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Attenuation of highly pathogenic porcine reproductive and respiratory syndrome virus by inserting an additional transcription unit

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ABSTRACT

Transcription regulatory sequences (TRSs) play a key role in the synthesis of porcine reproductive and respiratory syndrome virus (PRRSV) subgenomic mRNAs, which resembles similarity-assisted RNA recombination. In this study, genome instability was found when a highly pathogenic PRRSV (HP-PRRSV) strain was inserted by an additional transcription unit in which a foreign gene GFP was expressed from TRS2 while a copy of TRS6 drove ORF2a/b transcription. Structural protein gene-deleted genomes resulted from enhanced RNA recombinations were identified in the recombinant virus rHV-GFP. Moreover, rHV-GFP replicated slower than parental viruses, and caused less cell death in porcine alveolar macrophages. Pigs infected with rHV-GFP survived with no or mild syndromes, whereas all pigs infected with parental viruses died within 12 days. Our data showed that additional transcription unit insertion could confer genome instability and attenuation of HP-PRRSV.

1. Introduction

Arterivirus and coronavirus generate a 3′ co-terminal nested set of subgenomic mRNAs during virus replication. The subgenomic transcripts also contain a common 5′ leader sequence, which is derived from the 5′ end of the genomic RNA. Their synthesis involves a discontinuous transcription process that resembles similarity-assisted RNA recombination [1]. Transcription regulatory sequences (TRSs) consisting of conserved hexanucleotide motif and poorly conserved flanking sequences forming secondary structures are present at the 3′ end of the common leader (leader TRS) and upstream of each structural protein gene (body TRSs), and play a key role in the synthesis of subgenomic mRNAs [1,2]. The body TRSs could act as attenuation signals during minus-strand RNA synthesis, and then the nascent minus strand would be redirected to the 5′-proximal region of the template by a base-pairing interaction between the leader TRS and the antisense copy of the body TRSs. Then the completed subgenomic-length minus strands would serve as templates for the transcription of subgenomic mRNAs [1,3].

Porcine reproductive and respiratory syndrome virus (PRRSV) is an important arterivirus, causing respiratory diseases in piglets and severe reproductive failures in sows [4,5]. In May 2006, highly pathogenic PRRSV (HP-PRRSV), which was observed with a unique molecular hallmark namely a discontinuous deletion of 30 amino acids in nonstructural protein 2, emerged in China. HP-PRRSV infection is characterized by high fever, high morbidity, and high mortality in pigs of all ages [6–8]. In the past 5 years, HP-PRRSV has reemerged several times and caused immense economic losses for the swine industry in China.

Reverse genetics system provides a powerful tool to dissect the functions of viral proteins in viral life cycle and pathogenicity by gene manipulation. However, overlapped ORFs make it difficult to

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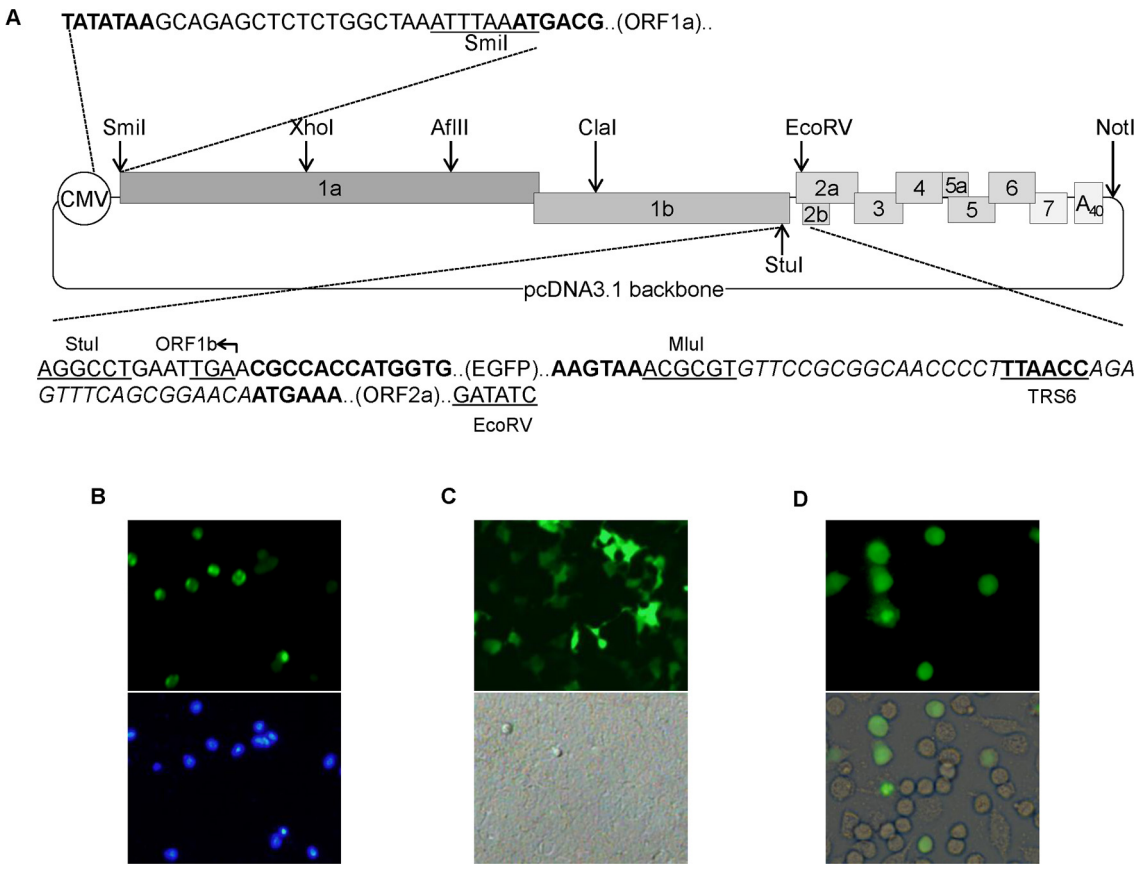

