Virus Research 178 (2013) 272–280

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Nonstructural proteins of *Torque teno sus virus 2* from O2AUG: Prediction to experimental validation

Yan Lu [a], Haisheng Yu [b], Xiaohua Nie [b], Yongqing Li [c], Liguo Zhang [b], Chengping Lu [a,*]

[a] College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, China
[b] Key Lab of Infection and Immunity, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China
[c] Institute of Animal Husbandry and Veterinary Medicine, Beijing Academy of Agricultural and Forestry Sciences, Beijing 100097, China

ARTICLE INFO

Article history:
Received 13 August 2013
Received in revised form
19 September 2013
Accepted 20 September 2013
Available online 30 September 2013

Keywords:
TTSuV2
Nonstructural protein
Monoclonal antibody
Genomic DNA clone
Expression profile

ABSTRACT

The expression profiles of nonstructural proteins (NSPs) in *Torque teno sus virus 2* (TTSuV2) have not yet been characterized. Here, we determined the coding sequences of the TTSuV2 NSPs ORF2, ORF2/2, and ORF2/2/3 by overlapping polymerase chain reaction (PCR) and subsequent expression in bacterial and mammalian cells. We generated two monoclonal antibodies (mAbs), 2E5 and 6F8, from mice immunized with mixed *Escherichia coli* expressing His-tagged ORF2 and ORF2/2. Enzyme-linked immunosorbent assay (ELISA) and western blot analysis revealed that, 2E5 mAbs bound to the consensus sequences of ORF2, ORF2/2, and ORF2/2/3, while 6F8 recognized the common sequences of ORF2/2 and ORF2/2/3. Immunofluorescence assay (IFA) revealed that ORF2 was localized in the cytoplasm, ORF2/2, in the nucleus but not the nucleolus, and ORF2/2/3, in the peri-nuclear region. To identify the expression profiles of TTSuV NSPs, a circular TTSuV2_ZJ (GenBank: KF660540) genomic DNA clone was constructed and transfected into HEK293T and HeLa cells. Splicing mRNAs and the expression and localization of ORF2/2 and ORF2/2/3 were identified by RT-PCR, western blot analysis, and IFA, respectively. However, ORF2 was not detected either at the RNA or protein level. Our study is the first to provide experimental evidence of the existence of ORF2/2 and ORF2/2/3 at the protein level. Moreover, the mAbs have potential applications in future research on TTSuV2 viral protein function and diagnosis of related diseases.

1. Introduction

Torque teno virus (TTV) was first isolated in 1997 from the serum of a Japanese patient (T.T.) with posttransfusion hepatitis of unknown etiology by using representational difference analysis (Nishizawa et al., 1997). Subsequent studies revealed that TTVs can infect not only human hosts but also nonhuman primates (Abe et al., 2000), tupaias (Okamoto et al., 2001), livestock and pets (Niel et al., 2005; Okamoto et al., 2002). *Torque teno sus virus* (TTSuV) includes two species (TTSuV1 and TTSuV2) and belongs to the genus *Iotatorquevirus* in the family *Anelloviridae*. TTSuV has been circulating in the pig population since at least 1985 (Segales et al., 2009). Although TTSuV has not yet been shown to be pathogenic, it appears to play an important role during co-infection with other pathogens. Infection with TTSuV appears to promote postweaning multisystemic wasting syndrome (PMWS) in *porcine circovirus-2* (PCV2)-infected gnotobiotic pigs (Ellis et al., 2008) and to facilitate the development of porcine dermatitis and nephropathy syndrome (PDNS) in *porcine reproductive and respiratory syndrome virus* (PRRSV)-infected gnotobiotic pigs (Krakowka et al., 2008). Moreover, TTSuV2 might be a potential indicator of immunosuppression in pigs (Aramouni et al., 2013). A previous study carried out by our group revealed that natural infection with TTSuV suppresses the host immune response and exacerbates porcine reproductive and respiratory syndrome (PRRS) in pigs (Zhang et al., 2012).

The genomic DNA of TTSuV is approximately 2.8 nt and was presumed to be circular and single-stranded (Okamoto et al., 2002). Although transcriptional analysis and protein expression of its

Abbreviations: BSA, Bovine serum albumin; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; ELISA, Enzyme-linked immunosorbent assay; FBS, Fetal bovine serum; GST, Glutathione S-transferase; HAT, Hypoxanthine-aminopterin-thymidine; MW, Molecular weight; NLS, Nuclear localization signal; OD, Optical density; ORF, Open reading frame; PAGE, Polyacrylamide gel electrophoresis; PBS, Phosphate buffered saline; PCR, Polymerase chain reaction; PDNS, Porcine dermatitis and nephropathy syndrome; PMWS, Postweaning multisystemic wasting syndrome; PRRS, Porcine reproductive and respiratory syndrome; PRRSV, Porcine reproductive and respiratory syndrome virus; RT, Room temperature; SDS, Sodium dodecyl sulfate; TTSuV, Torque teno sus virus; UTR, Untranslated region.

