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A DNA-based non-infectious replicon system to study SARS-CoV-2 RNA synthesis

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ABSTRACT

The coronavirus disease-2019 (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has seriously affected public health around the world. In-depth studies on the pathogenic mechanisms of SARS-CoV-2 is urgently necessary for pandemic prevention. However, most laboratory studies on SARS-CoV-2 have to be carried out in bio-safety level 3 (BSL-3) laboratories, greatly restricting the progress of relevant experiments. In this study, we used a bacterial artificial chromosome (BAC) method to assemble a SARS-CoV-2 replication and transcription system in Vero E6 cells without virion envelope formation, thus avoiding the risk of coronavirus exposure. Furthermore, an improved real-time quantitative reverse transcription PCR (RT-qPCR) approach was used to distinguish the replication of full-length replicon RNAs and transcription of subgenomic RNAs (sgRNAs). Using the SARS-CoV-2 replicon, we demonstrated that the nucleocapsid (N) protein of SARS-CoV-2 facilitates the transcription of sgRNAs in the discontinuous synthesis process. Moreover, two high-frequency mutants of N protein, R203K and S194L, can obviously enhance the transcription level of the replicon, hinting that these mutations likely allow SARS-CoV-2 to spread and reproduce more quickly. In addition, remdesivir and chloroquine, two well-known drugs demonstrated to be effective against coronavirus in previous studies, also inhibited the transcription of our replicon, indicating the potential applications of this system in antiviral drug discovery. Overall, we developed a bio-safe and valuable replicon system of SARS-CoV-2 that is useful to study the mechanisms of viral RNA synthesis and has potential in novel antiviral drug screening.

1. Introduction

As of July 2022, there are more than 572 million patients suffering from coronavirus disease-2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, COVID-19 has caused over 6.3 million deaths, and this death toll continues to increase [1]. Since antiviral treatments against SARS-CoV-2 are not readily available, it is urgent to develop

Abbreviations: COVID-19, coronavirus disease-2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; BSL-3, bio-safety level 3; HTS, high-throughput screening; ORF, open reading frame; nsps, non-structural proteins; RTCs, replication-transcription complexes; S, spike; M, membrane; E, envelop; N, nucleocapsid; sgRNAs, subgenomic RNAs; gRNA, genomic RNA; TRSs, transcription-regulatory sequences; sgmRNAs, subgenomic mRNAs; RNP, ribonucleoprotein; BAC, bacterial artificial chromosome; EGFP, enhanced green fluorescent protein; CMV, cytomegalovirus; HDV, hepatitis delta virus; Rz, ribozyme; YCp, yeast centromere plasmid; SNPs, single nucleotide polymorphisms; SR, serine/arginine.

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vaccines, antiviral agents, and treatments to prevent the spread of the pandemic. Due to the high pathogenicity and infectivity of SARS-CoV-2, SARS-CoV-2 research must be conducted in biosafety level 3 (BSL-3) laboratories. Considering that BSL-3 laboratory resources are relatively limited, many groups are investigating new methods on SARS-CoV-2 research to be accessible for BSL-2 conditions [2,3]. Consequently, developing a non-infectious replicon system for SARS-CoV-2 that avoids the risk of virus transmission can provide a useful tool for elucidating the mechanisms of SARS-CoV-2 replication and discovering antiviral drugs by high-throughput screening (HTS).

SARS-CoV-2 belongs to the genus betacoronavirus of *Cronaviridae* family and is an enveloped, single-stranded, plus-strand RNA virus with a larger than average RNA genome [4]. The SARS-CoV-2 genome is approximately 30 kb (NCBI ID: NC_045512.2), and the 5′-proximal two-thirds of the genome encodes polyprotein 1a and 1ab (pp1a and pp1ab) from the open reading frame (ORF) 1a and 1b, respectively [5]. Ribosomal frameshifting allows the transcriptional switch of ORF1a in ORF1b to generate pp1ab. Pp1ab is then processed into 16 non-structural proteins (nsps), most of which are responsible for viral replication and transcription in the replication-transcription complexes (RTCs) [6–9]. The other 3′ one-third end of the genome encodes four major structural proteins, including spike (S), membrane (M), envelop (E) and nucleocapsid (N) proteins, and several accessory proteins [10,11].

After SARS-CoV-2 viruses enter into host cells, the viral genome is released into the cytoplasm and translated into pp1a and pp1ab. Subsequently, RTCs initiate the replication and transcription of viral genome [12]. The typical process of CoV RNA synthesis contains two parts: replication of the whole genome and transcription of subgenomic RNAs (sgRNAs) [13,14]. The latter can be further divided into a discontinuous synthesis step (also termed template switch) for minus-strand production and a continuous synthesis step for plus-strand generation. When virus transcription is activated, viral genomic RNA (gRNA) acts as a template that is transcribed into a full-length minus-strand gRNA, or a series of sgRNA intermediates [15]. Notably, the transcriptional procedure of minus-strand sgRNAs characterized by 5′- and 3′-coterminals with the virus genome is discontinuous and guided by the transcription-regulatory sequences (TRSs), which are unique among known RNA viruses [14–16]. These sgRNAs in turn serve as templates for the continuous synthesis of plus-strand sgRNAs, generally called subgenomic mRNAs (sgmRNAs), that encode SARS-CoV-2 structural and accessory proteins from the 3′ one-third of the viral genome. Throughout the process of viral RNA synthesis, the N protein is an indispensable viral protein for viral transcription. It has been reported that the N protein has multiple functions including encapsulating the virus genome into a higher-order ribonucleoprotein (RNP) complex [17], coordinating viral replication and transcription [18–20], and suppressing host cell defense mechanisms against virus life cycle [21]. However, the functions of wild type and mutant N proteins of SARS-CoV-2 in viral RNA synthesis still need to be further explored.

