18 or 21 nt and an auxiliary region that extends from nt 36 up to 67 [9]. These two regions contain binding sites for the origin recognition protein (ORP-A), nuclear factor (NF) I (NFI) and NFIII. Between nt 51 and 103, the ITRs of human and simian adenoviruses also contain consensus motifs known as 'GC box' and 5'-TGACGT-3' for the binding of SP1 and ATF, respectively.

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The boundaries of the minimal *cis*-acting sequences in the ITR and the factors involved in viral DNA replication have been extensively studied for human adenoviruses, whereas only sequence comparisons and phylogenetic analyses have been reported for the ITRs of non-human adenoviruses. This short communication describes a 72 bp internal deletion in the left ITR of BAV3 that does not seem to affect virus replication in cultured cells.

Homologous recombination in bacteria is a powerful tool employed for the construction of human and non-human adenovirus recombinants [1,10-12]. We have used this method for the cloning of the full-length BAV3 genome [13], and recently we found out that PCR amplification of the left end of the genome generated a BAV3c29 mutant (fig. 1A). Initial comparison of DNA fragments generated by the *EcoRI* digestion of genomic DNA of wild-type (wt) BAV3 and BAV3c29 showed that the expected left end fragment for BAV3c29 was slightly smaller than that produced by the wt BAV3 genome (fig. 1B). The discrepancy in the size of the left end fragment was further confirmed by *AscI* digestion. The finding of a smaller than expected fragment corresponding to the left end of the BAV3c29 genome prompted us to locate the deletion by DNA sequencing. Nucleotide sequence analyses of the left ITR regions of wt BAV3, BAV3c29 and two plasmids containing the ITR of BAV3c29, pMvOBE1E4*PacI* [13] and pMvOBABV3c29, revealed a 72 bp deletion including nt 91 through 161 of the left ITR of pMvOBE1E4*PacI*, pMvOBABV3c29 and BAV3c29. The left ITR of wt BAV3 was without any deletion. Interestingly, the right ITRs in wt BAV3, pMvOBE1E4*PacI*, pMvOBABV3c29 and BAV3c29 were intact without any compensating deletion. In addition to the 72 bp deletion, the left ITR of BAV3c29 also had nt substitutions at positions 170 (G to T), 172 (G to T) and 173 (C to T). A detailed description and the specific regions of the ITR affected by these mutations are presented figure 2. The left end of wt BAV3 between 1 and 1156 nt was amplified using the same primers described in the legend to figure 1, with a number of changes in the PCR reaction conditions. On the basis of restriction enzyme digestion and agarose gel electrophoresis, all PCR products were slightly shorter than the expected fragment containing the left ITR (data not shown). Our results of restriction enzyme digestion and sequence analyses of wt BAV3, BAV3c29, pMvOBE1E4*PacI* and pMvOBABV3c29 indicated that the internal deletion in the left ITR was due to PCR amplification.

The first 13 bp of the BAV3 ITR are identical to their HAV5 counterpart and may represent the binding site for ORP-A (Fig. 2). A potential binding motif for NF1, TGGN₁₂GCCAAAT is located between 27 and 49 nt. In the BAV3 ITR, the sequences recognized by NFIII do not seem to be present with the same nt composition as in HAV5 ITR; however between 68 and 80 nt, there may be a potential NFIII recognition site. The ITR of BAV3 contains two potential ATF binding sites from 70 to 105 nt and five consensus SP1 binding sites from 142 to 195 nt. The left ITR of BAV3c29 (123 bp long) is still 20 bp longer than that of the HAV5 ITR (103 bp long). The 72-bp deletion removed at least three consensus SP1-binding sites and one potential ATF binding site; thereby, the remaining two SP1 sites came in to closer proximity to the auxiliary region of the left BAV3 ITR.

