Biochemical and Biophysical Research Communications 374 (2008) 138–142

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Construction of a non-infectious SARS coronavirus replicon for application in drug screening and analysis of viral protein function

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ARTICLE INFO

Article history:
Received 18 June 2008
Available online 11 July 2008

Keywords:
Severe acute respiratory syndrome (SARS)
Non-infectious replicon
Drug screening
Transcription
Replication

ABSTRACT

Severe acute respiratory syndrome virus (SARS-CoV) was the causative agent of the SARS outbreaks in 2002–2003. A safer in vitro system is desirable for conducting research on SARS-CoV and to screen for antiviral drugs against the virus. Based on the infectious cDNA clone of rSARS-CoV-ΔE, in which the E gene has been deleted, a safe non-infectious replicon was constructed by replacing the S gene with the enhanced green fluorescent protein (eGFP) gene. Successful replication was achieved as evident from continuous expression of eGFP detected by both fluorescence and Western blot. Treatment with antiviral drugs demonstrated that the replication could be significantly inhibited by 0.4 mg/ml of cysteine proteinase inhibitor E-64D, but not by ribavirin. The same replicons containing further deletion of the coding regions for non-structural proteins (nsp) 1, 2 or 16 confirmed previous observation that nsp16, but not nsp1 or nsp2, was essential for efficient viral replication or transcription.

Severe acute respiratory syndrome (SARS) illness which was caused by a novel coronavirus (SARS-CoV) [1] first arose in Guangdong province, China, in late 2002. The virus spread rapidly to 26 countries and killed 916 people out of 8422 infected patients with a fatality rate of 11% [2]. Though SARS epidemic was finally controlled by patient isolation and the ban of wild animal trading, the significant morbidity and mortality, especially the potential threat from SARS reemergence, reminds us the continuous risk facing the public health system [1]. Moreover, the wide distribution and genetic diversity of SARS-like CoVs discovered in China [3–5] also serve as a warning for potential human infection by related bat-borne CoVs.

SARS-CoV resembles to members of the three previously known groups of coronavirus and was classified as a member of group 2b in the family *Coronaviridae* based on phylogenetic analysis of genome sequences [6,7]. The SARS-CoV virion contains a single-stranded 29,700-nt RNA genome of positive polarity. The first two-thirds of the genome encode two overlapping polyproteins, ORF1a and ORF1b. Like other coronaviruses, these two polyproteins are processed into 16 mature replicase proteins by viral specific proteinases and most of them are believed to associate with a poorly characterized replication/transcription complex that mediates the synthesis of genome RNA (replication) and subgenomic mRNAs (transcription) [7–10]. Recently, the functions of several non-structural proteins (nsps) have been determined, including a non-canonical RdRp (nsp8), a single-stranded RNA-binding protein (nsp9), a RNA-dependent RNA polymerase (nsp12), a superfamily 1-like helicase (nsp13), a 3′ → 5′ exonuclease (nsp14), a uridylate-specific endoribonuclease (nsp15), and a 2′-*O*-ribose methyltransferase activities (nsp16) [11–14]. The complicated interactions network among these nsps makes it difficult, if not impossible, to fully understand their functions [15,16]. The rest of 3′ end genome codes for four main structural proteins, the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. The S protein, which mediates both cell attachment and membrane fusion, is responsible for virus host range and tissue tropism. Proteins E, M, N can assemble into non-infectious virus like particles (VLPs) in the absence of S [17]. Besides the nsps and major structural proteins, SARS-CoV possesses several group-specific proteins and their functions during virus life cycle remain elusive and deletion of genes for those proteins seems to have no effect virus replication and/or transcription [18]. Recent studies on these group-specific proteins revealed that some of them may be associated with virus pathogenesis and virus–host interaction [19–22].