* Corresponding author at: College of Veterinary Medicine, Nanjing Agriculture University, No. 6, Tongwei Road, Xuanwu District, Nanjing 210095, China. Tel.: +86 25 84396517.
E-mail addresses: luyan326@hotmail.com (Y. Lu), yuhaisheng_ibp@hotmail.com (H. Yu), niexiaohua2008@sina.com (X. Nie), liyngqng@yahoo.com (Y. Li), zhanglgf@hotmail.com (L. Zhang), lucp@njau.edu.cn (C. Lu).

0168-1702/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.virusres.2013.09.031

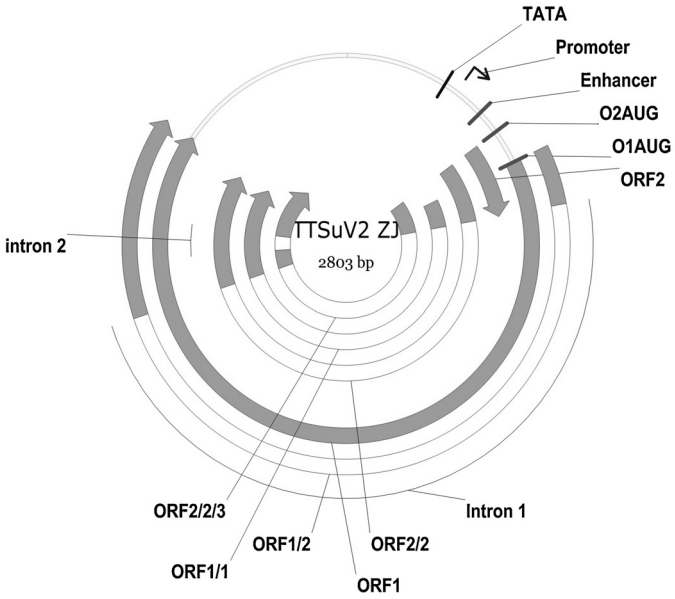

Fig. 1. Genome organization of TTSuV2 (isolate TTSuV2_ZJ). The locations of the TATA signal, promoter, enhancer, start codons O1AUG and O2AUG, and two introns are illustrated. The gray arrows represent six open reading frames (ORFs). The white boxes represent introns. The ORF1, ORF1/1, and ORF1/2 proteins use the start codon O1AUG. The ORF2, ORF2/2 and ORF2/2/3 proteins are initiated at O2AUG.

full-length genomic DNA have not been reported, we speculated that, similar to human anelloviruses, TTSuV encodes three mRNAs and generates at least six proteins (Fig. 1) (Kamahora et al., 2000; Qiu et al., 2005). ORF1, ORF1/1, and ORF1/2 initiate from the start codon O1AUG while ORF2, ORF2/2, and ORF2/3 start from O2AUG. ORF1 and ORF2 are encoded by continuous mRNA1. Removal of intron 1 generates mRNA2, which encodes ORF1/1, ORF1/2, and ORF2/2. Recent work by Huang et al. demonstrated the existence of a novel TTSuV mRNA transcript which encodes two more putative proteins, namely, ORF1/1/2 and ORF2/2/3 (Huang et al., 2012). However, the functions of TTSuV proteins remain largely unknown, limiting the understanding of the pathogenesis of TTSuV. A recent study constructed a full-length infectious TTSuV DNA clone (Huang et al., 2012) and detected the expression of the putative ORF1 capsid protein. However, there was no evidence of expression of other nonstructural proteins ORF2, ORF2/2, and ORF2/2/3.

In the present study, full-length ORF2, ORF2/2, and ORF2/2/3 proteins of TTSuV2 were expressed in *Escherichia coli*, and two different monoclonal antibodies (mAbs) that specifically recognized ORF2, ORF2/2 and ORF2/2/3 were designed. Thus, we used full-length DNA clones of TTSuV2 and the two mAbs, and for the first time, presented experimental evidence of the transcription, expression, and localization of the nonstructural proteins ORF2, ORF2/2 and ORF2/2/3. Therefore, our study will yield further insights into the function of the proteins in TTSuV.

