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

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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 protein-ase 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.

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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 $3' \rightarrow 5'$ 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

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′-<u>ACTAGT</u>ATGGTGAGCAAGGGCGA-3′ and eGFPr 5′-<u>ACTAGT</u>CTTGTACAGCTCGTCCATG-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′-TAT<u>GGTACC</u>GTTTAAACGACGGATCC<u>TCTAGA</u>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′-TGC<u>GGATCC</u>CCACGTGTTGAAAAGAA-3′ (1120–1136 nt) and Δnsp2R 5′-ATA<u>GGATCC</u>ACCTCCATTGAGCTCAC-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′-GTG<u>GTCGAC</u>AGGCTTATCATTAGAGAAAAC-3′ (21,423–21,443 nt) and Δnsp16R 5′-AGA<u>GTCGAC</u>CAAGTTAGGCATCGCAACACC-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 Fu-Gene6 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
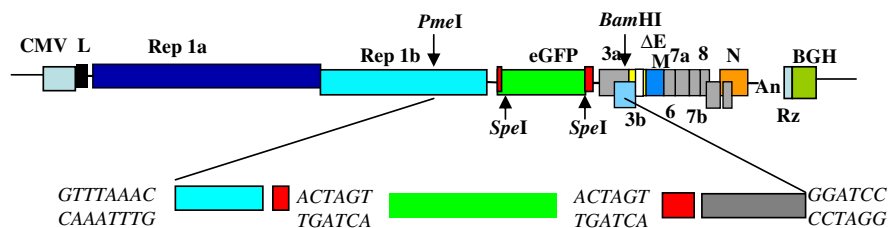

Fig. 1. Genetic organization of pBAC-SARS-ΔEΔS/eGFP. The core sequences of S and E gene were deleted. An eGFP gene was inserted to replace the S gene. Restriction sites PmeI, SpeI and BamHI used to construct the replicon are shown in italics. Letters and numbers indicate the viral genes. CMV, cytomegalovirus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

was conducted at 42 °C for 60 min, followed by heating to 70 °C for 5 min. Primers used for RT and quantitative PCR were as follows: qSARS-N-f: 5′-TACCGAAGAGCTACCCGACGAGTT-3′; qSARS-N-r: 5′-GCACGGTGGCAGCATTGTTATTAG-3′ (for N gene detection); qβ-actin-f: 5′-CAACTGGGACGACATGGAGAAAAT-3′; qβ-actin-r: 5′-CC AGAGGCGTACAGGGATAGCAC-3′ (for β-actin detection). Real-time quantitative PCR assays were conducted in a DNAEngine OPTICON machine (MJ). SYBR Green Real-time PCR Master Mix (Toyobo) was mixed with forward and reverse primers (0.4 μM each) in a final PCR mixture of 25 μl. The thermal cycling conditions were as follows: 1 cycle at 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 20 s, 72 °C for 20 s, 80 °C for 3 s. Fluorescence was measured after each cycle. A melting curve was generated after the last extension step from 55 °C to 95 °C. The experiment was repeated three times and the data represent the means of triplicates.

Results and discussion

Construction and characterization of a non-infectious SARS-CoV replicon

As described in Materials and methods, the eGFP gene was inserted to replace the S gene in the previously published construct

rSARS-CoV-ΔE to generate the non-infectious replicon plasmid pBAC-SARS-CoV-ΔEΔS/eGFP (Fig. 1). In this construct, none of the transcription regulation signals (TRSs) of SARS-CoV was interrupted and most ORFs were kept intact with the exception of the E and S genes. The eGFP gene was cloned under the control of the S gene TRS, retained the sequence of S gene at 5′ and 3′ end (30 and 102 nt, respectively). It is expected that the replicon can express most SARS-CoV ORFs at the same level as the original infectious cDNA clone. The resulting BAC clone was stably propagated in *Escherichia coli* DH10B cells.

When the purified pBAC-SARS-CoV-ΔEΔS/eGFP was transfected into 293T cells, eGFP expression was observed at 72 h post-transfection (Fig. 2A, left panel), indicating that this protein could be expressed from the S gene TRS. Western blot further confirmed the expression of eGFP and N proteins (Fig. 2B). These results demonstrated that the pBAC-SARS-CoV-ΔEΔS/eGFP replicon could replicate and transcribe in these cells.

