Virology 499 (2016) 170–177

Contents lists available at ScienceDirect

Virology

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

Identification and subcellular localization of porcine deltacoronavirus accessory protein NS6

Puxian Fang [a,b], Liurong Fang [a,b], Xiaorong Liu [a,b], Yingying Hong [a,b], Yongle Wang [a,b], Nan Dong [a,b], Panpan Ma [a,b], Jing Bi [a,c], Dang Wang [a,b], Shaobo Xiao [a,b,*]

[a] State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, China
[b] The Cooperative Innovation Center for Sustainable Pig Production, Wuhan 430070, China
[c] Department of Immunology and Aetology, College of Basic Medicine, Hubei University of Chinese Medicine, Wuhan 430065, China

ARTICLE INFO

Article history:
Received 5 August 2016
Returned to author for revisions
14 September 2016
Accepted 15 September 2016

Keywords:
Porcine deltacoronavirus
Accessory protein
NS6
Subgenomic RNA
Subcelluar localization

ABSTRACT

Porcine deltacoronavirus (PDCoV) is an emerging swine enteric coronavirus. Accessory proteins are genus-specific for coronavirus, and two putative accessory proteins, NS6 and NS7, are predicted to be encoded by PDCoV; however, this remains to be confirmed experimentally. Here, we identified the leader-body junction sites of NS6 subgenomic RNA (sgRNA) and found that the actual transcription regulatory sequence (TRS) utilized by NS6 is non-canonical and is located upstream of the predicted TRS. Using the purified NS6 from an *Escherichia coli* expression system, we obtained two anti-NS6 monoclonal antibodies that could detect the predicted NS6 in cells infected with PDCoV or transfected with NS6-expressing plasmids. Further studies revealed that NS6 is always localized in the cytoplasm of PDCoV-infected cells, mainly co-localizing with the endoplasmic reticulum (ER) and ER-Golgi intermediate compartments, as well as partially with the Golgi apparatus. Together, our results identify the NS6 sgRNA and demonstrate its expression in PDCoV-infected cells.

1. Introduction

Porcine deltacoronavirus (PDCoV) is an emerging swine enteric coronavirus that causes diarrhea in nursing piglets (Chen et al., 2015b; Hu et al., 2015; Jung et al., 2015; Ma et al., 2015). It was first detected in Hong Kong in 2012 (Woo et al., 2012). In early 2014, outbreaks of PDCoV were announced in swine populations in Ohio, Illinois, and Iowa, and it rapidly spread to multiple states in the United States (Li et al., 2014; Marthaler et al., 2014a; Marthaler et al., 2014b; Wang et al., 2014a, 2014b). Thereafter, PDCoVs were detected or caused outbreaks in Korea (Lee et al., 2016; Lee and Lee, 2014), China (Dong et al., 2015; Song et al., 2015; Wang et al., 2015) and Thailand (Janetanakit et al., 2016; Madapong et al., 2016), posing significant economic concerns and gaining considerable attention (Jung et al., 2016; Lorsirigool et al., 2016; Zhang, 2016).

PDCoV is an enveloped, single-stranded, positive-sense RNA virus belonging to the newly identified genus *Deltacoronavirus* within the family *Coronaviridae*. Its genome is approximately 25.4 kb in length, making it the smallest genome among the known coronaviruses (CoVs). The genome arrangement of PDCoV is similar to those of other CoVs with the typical gene order: 5′UTR-ORF1a-ORF1b-S-E-M-NS6-N-NS7-3′UTR (Song et al., 2015; Woo et al., 2012). Although the biological functions of PDCoV-encoded proteins have not been studied in detail, based on studies of other known CoVs, PDCoV ORF1a and ORF1b probably encode two viral replicase polyproteins, pp1a and pp1ab, which are predicted to be proteolytically cleaved into 15 mature nonstructural proteins responsible for viral replication and transcription; ORFs S, E, M, and N encode viral structural proteins, Spike (S), Envelope (E), Membrane (M), and Nucleocapsid (N), respectively (Chen et al., 2015a; Lee and Lee, 2014; Li et al., 2014; Woo et al., 2010). Additionally, NS6 and NS7 each encode a putative accessory protein (Fig. 1A).

Accessory proteins are genus-specific for coronavirus (Tan et al., 2006); however, different CoVs contain different numbers of accessory genes and proteins. For example, alphacoronavirus feline infectious peritonitis virus encodes five accessory proteins (p3a, p3b, p3c, p7a, and p7b), while only one accessory protein is encoded by porcine epidemic diarrhea virus (PEDV); betacoronavirus severe acute respiratory syndrome coronavirus (SARS-CoV) encodes a total of eight accessory proteins; and the most studied gammacoronavirus, infectious bronchitis virus (IBV), encodes four accessory proteins (Liu et al., 2014). Although coronavirus accessory proteins have generally been considered to be dispensable for viral replication *in vitro* (Haijema et al., 2004; Yount et al., 2005), extensive functional studies have shown that many accessory

* Corresponding author at: State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, China.
E-mail address: vet@mail.hzau.edu.cn (S. Xiao).