SARS-CoV is highly virulent and classified as a biosafety level 3 (BSL3) agent, which can only be handled in a BSL3 laboratory. For in-depth study of functions of different viral proteins and regulatory sequence elements by reverse genetics, full-length infectious cDNA clones have been established using various techniques including bacterial artificial chromosome (BAC) vector [23–25] and SARS-CoV replicon cell lines [26], which can also be used for antiviral drug screening. Among them, the SARS-CoV replicon cell lines which retain the genes necessary for replication and

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doi:10.1016/j.bbrc.2008.06.129

transcription represent the safest system available. However, these cell lines need to be continuously selected with antibiotics and is not readily amenable to introduction of mutations in different protein coding genes. Here, we report the construction of a plasmid-based replicon system which can be introduced into cells by transfection and can be easily manipulated for introduction of mutations. The usefulness of this replicon was demonstrated by its application in testing known antiviral drugs and in confirming the essential function of nsp16 in virus replication.

Materials and methods

Cells and viruses. 293T cell line was used in this study and maintained in DMEM medium (Gibco) supplemented with 10% (vol/vol) foetal bovine serum (FBS, Gibco). The plasmid rSARS-CoV-ΔE encodes an infectious attenuated virus derived from SARS-CoV Urbani strain (GenBank Accession No. AY278741) [25] and was kindly provided by Dr. Luis Enjuanes (CNB, CSIC, Madrid).

PCR amplification of eGFP gene and modification of pBlueScriptII. Primers eGFPf 5′-ACTAGTATGGTGAGCAAGGGCGA-3′ and eGFPr 5′-ACTAGTCTTGTACAGCTCGTCCATG-3′ (SpeI site is underlined) were used to amplify the eGFP gene from a vector pEGFP-N1 (Clontech). The PCR products were purified from agarose gels and cloned into pGEM-T Easy vector (Promega) for sequencing. Authentic clone with no mutation was used for subsequent cloning. The plasmid pBlueScriptII (Clontech) was modified by inserting a 35 bp sequence (5′-TATGGTACCGTTTAAACGACGGATCCTCTAGAC TG-3′) containing polycloning sites using the vector sites KpnI and XbaI (underlined), which resulted in the introduction of the PmeI (GTTTAAAC) site and the deletion of the SpeI from the original vector.

Construction of replicon pBAC-SARS-CoV-ΔEΔS/eGFP. The previously constructed infectious cDNA clone (plasmid rSARS-CoV-ΔE) [25] was digested with PmeI and BamHI producing a 7.5 kb fragment containing 3′ end of pp1b, S and 3a gene. This fragment was subcloned into a modified pBlueScriptII vector (described above), generating the plasmid pBS-SARS-CoV1b-3a. The eGFP gene was then inserted to replace S gene with single enzyme SpeI, generating pBS-SARS-CoV1b-3aΔS/eGFP. This GFP containing fragment was cloned back into pBAC-SARS-CoV-ΔE digested by PmeI and BamHI, generating pBAC-SARS-CoV-ΔEΔS/eGFP (Fig. 1).

In-frame deletion of nsp coding regions. To delete the junction region of nsp1–nsp2 which contains the C-terminus of nsp1 (139 aa) and the N-terminus of nsp2 (107 aa), pBAC-SARS-CoV-ΔEΔS/eGFP was digested by PmlI and self-ligated to generate pBAC-SARS-CoV-ΔEΔSΔnsp1-2J/eGFP. To delete the N-terminus of nsp2, firstly, primer pair nsp2F 5′-GCAGTCGATCATCAGCATACCTA-3′ (212–234 nt, forward) and nsp2R 5′-CAGGACATGGCATTTTCACTACAG-3′ (1355–1378 nt, reverse) were used to amplify a fragment containing partial nsp1 and nsp2 sequences. The amplified product was cloned into pGEM-Teasy to generate clone pGT-nsp1–2. Then, the N-terminus of nsp2 (107 aa) was further deleted by the following primers: Δnsp2F 5′-TGCGGATCCCCACGTGTTGAAAAGA A-3′ (1120–1136 nt) and Δnsp2R 5′-ATAGGATCCACCTCCATTGAG CTCAC-3′ (788–804 nt) (BamHI site is underlined). The amplified fragment was ligated through BamHI site to generate a clone pGT-Δnsp2. Last, the plasmid pGT-Δnsp2 was digested with PmlI and cloned back into the plasmid pBAC-SARS-CoV-ΔEΔS/eGFP. Using the similar deletion strategy for nsp2, based on the plasmid pBS-SARS-CoV1b-3aΔS/eGFP, core sequence of nsp16 (from 20,631 to 21,422) was deleted by PCR amplification with primers Δnsp16F 5′-GTGGTCGACAGGCTTATCATTAGAGAAAAC-3′ (21,423–21,443 nt) and Δnsp16R 5′-AGAGTCGACCAAGTTAGGCATCGCAACACC-3′ (20,610–20,630 nt) (SalI site is underlined), with a new SalI site as a genetic marker. The amplified fragment was ligated through the SalI site to generate a clone pBS-SARS-CoV1b-3aΔnsp16/eGFP. The generated plasmid was cloned back into the plasmid pBAC-SARS-CoV-ΔEΔS/eGFP between PmeI and BamHI sites to generate pBAC-SARS-CoV-ΔEΔSΔnsp16/eGFP.

