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Mini-genome rescue of Crimean-Congo hemorrhagic fever virus and research into the evolutionary patterns of its untranslated regions

Jiuru Zhao[a,b], Han Xia[a], Yujiang Zhang[c], Shiyu Yin[a,b], Zhong Zhang[a], Shuang Tang[a], Zheng Kou[a], Jingfeng Yu[a,b], Zhaojun Fan[a], Tianxian Li[a,*]

[a] State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, Hubei, China
[b] University of Chinese Academy of Sciences, Beijing 100039, China
[c] The Center for Disease Control and Prevention of Xinjiang Uygur Autonomous Region, Urumqi, China

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ABSTRACT

Crimean-Congo hemorrhagic fever virus (CCHFV) is a member of genus *Nairovirus*, family *Bunyaviridae*, which are distributed widely in Africa, Europe and Asia with several genotypes. As a BSL-4 level pathogen, the requirement of high-level biosafety facilities severely constrains researches on live virus manipulation. In this study, we developed a helper-virus-independent mini-genome rescue system for the Chinese YL04057 strain. Based on the enhanced green fluorescent protein (EGFP)-derived mini-genome plasmids, this polymerase I driven system permits easy observation and quantification. Unlike previous report, gradually reduced levels of activity of the CCHFV L, M and S untranslated regions (UTRs) were observed in our system. We also demonstrated that the UTRs at both ends were indispensable for mini-genome background expression. In addition, we phylogentically analyzed all six UTRs of CCHFV and showed that L-UTRs were clustered together approximately corresponding to their original geographical continents. The UTRs of M segment showed a similar branch structure to its open reading frames (ORFs), and nearly an identical tree was generated with 5′ UTRs of S segment compared with its ORFs. However, the 3′ UTRs of S segment formed new divergent groups. Compatibility tests of YL04057 strain nucleocapsid protein and L protein expression plasmids with Nigerian strain IbAr10200 mini-genomes revealed lower compatibility of L-UTRs without an obvious effect on M-UTRs. Moreover, we demonstrated that the L-UTRs could tolerate certain nucleotide mutations. This system may provide a foundation for future studies of the viral replication cycle, pathogenic mechanisms and evolutionary patterns of CCHFV.

1. Introduction

Crimean-Congo hemorrhagic fever is a tick-borne disease with outbreak records in more than 30 countries including Africa, the Middle East, Europe and Central Asia (Deyde et al., 2006). The CCHFV is the causative agent of this serious hemorrhagic fever in humans with a case fatality rate of 15–60% (Saksida et al., 2010) and is classified as a WHO Risk group IV pathogen. Currently, there is no FDA-approved vaccine or specific antiviral therapy (Ergonul et al., 2007; Keshtkar-Jahromi et al., 2011). CCHFV circulates in nature in an enzootic tick-vertebrate-tick cycle, and is transmitted mainly by *Hyalomma* genus ticks. However, it has also been isolated from other tick genera, such as *Rhipicephalus*, *Ornithodoros*, *Boophilus*, *Dermacentor* and *Ixodes* spp. (Hoogstraal, 1979). Infection has been demonstrated among smaller wildlife species, such as hares and hedgehogs. Antibodies have been also detected in the sera of horses, donkeys, goats, cattle, sheep and pigs (Lindeborg et al., 2012). In 1965, the first CCHFV case in China was identified in the Xinjiang province (Lindeborg et al., 2012; Papa et al., 2002; Sun et al., 2009). Now, serum antibodies have been tested positive in many parts of China including Yunnan, Qinghai, Sichuan and Anhui provinces (Xia et al., 2011).

As a member of the genus *Nairovirus* within the family *Bunyaviridae*, the genome of CCHFV consists of three negative-sense RNA strands designated S, M and L. The S segment encodes the nucleocapsid protein (NP) (Guo et al., 2012). The M segment encodes the glycoprotein precursor (GPC), which is further matured to Gn and Gc (Sanchez et al., 2002). And the L segment encodes the RNA-dependent RNA polymerase (Honig et al., 2004a). Similar to other negative-sense RNA viruses, the three genome segments associated with the polymerase L are encapsidated by NP to form ribonucleoprotein particles (RNPs), which was the minimal element for viral genome transcription and replication (Flick et al., 2003; Zhou et al., 2011).