http://dx.doi.org/10.1016/j.virol.2016.09.015
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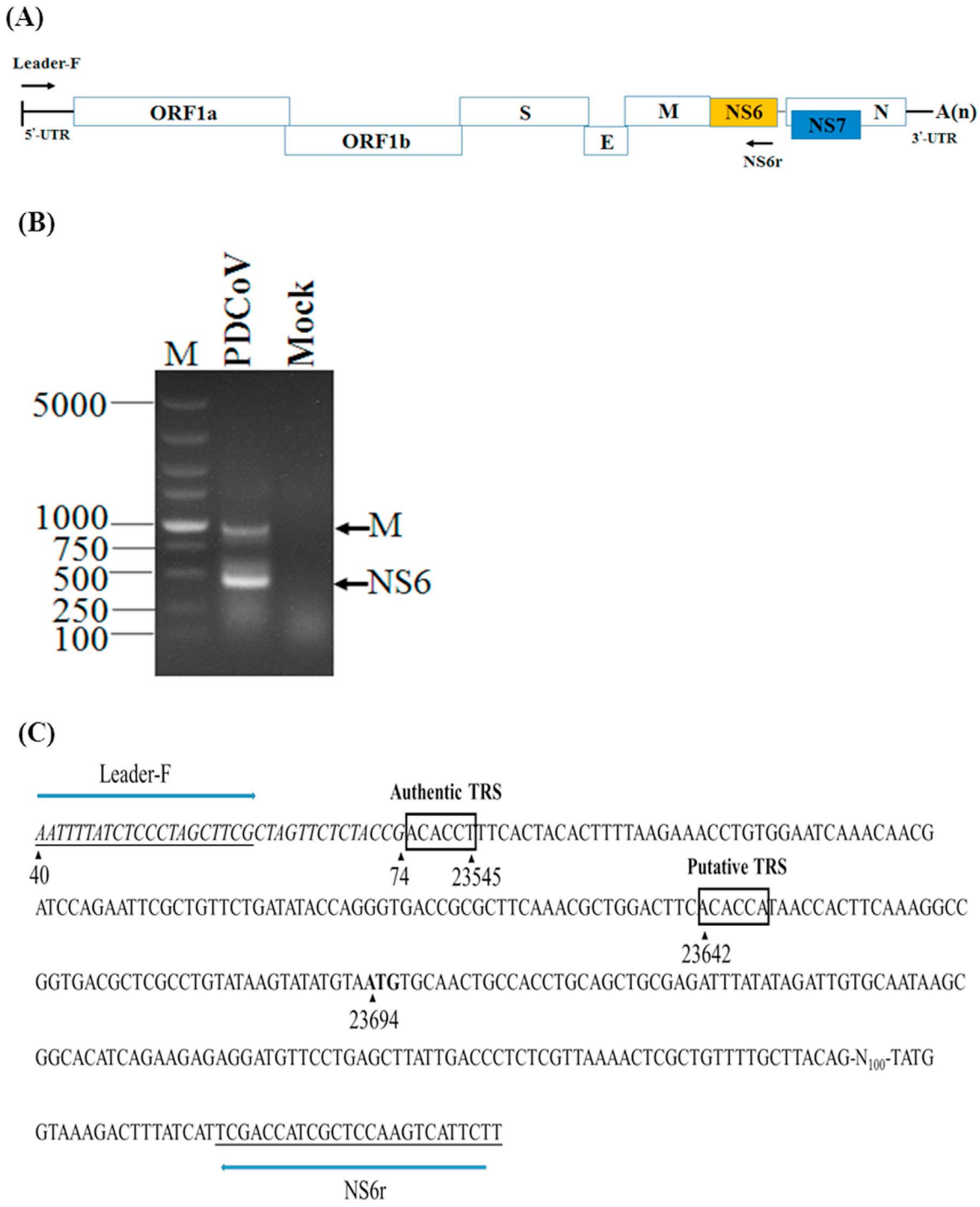

Fig. 1. *Analysis of the leader-body TRS junction of putative PDCoV NS6 sgRNA.* (A) The primer design for the leader-body junction RT-PCR analysis is shown in a schematic diagram of the PDCoV full-length genome. (B) A representative gel from agarose gel electrophoresis of RT-PCR products amplified from PDCoV mRNA is shown. M, molecular size ladder. (C) Analysis of the sgRNA NS6 sequence. The primers and the leader sequences are displayed as underlined and as italicized, respectively. The positions of the nucleotides in the genome sequences are indicated by black arrowheads. The start codon ATG in NS6 sgRNA is marked in bold. Boxed regions represent the authentic TRS and putative TRS used for sgRNA synthesis. The N_{100} indicates that the 100 nucleotides at that region are not shown.

proteins are involved in immune modulation (Kopecky-Bromberg et al., 2007) and viral pathogenesis *in vivo* (De Haan et al., 2002). The field of coronavirus accessory proteins has gained considerable attention in recent years.

In the PDCoV genome, there are two putative accessory genes, NS6 and NS7. NS6 is predicted to be located in the genome between M and N and to encode a 94-amino acid peptide, while NS7 is predicted to be located within the N gene (Lee and Lee, 2015; Woo et al., 2012). To date, there are no reports regarding the expression of PDCoV accessory genes or the identification of an associated transcription regulatory sequence (TRS) for production of these subgenomic RNAs (sgRNAs) in virus-infected cells.

Here, we identified the leader-body fusion site and TRS of NS6 sgRNA. By using monoclonal antibodies (MAbs) that recognize the putative NS6 protein, we demonstrated that the predicted NS6 could be expressed and localized to the cytoplasm in PDCoV-infected cells, providing the first biochemical evidence for the existence of PDCoV NS6.