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NOTE

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Whole-genome sequencing of live attenuated bovine adenovirus type 7 vaccine strain TS-GT suggests biomarkers for virulence attenuation

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ABSTRACT. Bovine adenovirus type 7 (BAdV-7) is one of the most important respiratory and enteric pathogens in the cattle industry. Although live attenuated vaccines are used to control the virus in Japan, limited information is available on the genomic regions that determine viral pathogenicity. We determined the complete genome sequence of the attenuated BAdV-7 strain TS-GT. The genome is 30,052 bp long and contains 45-bp inverted terminal repeats and 30 predicted genes. A genome sequence comparison showed that 99.9% of the TS-GT genome is identical to the prototypic and pathogenic BAdV-7 strain Fukuroi; however, the TS-GT genome contains a novel mutation and four indels. We describe here potential relationships between these genomic changes and the biological characteristics of BAdV-7.

KEYWORDS: attenuation, bovine adenovirus, inverted terminal repeat, pVI

Bovine adenovirus type 7 (BAdV-7) is a double-stranded linear genomic DNA virus that belongs to the genus *Atadenovirus* (family *Adenoviridae*) and is one of the most important respiratory and enteric pathogens in the cattle industry [1, 4]. BAdV-7 was first isolated in 1965 in Japan from blood samples of a Holstein cow with respiratory disease [4, 9, 10]. The virus was designated as strain Fukuroi and demonstrated to be pathogenic to cattle [4, 5]. In order to develop a Fukuroi-based live vaccine, serial passaging for 4 to 5 weeks with bovine kidney cells was performed at 30°C (10 passages in total), followed by short-term passaging at 30°C with bovine testicle cells (14 passages), and finally with goat testicle (GT) cells (5 passages) [5]. After plaque-purification (three passages with GT cells), a virus with the characteristic features of temperature-specific growth at 30°C and attenuated pathogenicity to cattle was developed; these features led to the development of the TS-GT for the strain [5]. Currently, multivalent live vaccines containing TS-GT are available in Japan (https://www.kyotobiken.co.jp/en/products/cow.html#respiration), but little is known about the genomic characteristics of the virus.

Herein we isolated BAdV-7 strain TS-GT from the hexavalent bovine respiratory disease vaccine Cattlewin-6 lot #40-1 (Kyoto Biken Laboratories Inc., Kyoto, Japan). The vaccine was treated with 10% chloroform at 20°C for 30 min to inactivate other constituent viruses, namely, bovine infectious rhinotracheitis virus, bovine viral diarrhea virus type 1 and 2, bovine respiratory syncytial virus, and bovine parainfluenza virus type 3. The suspension was then serially diluted and mixed with bovine embryonic testicle (BET) cells. Supernatants from the end-point dilutions showing cytopathic effects were collected. The isolate was confirmed to be positive for BAdV-7 and negative for the other five bovine viruses by PCR, and sequencing indicated that the strain was TS-GT [7, 12]. The virus was expanded by large-scale-culture with BET cells, followed by centrifugation to remove cellular debris. The stocks were semi-purified by discontinuous sucrose density gradient ultra-centrifugation and dissolved in phosphate-buffered saline. DNA was extracted using a QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany) and submitted to Macrogen Japan Co., Ltd. (Tokyo, Japan) for whole-genome sequencing. Briefly, sequence libraries were constructed using a TruSeq DNA PCR-Free kit (Illumina, San Diego, CA). DNA sequencing was performed with a deep sequencing protocol using Novaseq 6000 (Illumina). A total of 45 million paired-end reads (a total of 6.8 billion bases) were obtained with an average length of 151 bp. Bases with a phred quality score below 20 were trimmed from every read, and the reads were assembled using a *de novo* approach with Trimmomatic version 0.36 (http://www.usadellab.org/cms/?page=trimmomatic) and SPAdes version 3.13.0 (http://cab.spbu.ru/software/spades/), using default settings. A 29,941 bp contig was generated with an average base coverage depth of 217. To account for short nucleotide stretches lacking at