RNA viruses generally go through rapid evolution by mutation and recombination. For segmented viruses, reassortments also

* Corresponding author. Tel.: +86 27 87198465; fax: +86 27 87198465.
E-mail address: litx@wh.iov.cn (T. Li).

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arise frequently. A high level of CCHFV genome plasticity was reported (Deyde et al., 2006): nucleotide variations in the S, M and L RNA segments were estimated at 20, 31 and 22% while the amino acid variations for the NP, GPC, and L protein were estimated at 8, 27 and 10% respectively. Several studies have addressed the genetic variability and phylogenetic relationships of CCHFV strains based on the full-length sequences (Anagnostou and Papa, 2009; Deyde et al., 2006; Lukashev, 2005). According to the analysis of S segments, seven distinct groups were divided corresponding to their geographical origins and clustering. The group, Africa 1 represented isolates from Senegal; Africa 2 represented isolates from Uganda and South Africa; and Africa 3 represented isolates from South Africa and Western Africa. The group, Europe 1 represented isolates from Russia, Turkey and the Balkan region; Europe 2 represented a single isolate from Greece. The group, Asia 1 represented isolates from the Middle East, Pakistan and Iraq whereas Asia 2 represented isolates from China and Tajikistan. The high genetic variability together with high variation in recorded mortalities among diverse strains may indicate different efficiencies of transcription, replication, assembly and pathogenic ability of the virus in host cells. However, functional comparison between segments of different CCHFV strains with low nucleotide sequence identities remains unreported.

Additionally, UTRs of the *Bunyaviridae* family are presumed to contain signals for encapsidation by the viral NP to form RNPs, signals for regulation of transcription and replication and signals for packaging the RNPs into virions (Kohl et al., 2006; Mir and Panganiban, 2010; Vera-Otarola et al., 2010). Moreover, it was reported that viral UTRs also participated in host cell regulation networks (Flick et al., 2002; Habjan et al., 2008; Sriskanda et al., 1996). Furthermore, nucleotides are highly conserved at both ends of the segments of the *Bunyaviridae*. Their complement leads to non-covalently closed circular RNA molecules that provide the structure for interaction with the viral polymerase. Despite the importance of the UTRs, phylogenetic studies on them are lacking in CCHFV (Devereux et al., 1997; Gritsun and Gould, 2006; Sriskanda et al., 1996; Tzanetakis et al., 2009).

Reverse genetics system is a powerful tool for discriminating genetic determinants of signals relating to viral replication cycles of negative-strand RNA viruses, especially for highly virulent ones (Bergeron et al., 2010). In 2003, Flick et al. rescued the mini-genome of CCHFV using a helper-virus-dependent polymerase I (pol I) system (Flick et al., 2003). For biosafety purposes, it is necessary to develop rescue systems without helper viruses. However, cloning a mutation-free sequence of the longest L segment (~12,000 nt) limits the development of helper-virus-independent reverse genetics systems for CCHFV. To date, only one study regarding the T7-luciferase based helper-virus-independent mini-genome rescue system for the Nigerian IbAr10200 strain has been reported (Bergeron et al., 2010).

In this study, we developed a helper-virus-independent mini-genome rescue system for CCHFV strain YL04057 isolated from Xinjiang (China), which is phylogenetically distant from IbAr10200. We demonstrated that the UTRs at both ends were indispensable for mini-genome plasmids background expression, and that the UTRs evolved differently compared with the ORFs. Additionally, we tested the compatibilities of the YL04057-specific support plasmids (NP and L expression plasmids) with IbAr10200 strain mini-genomes, and showed lower compatibility of L-UTRs, but no obvious variation for the M-UTRs. We also proved that the L-UTRs could tolerate certain site-specific mutations. This study provides a basis for more comprehensive studies on the geographical transmission of CCHFV and functional comparisons of different strains. The technology developed in this study will be helpful for researches on viral replication cycles, pathogenic mechanisms and anti-viral drug development for CCHFV.