Demonstration of inhibition by known antiviral drugs

To explore the feasibility of using the replicon system as a tool for drug screening, an inhibition assay with two known antiviral drugs, ribavirin and E-64D, was conducted at 12 h post-transfection. The results showed a significant reduction in fluorescence in
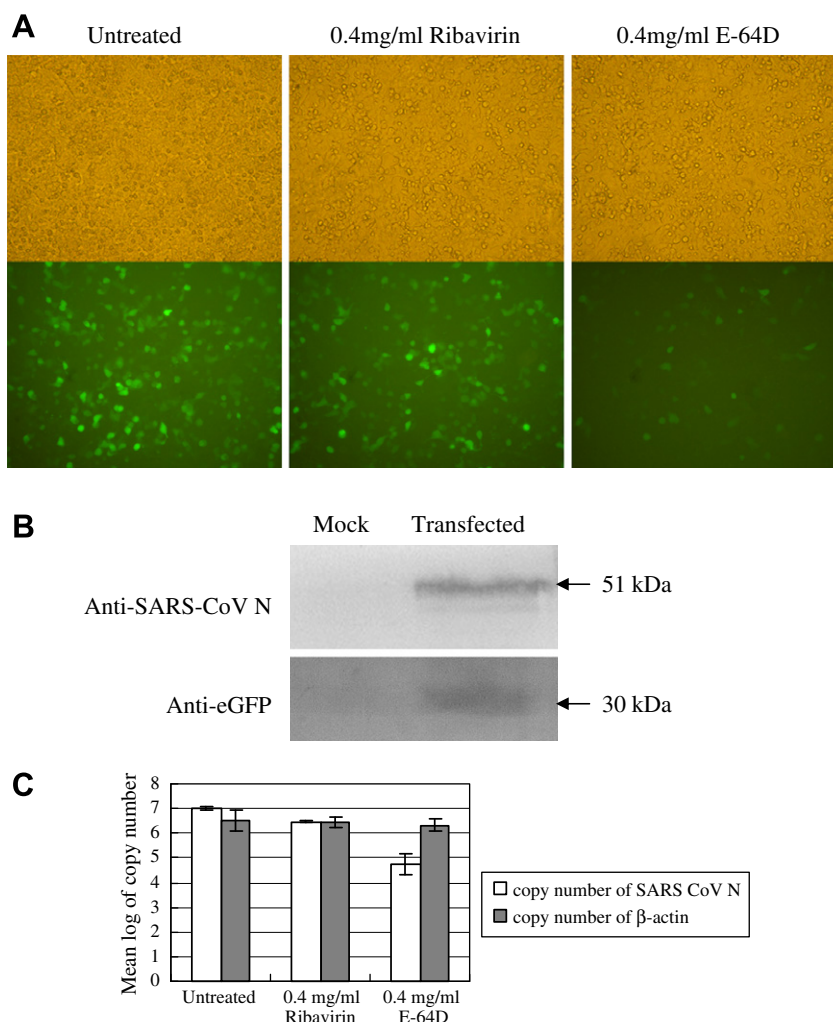

Fig. 2. Transfection and inhibition assay. (A) Fluorescence microscopy observation. The 293T cells were transfected with pBAC-SARS-CoVΔE-ΔS/GFP and either observed at 72 h post-transfection (hpt) (first panel) or treated with 0.4 mg/ml ribavirin (second panel) and 0.4 mg/ml E-64D (third panel) at 12 hpt and then observed at 72 hpt. (B) Western blot detection of eGFP and SARS-CoV N protein in transfected and non-transfected cells (mock) at 72 hpt. (C) Quantitative analyses of SARS-CoV replicon RNA (N gene) from antiviral drugs treated and untreated cells; β-actin was monitored as an internal control. The data presented are the means of three independent experiments.

the cells at 60 h after treatment with 0.4 mg/ml E-64D. (Fig. 2A, right panel). However, cells treated with ribavirin, a widely used broad-spectrum replicase inhibitor, did not have obvious effect on the fluorescence signal compared with those of the untreated cells (Fig. 2A, middle panel). It is worth noting that no obvious cytotoxic effect was observed for either ribavirin or E-64D at the concentrations used in this study.

Quantitative real-time RT-PCR was used to evaluate the inhibition of SARS-CoV N gene messenger RNA production. The N gene copy number was reduced by more than 2 logarithmic units after treatment with 0.4 mg/ml E-64D (Fig. 2C), indicating an efficient inhibition of SARS-CoV replication. In contrast, the mRNA level for the house keeping gene from both treated and untreated cells remained at a similar level. While the fluorescence intensity detected by microscope showed no significant difference between control and ribavirin treated cells, real-time RT-PCR revealed that ribavirin might have a small, but invisible, inhibitory effect on SARS-CoV replication.