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both ends of the genome, we performed 5′ and 3′ adapter ligation, followed by PCR with specific primers, and Sanger sequencing was performed [6]. The full-length TS-GT genome sequence of 30,052 nucleotides, with 33.58% GC content, was obtained and deposited in DDBJ under the accession number LC606503.1. Inverted terminal repeat (ITR) sequences of 45 bp in length were present. Putative open reading frames and functions of the translated products were predicted using the DNA data bank of Japan fast annotation and submission tool (https://dfast.nig.ac.jp/). The obtained sequence encoded 30 predicted genes.

Compared with the complete sequence of Fukuroi (GenBank accession number LC597488), TS-GT had a nucleotide mutation (at 14,234 nt), two deletions (at 14 nt and 30,021 nt), and two insertions (at 13,685 nt and 29,999 nt) (Table 1). The 14,234 nt mutation is located in the central region of the minor capsid pVI protein and is expected to induce an amino acid change at position 96 from lysine to asparagine (Fig. 1A). The amino acid change may affect the biological function of the pVI protein. It has been reported that adenovirus pVI is a multifunctional protein that is crucial for decapsidation and release from the endosome in the early phase of infection [2]. It has also been reported that temperature-sensitive (ts) mutants of human adenovirus type 5 produce a very stable form of the pVI proteins in the endosomal environment, and that ts mutants significantly reduce membrane lytic activities, in comparison to wild-type viruses [15]. This behavior underlies the characteristic ts biotype. On the basis of previous reports, the mutation in pVI is likely to play an important role in the ts dependent growth of TS-GT. The deletions at 14 nt and 30,021 nt are located in the core origins of the 5′ and 3′ ITR promoter regions (Fig. 1B and 1C). Zhu *et al*. reported that similar deletions are found in the ITR of BAdV type 3 (BAdV-3) strain HLJ0955, in comparison to the prototype BAdV-3 strain WBR-1 [17]. Indels in the ITR regions seems to be common in BAdV. Whether or not these nucleotide changes affect adenoviral promoter function is still controversial and requires further investigations [13, 16]. The insertion at 13,679 nt is located in the non-coding region between pVII and pX, and does not change the protein-coding sequence (Table 1). Interestingly, the ITRs of TS-GT, at both ends of the genome, were nine bp longer than in Fukuroi (Fig. 1B and 1C). Notable changes were found in the 3′ ITR region. Next to an SP1-transcription-factor binding site, a 19-bp DNA fragment is inserted in the TS-GT genome, but not in Fukuroi (Fig. 1C). This region is thought to regulate the promoter activity of the 3′ ITR. The region downstream of the 3′ ITR promoter encodes five predicted proteins of unknown function, RH1 to

Table 1. Location, genomic position and specfication of the five nucleotide changes in the TS-GT genome compared with the sequence of prototype Fukuroi. Amino acid changes are also shown

Location (nt)	Genomic position	Specification	Amino acid changes
14	5′ ITR[a]	Deletion (thymine)	-
13,685	Non-coding region	Insertion (adenine)	-
14,234	Coding region of pVI	Mutation (from thymine to adenine)	K96N
29,999	3′ ITR	19 bp insertion (AGCCACGCCCAAAACTGTC)	-
30,021	3′ ITR	Deletion (adenine)	-

[a] Inverted terminal repeat.