2. Materials and methods

2.1. Cells and viruses

Baby hamster kidney cells (BHK-21) and human adrenal cortical tumor cells (SW-13) were obtained from China Center for General Viruses Culture Collection. BHK-21 cells were cultured at 37 °C in Dulbecco's modified essential medium (DMEM) and SW-13 in Leibovitz's L-15 medium, both supplemented with 10% bovine calf serum (BCS). CCHFV strain YL04057 was kindly provided by the Center for Disease Control and Prevention of Xinjiang Uygur Autonomous Region, Urumqi, China. All operating of infectious material with CCHFV was performed in a BSL-3 laboratory and complied with the national biosafety regulations.

2.2. Support plasmids construction

RNA was isolated from infected SW-13 cells by RNeasy Mini Kit (Qiagen). Reverse transcription reactions were conducted with the RevertAid First Strand cDNA Synthesis Kit (Fermentas) using the random hexamer primers. Full-length S, M and two partial L (1-7197 and 4489-12156) segment cDNAs were amplified by LA Taq DNA Polymerase (Takara) with strand-specific primers. Purified PCR products were ligated to pGEM-T vector (Promega) using the DNA Ligation Kit LONG (Takara) and denominated as pGEM-T-S, pGEM-T-M, pGEM-T-L1 and pGEM-T-L2. Whole length pGEM-T-L was constructed by digestion of pGEM-T-L1 and pGEM-T-L2 by *Eco91*I and *Sal* I. The ORFs of the S and L segments were cloned into eukaryotic expression vector pcDNA3.1 (+) (Invitrogen), resulting in pcDNA3.1 (+)/S and pcDNA3.1 (+)/L. *Bam*H I with *Not* I and *Kpn* I with *Not* I were used for digestion respectively. All restriction enzymes were obtained from Fermentas.

2.3. Mini-genome plasmids construction

Mini-genome gene fragments were amplified mainly by overlapping PCR. Briefly, the *Bbs* I restriction site, 3′ UTRs and nucleotides identical to EGFP gene 5′ end were incorporated into the forward primers, along with the primer complemented to the EGFP 3′ end, 3′-UTR-GFP segments were amplified with pEGFP-N3 (Clontech) as the template. The 5′ UTR regions with about 16 extra nucleotides identical to the EGFP gene 3′ end were amplified from corresponding pGEM-T clones. Then, the 3′-UTR-GFP-UTR-5′ fragments were generated by following cycling conditions: 94 °C for 5 min; five cycles of 94 °C for 30 s, 58 °C for 30 s and 68 °C for 1 min 30 s; 15 cycles of 94 °C for 15 s, 68 °C for 1 min. Besides, to keep fidelity, any primers longer than 100 nt were synthesized by dividing into two parts with about 16 nt coincides, and both were added into the reaction mixtures during PCR. Next, fragments were introduced into pRF42 or pRF207 by *Bbs* I digestion. For mutations' construction, similar strategy was used except for introducing mutations into corresponding primers. For mini-genome plasmids of IbAr10200 strain, all UTR regions were synthesized by primers and also overlapping method was adopted. All clones were verified by sequencing and all primer sequences are available upon request.

2.4. Transfection

BHK-21 cells were plated into the 24-well the day before transfection and grown to 90% confluence. For single plasmid transfection, a total of 500 ng/well of each plasmid DNA was used, and operated according to the manufacturer's instruction of Lipofectamine LTX (Invitrogen). For co-transfection, 0.8 μg pcDNA3.1(+)/L, 200 ng pcDNA3.1(+)/S and 300 ng mini-genome plasmids along with 2.5 μl Lipofectamine LTX and 1.5 μl PLUX reagent (Invitrogen)