Due to the large size of the CoV genome and the instability and toxicity of the CoV sequence in *Escherichia coli*, bacterial artificial chromosomes (BACs) have been commonly used to clone CoV cDNA fragments [22–24]. The amplification of BACs is strictly synchronous with bacterial reproduction, leading to one or two copies per cell. Compared with high-copy-number plasmids, the BAC plasmid presents relatively stable maintenance of large DNA fragments and low toxicity to bacteria [25–27]. Except for the low-copy-number case, the preparation and manipulation of BACs are similar to that of conventional plasmids, allowing scientists to conveniently study the mechanisms of viral RNA synthesis. Furthermore, the BAC system makes it feasible to screen antiviral drugs against CoVs and many other viruses such as *Flaviviridae* (eg., dengue virus (DENV)) [28], *Herpesviridae* (eg., human cytomegalovirus (HCMV) [29], and murine cytomegalovirus (MCMV) [30]), and *Baculoviridae* (eg., autographa californica multiple nucleopolyhedrosis virus (AcMNPV)) [31] under BSL-2 conditions.

Here, we designed a non-infectious replicon system of SARS-CoV-2 by cloning viral cDNA fragments into a BAC vector while retaining the 5′ leader sequence, ORF1ab, nucleocapsid (N), and 3′ terminal sequence and inserting an enhanced green fluorescent protein (EGFP) as a reporter gene between ORF1ab and N. Our experiments showed that a conventional cell transfection method was sufficient to make SARS-CoV-2 replicons enter cells and carry out viral RNA synthesis, which can be detected by the expression of EGFP. Furthermore, we applied an improved real-time quantitative reverse transcription PCR (RT-qPCR) method to distinguish replication, discontinuous and continuous synthesis step of transcription. In our study, we found that the SARS-CoV-2 N protein elevated the level of sgRNAs by optimizing both steps of transcription. In addition, four of the six highest frequency spontaneous mutations within the N protein resulted in alterations in sgRNAs transcription, indicating that they can likely influence SARS-CoV-2 adaptability. Furthermore, two known candidate drugs against SARS-CoV-2, remdesivir and chloroquine, also showed inhibitory activity during viral transcription in our replicon system, suggesting that the replicon system can be potentially used to discover antiviral drugs through HTS. Overall, our work provides a feasible, comprehensive, and valuable tool for fundamental research in viral RNA synthesis and antiviral drug discovery.

2. Materials and methods

2.1. Cell culture

Vero E6 cells were cultured in DMEM medium (gibco, C12430500BT) supplemented with 10% fetal bovine serum (FBS, gibco, 10099-141) and 1% penicillin-streptomycin (gibco, 15140-122) at 37 °C with 5% CO_2. Cells were digested with 0.25% Trypsin-EDTA (gibco, 25200-056) and subcultured at a ratio of 1:3 when the abundance of them reached 90%.

2.2. Plasmid purification

The pBAC-SARS-CoV-2 plasmid was purified using QIAGEN Plasmid Midi Kit (QIAGEN, 12143) and other plasmids were extracted with HiPure Plasmid EF Mini Kit (Magen, P1112-02), according to the corresponding protocols.

2.3. Liposome transfection

Plasmid transfection was performed according to the protocol of ViaFect™ Transfection Reagent (Promega, E498A). Vero E6 cells were plated one day before transfection experiment with approximately 75% confluence in growth medium. On the day of transfection, cells were washed once with PBS (gibco, C10010500BT), followed by the addition of the antibiotic-free medium. The pBAC-SARS-CoV-2 alone or combined with pcDNA3.1-mCherry-N plasmid were mixed well in given proportion in Opti-MEM (gibco, 31985-070) with the same amount of total DNA in each group. Then the transfection reagent was added in above mixture with appropriate amount to achieve the ratio of reagent to DNA as 3:1, mixed immediately and incubated at room temperature for 10 min. Finally, the mixture was added into cells and incubated for 24 – 72 h.

2.4. Flow cytometry analysis

The transfection efficiency of the pBAC-SARS-CoV-2 plasmid in HEK293T cells was identified with flow cytometry analysis. More than 1×10^6 cells were collected and washed once with PBS, fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature, resuspended in PBS containing 2% FBS, and analyzed by Sony ID 7000 and FlowJo software.

2.5. Cell viability assays

Cell viability assays were performed by the Cell Counting Kit-8 (CCK-8) according to the manufacturer's instructions. Vero E6 cells were transferred into 96-well plate (5×10^3 cells per well) with 100 μl complete medium. After the cells were completely adhered, plasmid transfection was conducted for 24, 36, 48 and 72 h. At the indicated time point of incubation, the CCK-8 reagent was added (10 μl per well) and 450 nm OD values were determined with a multifunctional microplate reader (Synergy H1, BioTek) after 1 h of incubation.