2. Materials and methods

2.1. Tissue samples and DNA extraction

Diseased pigs exhibiting typical clinical signs of PMWS, including progressive wasting, dyspnea and jaundice (Hamel et al., 1998) were obtained from a commercial farm in Zhejiang Province, China. Liver, spleen, and lymph node samples were obtained from the pigs. Tissues were stored at −80 °C immediately after necropsy, until use. Total DNA was extracted from tissue homogenate using the Viral Nucleic Acid Extraction Kit II (Geneaid Biotech, Nanjing) according to the manufacturer's instruction.

2.2. PCR amplification of full-length genome

The extracted total DNA was used as a template for PCR amplification with inverted primers targeting a conserved domain within the untranslated region (UTR) of the viral genome (Liu et al., 2011). PCR was carried out using PrimeSTAR HS DNA polymerase in accordance with the manufacturer's protocol (TaKaRa Bio, Dalian). Briefly, two-step PCR (98 °C for 10 s and 68 °C for 4 min) was performed for 30 cycles using primers LG567 and LG568. The amplification product was 2815 bp. Amplicons with expected sizes were purified after separation using 1.2% agarose gel electrophoresis by using TIANgel Midi Purification Kit (TIANGEN Biotech, Beijing) and cloned into the pMD19-T vector (TaKaRa Bio, Dalian), resulting in T-*pTTSuV2_ZJ*. Plasmids containing full-length TTSuV2 genomic DNA were sequenced for both strands at Invitrogen (Life Technologies, Beijing). The sequences were analyzed with Vector NTI software package (Invitrogen Life Technologies, Beijing). The TTSuV2 sequences reported earlier were compared with sequences obtained following a search of the BLAST database (http://blast.ncbi.nlm.nih.gov).

2.3. Construction of full-length genomic DNA clone of TTSuV2

A full-length genomic DNA clone of TTSuV2 was constructed as described previously (Huang et al., 2012) with some modifications. Briefly, the two PCR fragments, K1 (primers LG1012 and LG568) and K2 (primers LG567 and LG1013), which covered the full-length genome of TTSuV2 were amplified from plasmid T-*pTTSuV2_ZJ*. The two fragments were assembled into a full-length genomic DNA clone by overlapping PCR (primers LG1012 and LG1013), cloned into a p*EASY*-Blunt Simple Cloning Vector (TransGen, Beijing), and designated *pEASY-TTSuV2_ZJ*. As the full-length DNA was flanked by a *Kpn*I restriction site at both ends, the full-length TTSuV2_ZJ genome was excised from *pEASY-TTSuV2_ZJ* by *Kpn*I digestion, then purified and circularized by ligation.

2.4. Molecular cloning of the ORF2, ORF2/2, and ORF2/2/3 genes and construction of expression plasmids

The corresponding DNA sequence encoding ORF2 was amplified by using T-*pTTSuV2_ZJ* as the template. The forward primer was LG758 (5′-CCG GAA TTC ATG GAA GAA AGA TGG CTG ACG-3′) and the reverse primer was LG759 (5′-CGA GCG GCC GCT TAC CTT TGT GCG GCG GCG GCG AG-3′). Two restriction sites *Eco*RI and *Not*I were added into the forward and reverse primers (underlined), respectively.

ORF2/2 encoding sequence was amplified by overlapping PCR in accordance with *Nature Protocols*(Heckman and Pease, 2007). Briefly, the coding sequence of the ORF2/2 protein was divided into two segments. The N-terminal segment was upstream of the intron, while the C-terminal segment was downstream of the intron. The N- and C-terminal genes were amplified from template T-*pTTSuV2_ZJ* with the primer sets LG758 and LG761 (5′- *GTG GCC TCC CCA* CTT TGT GCG GCG GCG GCG AG-3′), and LG762 (5′-*CGC CGC ACA AAG* TGG GGA GGC CAC GGA ACC GAA AG-3′) and LG760, respectively. The internal primers LG761 and LG762 contain 12-bp nucleotide sequences (italics) from each gene segment. These overlapping segments will hybridize with one another during the second PCR with the primer set LG758 and LG760 to generate the encoding gene of ORF2/2. Two restriction sites *Eco*RI and *Not*I were added to the flanking primers LG758 and LG760 (underlined), respectively. The same method was used to amplify the ORF2/2/3 sequence with LG1733 and LG1734 for the N-terminal sequence, LG1735 and LG1736 for the C-terminal sequence, and LG1733 and LG1736 for the entire ORF2/2/3.