Plasmid purification and transfection. The above constructed plasmids were purified with the Qiagen Large-Construct Kit (QIAGEN) according to the manufacturer's protocol. Transfection was performed when 293T cells were grown to 85% confluence with FuGene6 transfection reagent (Roche) according to manufacturer's instructions. The transfected cells were incubated at 37 °C with 5% CO_2 for 12 h and then cultured with fresh DMEM containing 10% FBS at 37 °C with 5% CO_2 either until the observation at 72 h.p.t. or used for antiviral drug test with 0.4 mg/ml ribavirin (Sigma) or 0.4 mg/ml E-64D (Sigma).

Western blot analysis. Cell lysates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were transferred onto Immobilon-P Transfer membranes (Milipore) by semi-dry electrophoresis transfer (Bio-Rad). The membrane were blocked overnight in TBS (0.2 M NaCl, 50 mM Tris–HCl, pH 7.5) with 3% (w/v) BSA (BIOSHARP) and then probed with rabbit polyclonal antiserum against SARS-CoV N protein (produced at the Australian Animal Health Laboratory) or GFP (Beyotime, China) diluted 1:2000 in Immunoreaction Enhancer Solution I (Toyobo) for 1 h at 37 °C, followed by washing in TBS-T (0.1% Tween 20 in Tris-buffered saline) for three times. After incubation with the alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (diluted 1:2000 in Immunoreaction Enhancer Solution II (Toyobo) for 1 h at 37 °C, the signal was developed a BCIP/NBT substrate mix (Sino-American).

Quantitative real-time PCR analyses. Total cellular RNAs from transfected 293T cells were extracted with RNAprep Cell Kit (TianGen) at 72 hpt. RQ1 RNase free DNase (promega) was used to digest DNA at 37 °C for 30 min followed by incubating at 65 °C for 10 min. Reverse transcription was performed in a 25-μl volume with 5 μl of purified RNA solution. Primers and RNA were firstly denatured at 70 °C for 10 min, then immediately quenched on ice and subsequently added to the RT mixture, consisting of 0.6 mM each of the 4-deoxynucleoside triphosphates, 8 U RNasin (BioStar) and 80 U M-MLV reverse transcriptase (Promega). The reaction

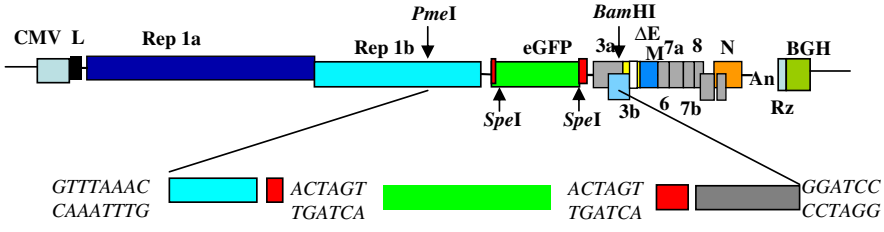

Fig. 1. Genetic organization of pBAC-SARS-ΔEΔS/eGFP. The core sequences of S and E gene were deleted. An eGFP gene was inserted to replace the S gene. Restriction sites PmeI, SpeI and BamHI used to construct the replicon are shown in italics. Letters and numbers indicate the viral genes. CMV, cytomegalovirus promoter; L, leader sequence; An, poly(A) tail; Rz, hepatitis delta virus ribozyme; BGH, bovine growth hormone termination and polyadenylation sequences.