2.6. Drug treatment

Vero E6 cells were pre-treated with various concentrations of remdesivir (MCE, GS-5734) or chloroquine (MCE, HY-17589) for 2 h at 37 °C with 5% CO_2 before plasmid transfection. While chloroquine was dissolved in water, dimethyl sulfoxide (DMSO) was used to dissolve remdesivir at the final concentration (v/v) of 0.05% in the culture medium, which was held constant in all remdesivir sensitivity tests. Cells were then transfected with the plasmid mixture through the method mentioned above. Lastly, the culture medium was replaced with fresh antibiotic-free media supplemented with the same concentration of each drug after 12 h post-transfection (hpt). Then, cells were continued to culture for 24 h.

2.7. Construction of plasmids of N variants

The N variants vectors were constructed in the following steps. First, we designed 6 primers for different site-directed mutations (**Table S1**) based on the plasmid of pcDNA3.1-mCherry-N, which was constructed in our previous study [32]. PCR reactions were performed according to the manufacturer's instructions (TSINGKE, TP001) with some modification (**Table S2, S3**), followed by 1 μl of DpnI (NEB, R0176S) being added to the PCR products and incubated at 37 °C for 30 min to digest the template plasmids. Finally, 10 μl of the reaction mixture was added into DH5α (*E. coli* strain) competent cells for transformation and colonies on the LB plate were identified with sequencing analysis.

2.8. RT-qPCR analysis

Total RNAs from transfected Vero E6 cells were extracted with RNAprep Mini Kit (AXYGEN, AP-MN-MS-RNA-250G) and followed by reverse transcription conducted with TIANScript II RT Kit (TIANGEN, KR107) (**Table S4**). RT-qPCR was performed with SYBR Green SuperReal PreMix Plus (TIANGEN, FP205) according to the manufacturer's protocol (**Table S4, S5**). Primers used in above reactions were listed in **Table S1**.

2.9. Statistical analysis

In the ratio and time gradient of plasmid co-transfection assays, total numbers of cells were counted manually based on bright-field microscopic imaging data. A total of 61,003 SARS-CoV-2 genome sequences from the China National Center for Bioinformation, 2019 Novel Coronavirus Resource (July 6th, 2020) [33] were used to calculate the frequency of non-synonymous mutations within the N protein region (from positions 28,274 to 29,530 in the genome).