Fig. 1. Construction of a recombinant HP-PRRSV expressing an additional transcription unit (rHV-GFP). (A) Strategy for the construction of the recombinant cDNA clone. Five overlapping fragments amplified from the HV genome were ligated into the modified pcDNA3.1 vector using the listed restriction enzyme cleavage sites. GFP gene and a copy of TRS6 were introduced into the non-coding region between ORF1b and ORF2a using restriction sites Stu I and Mlu I. TTAACC, the core hexanucleotide of TRS6; the sequence in italic indicates its flanking sequences. (B) The plasmid containing assembled HV genome was transfected into 293FT cells. The reused virus rHV was passaged on PAMs and immunofluorescence assay was performed for virus detection. Magnification, 200×. (C) The recombinant cDNA clone containing GFP gene and a copy of TRS6 was transfected into 293FT cells and directly examined for GFP expression at 48 h post transfection under an immunofluorescence microscope. Magnification, 200×. (D) rHV-GFP was serially passaged on PAMs and green fluorescence was directly examined under an immunofluorescence microscope. Magnification, 400×.

manipulate PRRSV genome [9]. Recently, PRRSV gene expression vectors capable of expressing a foreign gene from an additional transcription unit were generated by inserting a copy of the transcription regulatory sequence for ORF6 (TRS6) [10–13]. This novel approach was used to construct recombinant viruses expressing antiviral cytokines as adjuvants to overcome immune subversion of PRRSV and enhance the viral specific immune response following vaccination [13], or expressing reporter proteins to monitor virus replication, as well as elucidating the role of host factors and screening for novel antiviral drugs [11,13]. This PRRSV reverse genetics system may also function as a vector for development of recombinant multivalent vaccines against swine diseases by encoding immunogenic antigens from other swine pathogens [10]. The inserted foreign gene was supposed to be tolerated by the virus and could be stably expressed in cell culture. In this study, a recombinant HP-PRRSV expressing an additional transcription unit (rHV-GFP) was constructed. RNA recombination resulted from the additional transcription unit insertion conferred high attenuation both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cells and virus

Porcine alveolar macrophages (PAMs) were collected by post-mortem lung lavage of 8-week-old specific-pathogen-free pigs, and maintained in RPMI 1640 supplemented with 10% FBS [14]. Cells of 293FT line (Invitrogen) were cultured in DMEM containing 10% FBS. HP-PRRSV strain HV (GenBank accession no. JX317648) was used in this study [15]. Viruses were propagated and titrated on PAMs.

2.2. Construction of a full-length cDNA clone

The assembly strategy is illustrated in Fig. 1. Briefly, viral RNA was extracted and reverse transcribed. Then, five overlapping fragments spanning appropriate restriction sites were amplified, and cloned into pMD®18-T vector. Primer sequences were listed in Table 1. Eukaryotic expression vector pcDNA3.1(+) was modified into backbone for assembly. The number of nucleotides between the TATA box and the viral genome was adjusted to 24 as described previously [16]. The plasmid containing assembled genome of HV (pcDNA3.1-HV) was transfected into 293FT cells. Cell culture supernatant obtained at 48 h post transfection was inoculated on PAMs and immunofluorescence assay was performed for virus detection [17]. The rescued virus was designated as rHV.

2.3. Insertion of an additional transcription unit

Enhanced green fluorescent protein (EGFP) gene including Kozak consensus translation initiation site was amplified from pEGFP-N1 vector using primer set of StG-F (containing a StuI site) and StG-R (containing a MluI site). TRS6 or TRS2 sequence was amplified using primer set of TRS6-F or TRS2-F (containing a MluI site) and HV4-R (containing an EcoRV site). The amplification