We also examined the ability of BAV3c29 to replicate in MDBK cells and compared its growth characteristics with those of wt BAV3. The time needed for plaque formation after infection and the size and morphology of plaques were similar to those produced by wt BAV3 (fig. 3). To determine whether the deletion in the left ITR of BAV3c29 affects virus replication, the kinetics of BAV3c29 replication was compared with that of wt BAV3 in MDBK cells infected at an MOI of 2 PFU per cell (fig. 4). The replication kinetics and virus titers of BAV3c29 seemed to be similar to that of wt BAV3. To quantitate the total amount of DNA incorporated into newly synthesized virions of mutant compared to wt BAV3, a fixed number of MDBK cells were infected with BAV3c29 or wt BAV3 at a MOI of 5 PFU per cell, and virus-infected cells were harvested 48 h after infection. The viral samples were purified by cesium chloride

density gradient centrifugation, and the viral DNA from purified virions was extracted using an SDSpronase method [14]. The DNA concentration was monitored by measuring optical density at 260 nm using a spectrophotometer and also by restriction enzyme analysis. Similar amounts of DNA were recovered from purified preparations of BAV3c29 and BAV3 (data not shown) suggesting that BAV3c29 DNA replication was not affected.

The elimination of the 72-bp internal deletion in the BAV3 left ITR did not seem to affect the replication of the viral DNA, probably due to the following reasons. Since both the left and right *ori* are intact, replication of the first strand from each end may not be affected. The first 89 nt that are complementary between opposite ITRs may be sufficient for the formation of the panhandle structure, therefore, synthesis of the second strand by type II replication [15] is not impaired. The deletion did not remove the auxiliary region of the *ori*, and at least one potential ATF-binding site and two Sp1-binding sites were intact.

It has been demonstrated that complete deletion of the ITR of adenoviruses resulted in non-infectious DNA [16,17]. Flanking of ITR sequences rendered the *ori* inactive, and consequently, infectious progeny virions were not produced, indicating that the presence of a free terminal *ori* is essential for initiation of DNA replication [18]. The formation of the panhandle in HAV5 requires a minimum of the first 31 complementary nt of the ITR [19]. In contrast, deletions within the left ITR of HAV2 starting beyond nt 45 were stable and did not affect virus replication [16]. A virus carrying a deletion including the NFIII, Sp1 and ATF sites within the ITR replicated to similar titers as those obtained with wt HAV5 [20], thereby suggesting that there is functional redundancy between different transcription factors that interact with the left ITR of the HAV5 genome.

Further mutational and functional analyses are required to precisely define the minimal ITR sequences that are necessary for BAV3 replication and to identify the exact positions of binding motifs for various transcription factors. Our results support the exploration of this site as a novel location for the insertion of foreign genes.

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Fig. 1. Generation of BAV3c29 and restriction enzyme analysis of genomic DNA extracted from wt BAV3 and BAV3c29. A) Diagram representing the generation of the internal deletion in the left ITR during PCR amplification of the EcoRI fragment of BAV3. PCR was performed using an oligonucleotide encoding the PacI recognition site and the first 21 bp of the BAV3 ITR (PacI-1-21) as a forward primer and the nucleotide sequence from 1138 to 1159 as a reverse primer. The purified PCR product was subsequently cloned in pUC18 to form pMvOBE1E4PacI (van Olphen & Mittal, 1999), and it was subsequently then used with the BAV3 genome to generate plasmid pMvOBAB3c29 by recombination in *Escherichia coli* BJ5183. Genome-length DNA obtained from pMvOBAB3c29 was linearized with PacI and transfected into MDBK cells using a iLipofectin-mediated transfection protocol (Life Technologies). Two weeks post-after transfection, an isolated viral plaque was collected and propagated to produce the BAV3c29 stock. The left-end AscI fragment of wt BAV3, pMvOBAB3c29, and BAV3c29 depicting the nt nucleotide position of the ITR boundaries, and the locations of EcoRI and AscI recognition sites are shown. ITRs are shown as open-boxes and the position and length of the internal ITR deletion are also shown. B) DNA samples obtained from purified preparations of wt BAV3 and BAV3c29 were digested with restriction enzymes, AscI and EcoRI. The resultant DNA fragments were loaded onto a 1.5% agarose gel and, separated by electrophoresis and the ethidium bromide-stained bands were visualized with an UV transilluminator. The sizes of the terminal fragments containing the left ITR generated with the digestion of AscI and EcoRI are shown on the left and right side of the panel, respectively. MWM = 1 kb DNA molecular weight marker.