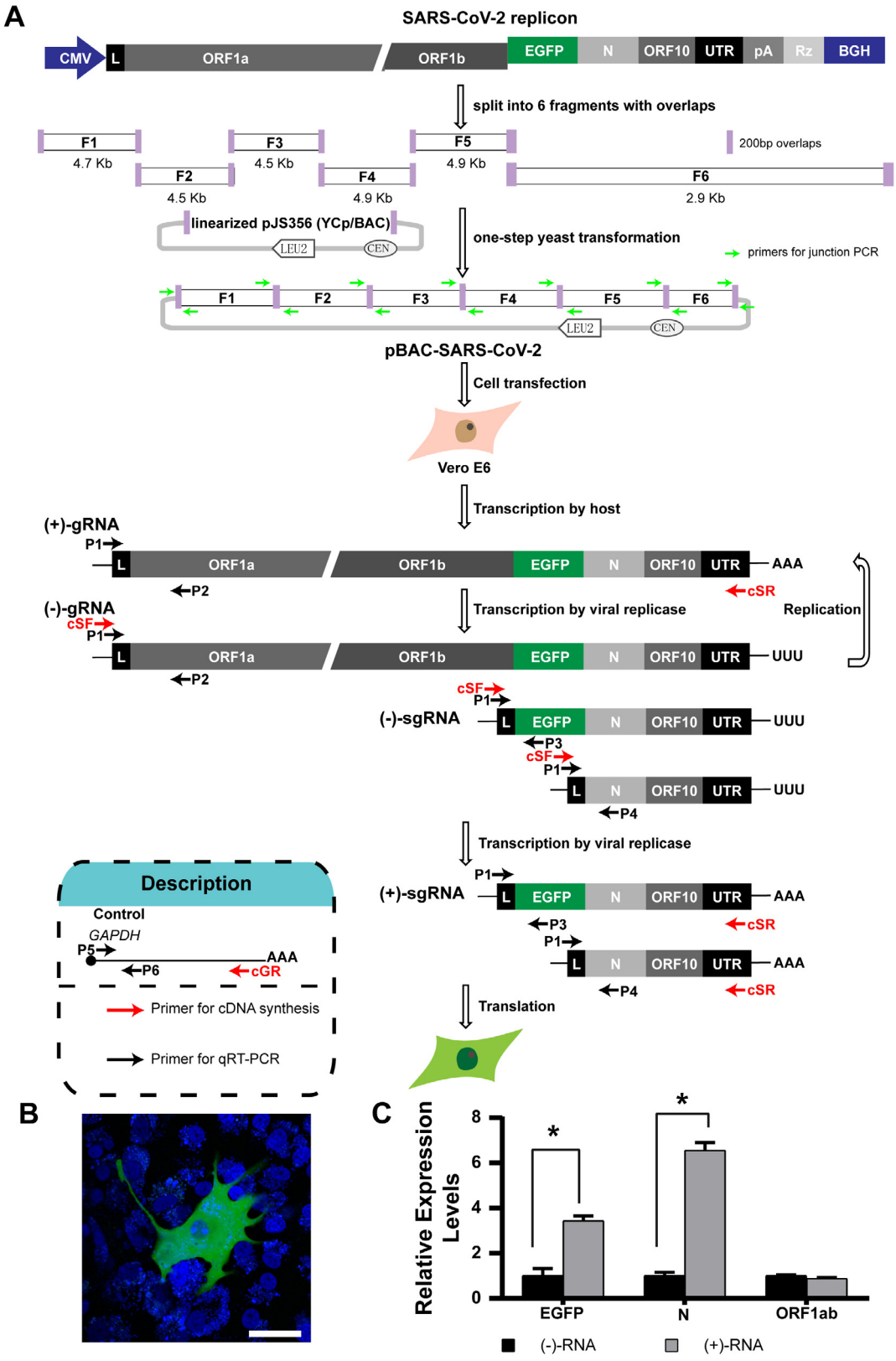
3. Results

3.1. Design and construction of a SARS-CoV-2 non-infectious replicon system in bacterial artificial chromosome (BAC) backbone

The SARS-CoV-2 genome contains about ten ORFs, ORF1a and ORF1ab are translated into two large polyproteins, pp1a and pp1ab, which are the main components of RTCs. Four structural proteins, S, E, M, and N protein, are essential for SARS-CoV-2 to generate a complete viral particle [13,34]. In addition, ORF3a, ORF6, ORF7a, ORF7b, and ORF8 encode five accessory proteins [35]. Unlike viral replication, CoV sgRNA transcription involves the discontinuous synthesis of a nested set of minus-strand sgRNAs that share the same 5′ leader sequence and the 3′ terminus within the viral genome (Fig. 1**A**) [12,36]. The discontinuous RNA synthesis results in a 3′ body sequence fused with a 5′ leader sequence via *cis*-acting RNA elements TRSs [37]. TRSs consist of a conserved core sequence of 6 or 7 nucleotides (nt) and variable 5′ and 3′ flanking sequences. In SARS-CoV-2, the conserved core sequence of TRSs is 5′-ACGAAC-3′ [7]. Also, the N protein is an RNA chaperone that modulates the TRS-mediated template switch in transmissible gastroenteritis virus (TGEV), one of the α-CoVs [20], implying that the N protein should be essential for the replicon system of CoVs.

Fundamentally, the sequence of the SARS-CoV-2 replicon system that we designed was synthesized according to the published sequence (NCBI ID: NC_045512.2). To block the production of progeny virions, three of the structural proteins (S, E, and M proteins) and all accessory proteins were excluded except ORF10 (Fig. 1**A**). But, it is unclear whether ORF10 encodes protein since sgmRNA of ORF10 are not detected in cell and there is no homology to known proteins [38]. In order to detect replication of the replicon, an EGFP was inserted between ORF1ab and N. The EGFP expression was guided by the TRS of S protein located in the front of it. The SARS-CoV-2 replicon DNA was cloned downstream of the cytomegalovirus (CMV) promoter which enables the replicon RNA to be expressed in the host cell nucleus mediated by host RNA polymerase II. At the 3′ end, the replicon was assembled with a 25 bp poly(A) tail (pA25), followed by a hepatitis delta virus (HDV) ribozyme (Rz) and a bovine growth hormone (BGH) termination to generate replicon RNAs bearing authentic 3′-ends.

Because of the large size of the replicon, it was divided into six fragments with 200 bp overlaps for DNA synthesis (Fig. 1A). These fragments were assembled into a linearized yeast centromere plasmid (YCp)/bacterial artificial chromosome (BAC) vector pJS356 through homologous recombination in BY4742 cells. The assembled plasmids were isolated from yeast strains with correct results of junction PCR analysis, enriched in EPI300 cells and sequenced for final verification. We named the verified plasmid pBAC-SARS-CoV-2 (Fig. 1**A**), and its size was approximately 26 kb. The results of flow cytometry analysis showed that the proportion of EGFP-positive cells was about 13% in the bulk of HEK293T cells transfected with the pBAC-SARS-CoV-2 for 48 h, demonstrating that the replicon could enter cells through common liposome transfection method and express normally (**Fig. S1**). After the plasmid was transfected into Vero E6 cells with the same approach, cell viability assays indicated that the replicon had no significant effect on the survival of Vero E6 cells (**Fig. S2**). EGFP fluorescence could be detected with confocal microscopy at 72 h post-transfection (hpt) (Fig. 1**B**), suggesting there was efficient amplification of the


A
SARS-CoV-2 replicon
CMV
L
ORF1a
ORF1b
EGFP
N
ORF10
UTR
pA
Rz
BGH
split into 6 fragments with overlaps
F1
4.7 Kb
F2
4.5 Kb
F3
4.5 Kb
F4
4.9 Kb
F5
4.9 Kb
F6
2.9 Kb
200bp overlaps
linearized pJS356 (YCp/BAC)
LEU2
CEN
one-step yeast transformation
primers for junction PCR
pBAC-SARS-CoV-2
Cell transfection
Vero E6
Transcription by host
(+)-gRNA
P1
P2
AAA
cSR
Transcription by viral replicase
Replication
(-)-gRNA
cSF
UUU
(-)-sgRNA
P3
P4
(+)-sgRNA
Translation
Description
Control
GAPDH
P5
P6
cGR
Primer for cDNA synthesis
Primer for qRT-PCR
B
C
Relative Expression Levels
EGFP
N
ORF1ab
(-)-RNA
(+)-RNA

replicon in the transfected Vero E6 cells. Nevertheless, the EGFP intensity only represented the global level of replicon RNA synthesis throughout the cell cycle, so we still need to develop a more specific measurement to detect each step of replicon replication and transcription.