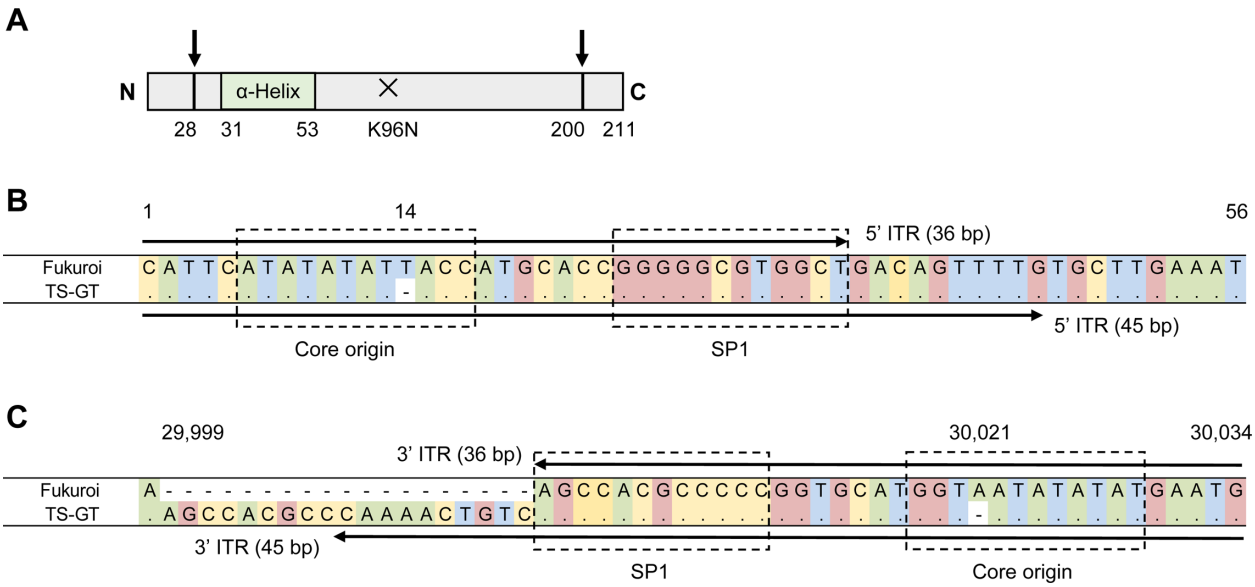

Fig. 1. (**A**) Schematic representation of the pVI protein. The green box shows the α-helix domain. Proteolytic cleavage sites in both the N and C terminal regions are indicated by vertical arrows. Numbers indicate the amino acid positions, and X represents the position of mutation. Alignment of the (**B**) 5′ and (**C**) 3′ terminal sequences of Fukuroi and TS-GT. Dotted boxes indicate the core origin and the SP1 binding site. Horizontal arrows above and below the alignment indicate the inverted terminal repeat regions of Fukuroi and TS-GT. Numbers represent nucleotide positions in the Fukuroi genome.

RH5, and three E4 proteins, E4.1 to E4.3 that are key viral and cellular regulatory factors involved in transcription, apoptosis, cell cycle control, and repair [14]. It is possible that these genes function as trans-acting regulators by modulating the host immune response to viral infection and are involved in attenuation. In some adenoviruses, for example egg drop syndrome virus, virus-associated RNA (VA RNA) is also encoded in the RH region [3]. VA RNAs are non-protein coding sequences that can antagonize the antiviral activity of interferons [8]. However, in our study, we did not identify any sequences with homology to VA RNA in the corresponding region of the TS-GT genome.

It is noteworthy that the adenovirus solution used in our genetic analysis was prepared in a different production lot from that which previous researchers used in the injection experiments of cattle to confirm its pathogenicity in the 1960s [5]. It is possible that several additional passages with BET cells could induce unexpected mutations in the BAdV-7 genome. However, the adenoviral genome is remarkably stable, having a calculated mutation rate per cell infection cycle of 1.3×10^{-7}, equivalently, 0.0039 per 30.0 kbp genome [11]. Therefore, mutations are unlikely to be introduced in the BAdV-7 genome by the additional cell-culture passages required here. Although further mutational and functional analyses are required to clarify the effect of these mutations and indels in the TS-GT genome, our results provide insight into the molecular basis of BAdV-7 attenuation, and open new avenues for future analyses.

CONFLICT OF INTEREST. The authors declare no conflicts of interest.

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