	1		50
wt BAV3	5'	CATCATCAAT AATCTACAGT ACACTGAT <u>TGG</u> CAGCGGTCCA ACT <u>GCCAATC</u>	
BAV3c29	5'	CATCATCAAT AATCTACAGT ACACTGAT <u>TGG</u> CAGCGGTCCA ACT <u>GCCAATC</u>	
HAV5	5'	CATCATCAAT AATATACCTT ATTT <u>TGGATT</u> GAA <u>GCCAATA</u> TGATAATGAG	
		ORP-A NFI NFIII	
	51		100
wt BAV3	5'	ATTTTTGCCA CGTCATTTAT GACGCAACGA CGGCGAGCGT GCGTGCTGA	
Bav3c29	5'	ATTTTTGCCA CGTCATTTAT GACGCAACGA CGGCGAGCG~ ~~~~~	
HAV5	5'	GGGGTGGAGT TTGTGACGTG GCGCGGGGCG TGGGAACGGG GCGGGTGACG	
		SP1 ATF SP1 SP1 ATF	
	101		150
wt BAV3	5'	CGTAACTGTG GGGCGGAGCG CGTCGCGGAG GCGGCGGCGC TGGGCGGGGC	
BAV3c29	5'	~~~~~ ~~~~~ ~~~~~ ~~~~~ ~~~~~	
HAV5	5'	TAG	
	151		195
wt BAV3	5'	TGAGGGGCGGC GGGGGCGGCG CGCGGGGCGG CGCGCGGGGC GGGGC	
BAV3c29	5'	~~~~~ ~GGGGCGGC T CTT GGGGCGG CGCGCGGGGC GGGGC	

Fig. 2.

Nucleotide sequence comparison of the left ITR of wt BAV3, BAV3c29 and HAV5. On the basis of sequence similarities with HAV5, the potential binding sites for a number of transcription factors were identified in the left BAV3 ITR. The potential binding motifs for ORP-A, NFI, NFIII, SP1 and ATF are depicted as gray underlined, underlined, double underlined, gray background and thick underlined, respectively. The deleted nt in the left ITR of BAV3c29 are depicted by the symbol '~'. The conserved nt in the binding motif for NFI are shown in bold. The nt substitutions in BAV3c29 are shown in bold face with lines above and below.

Fig. 3.

Development of plaques by BAV3 and BAV3c29 on MDBK cells. Confluent monolayers of MDBK cells in 60 mm dishes were infected with various log dilutions of either BAV3 or BAVc29 and the virus was allowed to adsorb for 1 h at 37°. Subsequently monolayers were covered with an agarose overlay (1 x MEM, 5% fetalCloneIII, 0.5% agarose, 0.05% yeast extract, 50 µg/ml gentamicin and 25mM MgCl₂). Plaques were stained with crystal violet 12 days after infection. Dishes infected with virus dilutions of 10⁻⁸, 10⁻⁹ and 10⁻¹⁰ are shown.

Fig. 4. Kinetics of replication of wt BAV3 and mutant BAV3c29. MDBK cell monolayers were grown in 60mm culture dishes and infected with wt BAV3 or BAV3c29 at an MOI of 2 PFU per cell. Infected cells along with the medium were collected 6, 12, 24, 48, 72 and 96 h after infection and the virus titration was done by plaque assay on MDBK cells.