3.2. An improvement of real-time quantitative reverse transcription PCR (RT-qPCR) methods

A distinctive feature of CoV RNA synthesis is the co-existence of sgmRNAs and minus-strand sgRNAs, produced by continuous and discontinuous synthesis step, respectively. Given the nested set of sgRNAs sharing the same 5′ leader and 3′ UTR sequence with the viral gRNA, we designed a series of gene-specific primers for cDNA synthesis and RT-qPCR (**Table S1**) to facilitate the detection of sgmRNAs, minus-strand sgRNAs and gRNA of the replicon. Specifically, total RNAs were reverse-transcribed into the first-strand cDNA using the gene-specific primers to distinguish viral plus and minus-strand RNAs from all RNAs. The cDNA synthesis forward (cSF) primer only recognized the anti-leader region of the minus-strand RNAs through complementary base pairing (Fig. 1**A**). Correspondingly, the cDNA synthesis reverse (cSR) primer was used for cDNA synthesis from the viral sgmRNAs (Fig. 1**A**). To calculate the ratio of sgmRNAs to minus-strand sgRNAs, we introduced a specific primer of GAPDH (cGR) as a control to ensure that the detection of cDNA synthesis was reliable (Fig. 1**A**).

RT-qPCR was used as a simple, quick, and sensitive method to identify different-sized sgRNAs and gRNA of the replicon. Considering the viral RNA shared the same 5′ leader region, we designed a common forward primer (P1) on the leader region, without overlap with the cSF, and synthesized the strand-specific reverse primers (P2 for replicon gRNA, P3 for EGFP sgRNA, and P4 for N sgRNA) at the downstream of the different 3′ body regions (Fig. 1**A**). These primer pairs were restricted to amplifying the corresponding sgRNAs, but were unable to detect other longer sgRNAs as the insert fragments were too long to be amplified by PCR.

To validate our method, we amplified several viral DNA segments characterizing different steps of RNA synthesis of the replicon. In our results, the level of sgmRNAs indicates the abundance of viral mRNAs, and the level of minus-strand RNAs represents the discontinuous synthesis step of transcription or the template switch. The results showed that the transcriptional levels of plus and minus-strand replicon gRNA (named with ORF1ab), EGFP sgRNA, and N sgRNA can be well detected in Vero E6 cells at 72 hpt. We observed that the abundance of sgmRNAs was greater than that of minus-strand sgRNAs (Fig. 1**C**), which was consistent with the previous study [36]. In general, the improved RT-qPCR method is convenient for researchers to distinguish sgmRNAs and minus-strand RNAs through the primers for cDNA synthesis and detect distinct sgRNAs through corresponding RT-qPCR primers. These results further demonstrate that the pBAC-SARS-CoV-2 plasmid provides an effective and valuable tool to mimic the replication and transcription processes of SARS-CoV-2 in Vero E6 cells.

3.3. Nucleocapsid (N) protein promotes discontinuous minus-strand synthesis

Previous studies have revealed that the N protein can promote the discontinuous synthesis step of SARS-CoV transcription [18,39,40] and that the interaction between N and RTCs is essential for viral survival [41,42]. One advantage of our DNA-based replicon is that it can be co-transfected with other purified plasmids (Fig. 2**A**). Given the incompatibility between two plasmids in one cell, we tested different co-transfection ratios of the pBAC-SARS-CoV-2 to pcDNA3.1-mCherry-N (ranging from 1:2 to 10:1) in order to achieve a balance between the co-transfection efficiency and the enhancement of N on replicon transcription (**Fig. S3**). By comparing the percentage of EGFP and mCherry-positive cells respectively at a series of time intervals, we found that the ratio of 4:1 was optimal (**Fig. S3**). Accordingly, the pBAC-SARS-CoV-2 and pcDNA3.1-mCherry-N plasmids were co-transfected into Vero E6 cells at a ratio of 4:1 for 36 – 48 h in our subsequent experiments. We observed that the EGFP fluorescence intensity in the cells co-transfected with N protein was significantly higher than that without N protein (Fig. 2**B-C**). Correspondingly, the proportion of EGFP-positive cells rose to approximate 40% in HEK293T cells co-transfected with N, which was about three times that of the group without N, indicating that the N protein promoted the expression efficiency of the replicon in cells (**Fig. S1**). RT-qPCR analysis further demonstrated that the expression levels of both sgmRNAs and minus-strand sgRNAs with extra N protein were much higher than those without N (Fig. 2**D-E**). The increased level of minus-strand sgRNAs suggested that the N protein can promote the discontinuous synthesis process of replicon transcription in cells. Furthermore, the ratio of sgmRNAs to minus-strand RNAs indexes the efficiency of continuous step, taking minus-strand RNAs as templates. Our results showed that the proportion of sgmRNAs to minus-strand sgRNAs was also increased markedly (Fig. 2**F**), indicating that the N protein also promotes the continuous synthesis of sgmRNAs generated from minus-strand sgRNAs.