Functional analysis of the SARS-CoV nsp1, nsp2 and nsp16

SARS-CoV polyprotein is processed into 16 non-structural proteins which form the replication/transcription complex during viral life circle. In previous studies, nsp16, but not nsp1 and nsp2, was reported to be required for virus replication [27,28]. In this study, we constructed three recombinant variants to test whether the same conclusion could be obtained using this new replicon system, the first one with a deletion in the junction domain between nsp1 and nsp2 genes (pBAC-SARS-CoV-ΔEΔSΔnsp1-2J/eGFP), the second one with a deletion within nsp2 N-terminus (pBAC-SARS-CoV-ΔEΔSΔnsp2N/eGFP), and a third in which nsp16 was deleted (pBAC-SARS-CoV-ΔEΔSΔnsp16/eGFP) (see Materials and methods). The results presented in Fig. 3 indicate that fluorescence intensity did not change for the first two deletion constructs compared with the original replicon, but there was a sharp reduction for the third construct with the nsp16 deletion. This was further confirmed by real-time RT-PCR evaluation of the N gene transcript, which revealed a reduction of close to 3 logs (Fig. 3B).

In this study, a non-infectious SARS-CoV replicon was generated based on the SARS-CoV-ΔE BAC clone. The replicon retains most SARS-CoV genes except the S and E genes. The insertion of eGFP gene renders it convenient to monitor virus replication by measuring fluorescence intensity. The deletion of S gene allows the manipulation of the replicon in a conventional laboratory instead of using a BSL3 facility. Our results indicated that the replicon could transcribe and express heterogeneous genes in vitro and can be used for antiviral drug screening and analysis of viral protein functions. Moreover, our newly constructed replicon is a stable BAC system and can be easily amplified in *E. coli* and manipulated by reverse genetics. As indicated in the results, it will be possible to insert any mutation in this replicon for gene function analysis.

Ribavirin has been widely used as viral replicase inhibitor including SARS-CoV [29]. However, our results showed that ribavirin is not an effective inhibitor of SARS-CoV replication at a noncytotoxic concentration at least in the cell line used in this study. Our observation is in agreement with previous published studies [26,30,31]. In contrast, E-64D was found quite effective against SARS-CoV infection, again in consistence with previous studies based on live viruses [23,26].

Bats were identified as the natural reservoirs of SL-CoVs. Bat SL-CoVs and SARS-CoV have a high similarity in genome organization, gene and gene product sequences, particularly in replicase proteins (96–98% amino acid identity) [3–5], suggesting that the function of most of these replicase proteins is conserved among different SARS and SARS-like CoVs. Even though the pathogenesis of bat SL-CoVs in human is unknown, there is a high potential of other related CoVs of bat origin to infect and cause diseases in human. In this regard, it is important to stress that our replicon system can be used to screen for drugs with equal effect to SARS-CoV or other un-
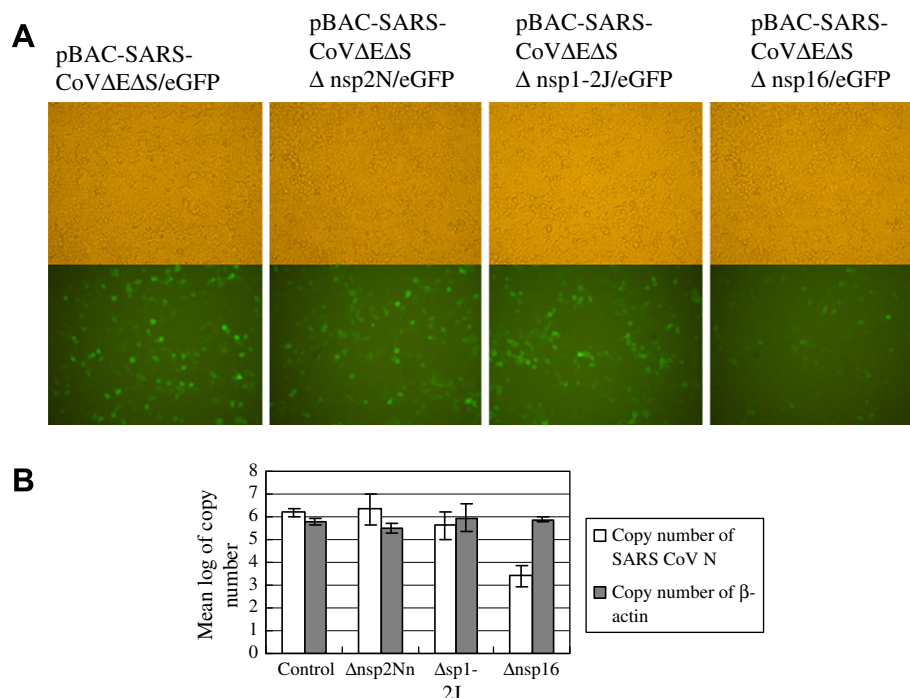

Fig. 3. Construction and functional analysis of a set of SARS-CoV nsp deletion mutants. (A) Fluorescence microscopy observation. Plasmids used for transfection were labeled on the top of each panel. (B) Quantitative real-time RT-PCR analysis of copy N gene transcripts from all four groups. The β-actin gene was monitored as an internal control. Data presented are the means of three independent experiments.

known but closely related bat CoVs which could potentially emerge in the future.

Acknowledgments

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