3.4. Identification of the function of single nucleotide polymorphisms (SNPs) within N on viral transcription

To date, a large number of SNPs in the SARS-CoV-2 genome have been identified by high-throughput sequencing [43,44]. However, the functions of these SNPs remain elusive. Based on the replicon system, we attempted to explore the functions of SNPs within the N protein in viral RNA synthesis. The N protein has been

Fig. 1. Schematic diagram of the construction of the SARS-CoV-2 replicon. (A) The sequence of the SARS-CoV-2 replicon was split into six shorter fragments for synthesis, each of which had 200 bp overlaps with adjacent fragments or the backbone plasmid pJS356. Cytomegalovirus promoter (CMV) was integrated into the fragments. An enhanced green fluorescent protein (EGFP) replaced the S protein and was used as a reporter gene. The 3′ viral genome was flanked with 25 bp poly(A) tail (pA25), a hepatitis delta virus (HDV) ribozyme (Rz), and a bovine growth hormone termination (BGH). These fragments and pJS356 were linearized and introduced into BY4742 cells through a one-step yeast transformation. Seven pairs of primers were used for junction PCR analysis to screen positive clones (marked as green arrows). The correctly assembled pBAC-SARS-CoV-2 was isolated from yeast and transformed into EPI300 cells for large-scale purification. Purified plasmids were further verified with sequencing and the correct ones were used in downstream experiments. The pBAC-SARS-CoV-2 plasmid was transfected into Vero E6 cells by liposome reagents. After 72 h, the cells were collected through low-speed centrifugation for RNA extraction. Two gene-specific reverse primers, cSF and cSR, were used to reverse-transcribe the minus and plus-strand replicon RNA, respectively. cGR was used to synthesize GAPDH mRNA as control. P1 – P6 primers were designed for detecting distinct target RNA. The red arrow indicates the gene-specific reverse primer; black arrows represents RT-qPCR primer. (B) EGFP fluorescence intensity of Vero E6 cells transfected with the pBAC-SARS-CoV-2 plasmid was detected by confocal microscopy at 72 h post-transfection (hpt). Cell nuclei were stained with Hoechst. Scale bar, 50 μm. (C) Quantitative RT-PCR analysis of expression levels of sgmRNAs and minus-strand RNAs of EGFP, N, and ORF1ab in Vero E6 cells transfected with the pBAC-SARS-CoV-2 at 72 hpt. Data presented were normalized with GAPDH as an internal reference. Error bars indicate the standard deviations over three replicates. *, P<0.05; significance was assessed by Mann-Whitney *U* test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

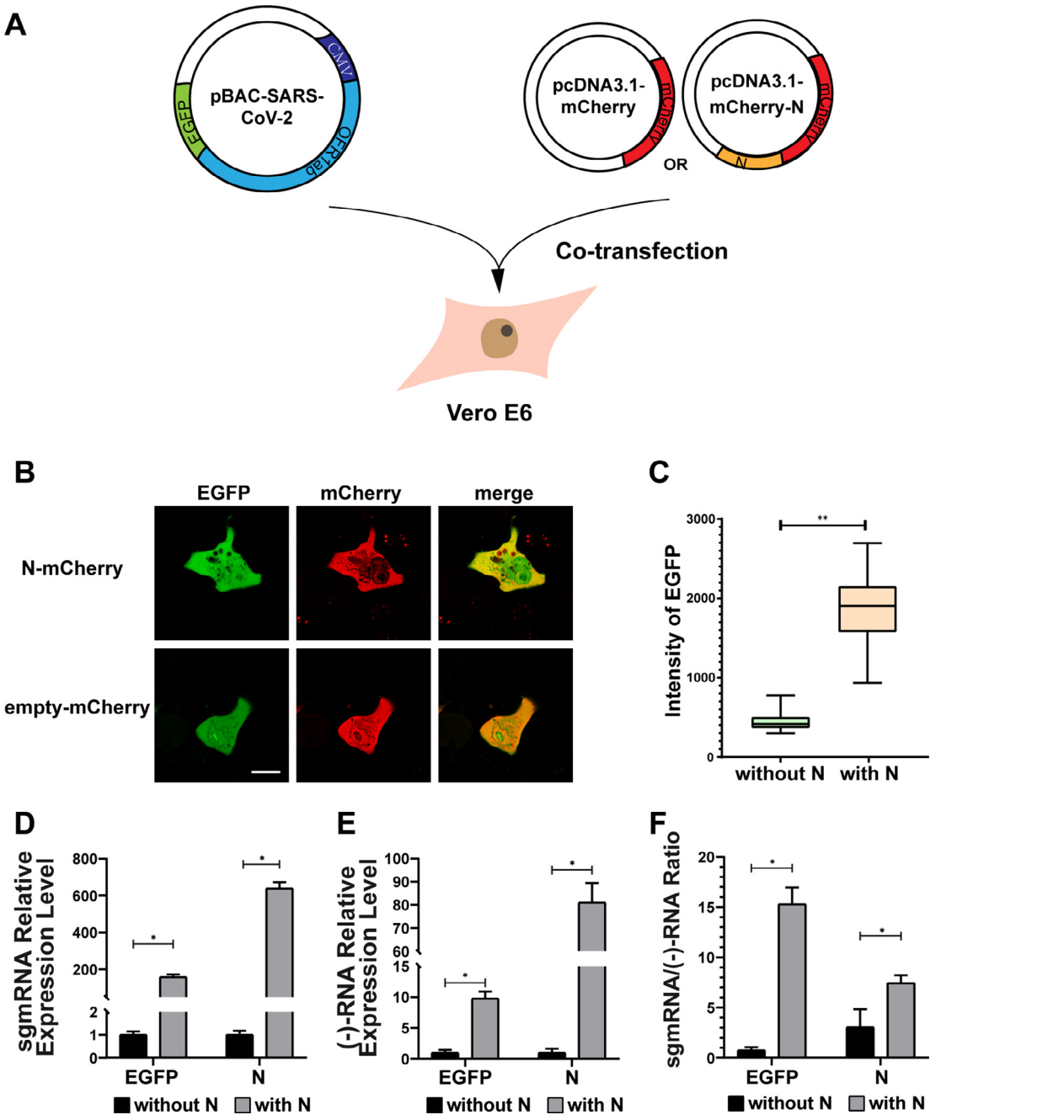


Fig. 2. Up-regulation of replicon transcription with N. (A) Schematic illustration of the co-transfection experiment performed in Vero E6 cells. (B) Vero E6 cells were co-transfected with the pBAC-SARS-CoV-2 and pcDNA3.1-mCherry or pcDNA3.1-mCherry-N plasmid. Fluorescence intensity of co-transfected cells was measured with microscopy at 36 hpt. Scale bar, 20 μm. (C) Quantitative statistics of EGFP intensity presented in (B), a total of 32 cells were considered in the group with or without N. P values were calculated by Mann-Whitney *U* test. *, P<0.05. (D-F) Total RNAs from co-transfected cells were extracted and used for quantitative analysis of expression of EGFP and N. The level of ORF1ab gRNA was used as control. Error bars indicate standard deviations over three replicates. *, P<0.05; significance was assessed by Mann-Whitney *U* test.

shown to accumulate many SNPs so far [45,46], and a large proportion of these SNPs are located in the serine/arginine (SR)-rich region (Fig. 3**A**). Generally, the N protein of SARS-CoV is mainly phosphorylated within the SR region [47,48]. The phosphorylated SARS-CoV N protein compromises its formation of the highly-ordered structure of RNP complexes, decreases its inhibitory activity on host translation, and attenuates the oligomerization of N protein, which prevents its aggregation in stress granules (SGs) [49,50]. Moreover, if the SR-rich motif was deleted from the N protein, the replication capacities of SARS-CoV appeared to be diminished [49], hinting that the SR-rich region is essential for the regulation of N protein function.

To illustrate the effect of SNPs of N protein on viral transcription, we analyzed 61,003 SARS-CoV-2 genome sequences published from the China National Center for Bioinformation, 2019 Novel Coronavirus Resource as of July 6th, 2020. We selected the top six SNPs (R203K, S197L, P13L, S194L, S188L and S202N) that most frequently had natural mutations for further experiments and constructed corresponding mutant plasmids with single point mutations (Fig. 3**A-B**). Interestingly, R203K, S197L, S194L, S188L,

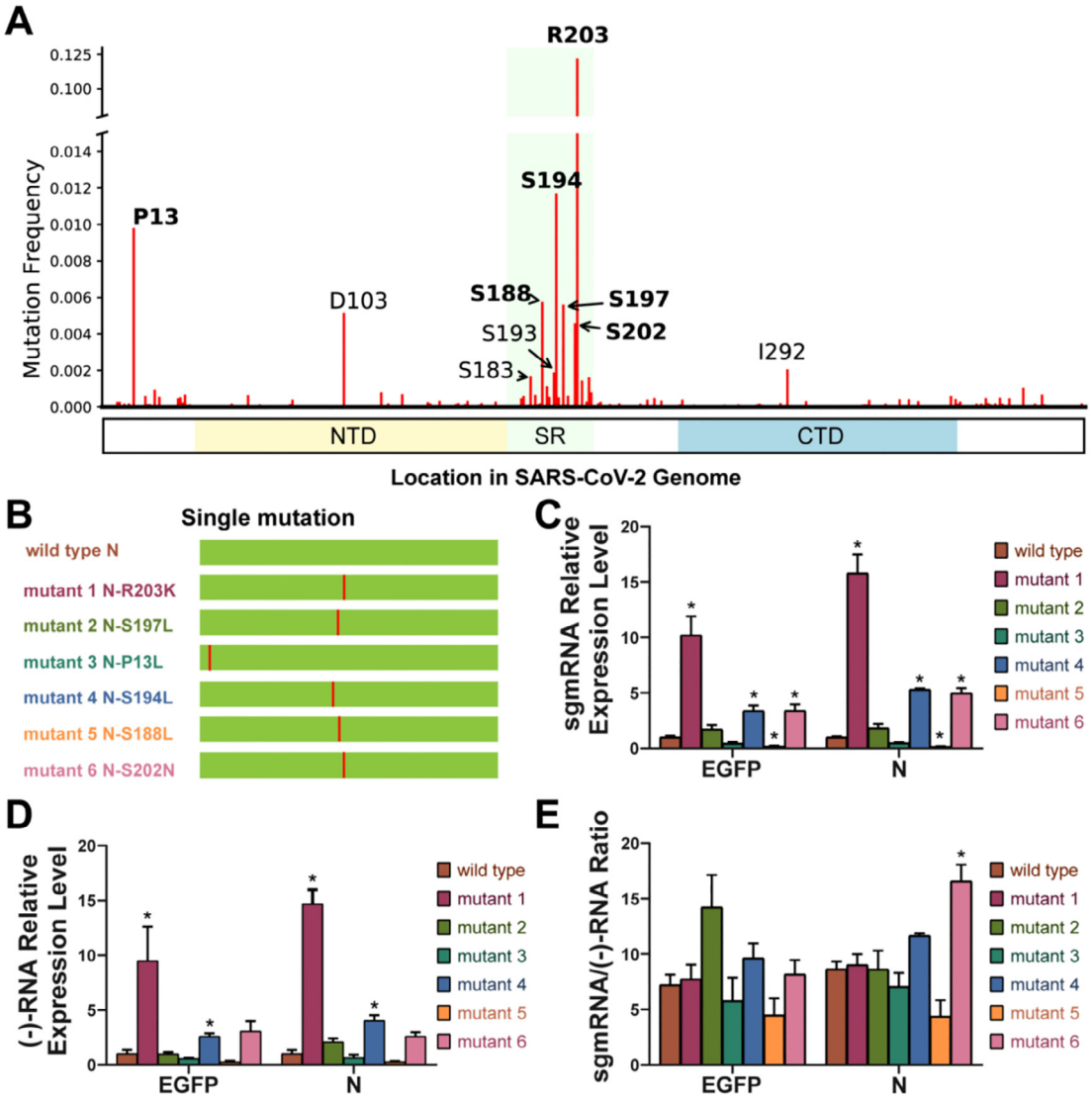


Fig. 3. Influence of SNPs within N on viral RNA expression. (A) Distribution and frequency of SNPs in the gene structure of N. The top 10 SNPs within N were marked on the mutation frequency map and the top 6 ones used in our study were in bold. NTD, N-terminal domain; SR, serine/arginine; CTD, C-terminal domain. (B) Location of the six N mutants used in this study. (C-E) Quantitative analysis of the levels of sgmRNAs and minus-strand RNAs of EGFP and N in Vero E6 cells co-transfected with the replicon and the six N variants individually. The level of ORF1ab gRNA was used as control. Error bars indicate standard deviations over at least three replicates. *, P<0.05; significance was assessed by Mann-Whitney *U* test, compared with the wild type N group.

and S202N are all located in the SR-rich region of N protein, demonstrating that the SR-rich region is a mutation hotspot. Among them, R203K is the site with the highest mutation frequency (Fig. 3**A**). Next, we co-transfected the pBAC-SARS-CoV-2 plasmid with the six N mutants individually into Vero E6 cells and collected cells at 36 hpt. RT-qPCR results showed that the N mutants 1 (R203K), 4 (S194L) and 6 (S202N) significantly improved the levels of the sgmRNA of EGFP and N (Fig. 3**C**). Additionally, the levels of the minus-strand sgRNAs with the N mutants 1 and 4 were also significantly increased (Fig. 3**D**). These results suggested that in our replicon system, the R203K mutation on N protein has the strongest effect on the viral transcription process. On the contrary, the mutant 5 (S188L) compromised viral transcription and presented the opposite function (Fig. 3**C-D**). The reduction of the sgmRNAs of S188L mutation is primarily due to the mild effect on both template switch and continuous synthesis step of transcription, represented by the reduced minus-strand RNAs amount (Fig. 3**D**) and the impaired transformation efficiency from minus-strand sgRNAs to sgmRNAs (Fig. 3**E**). Recent study has shown similar results that the double mutations of S188A and S206A completely abolished N functions [51]. The above results further elucidate that our non-infectious SARS-CoV-2 replicon is an easy-used and powerful tool for fundamental studies of viral RNA synthesis.

3.5. Application of SARS-CoV-2 replicon in drug discovery

To explore the availability of the pBAC-SARS-CoV2 in antiviral compound discovery, we performed an inhibition assay with two known drugs, remdesivir and chloroquine, which have been proven to be effective against SARS-CoV-2 in previous studies [52–54]. While remdesivir has been approved for clinical treatment of COVID-19, chloroquine shows inhibitory effect only *in vitro*. Our drug treatment is based on the "full-time" strategy used in previous study [52] with some adjustments. At first, Vero E6 cells grown in fresh antibiotic-free medium were pre-treated with each drug at various concentrations for 2 h, the pBAC-SARS-CoV-2 and pcDNA3.1-mCherry-N plasmids were then co-transfected into cells

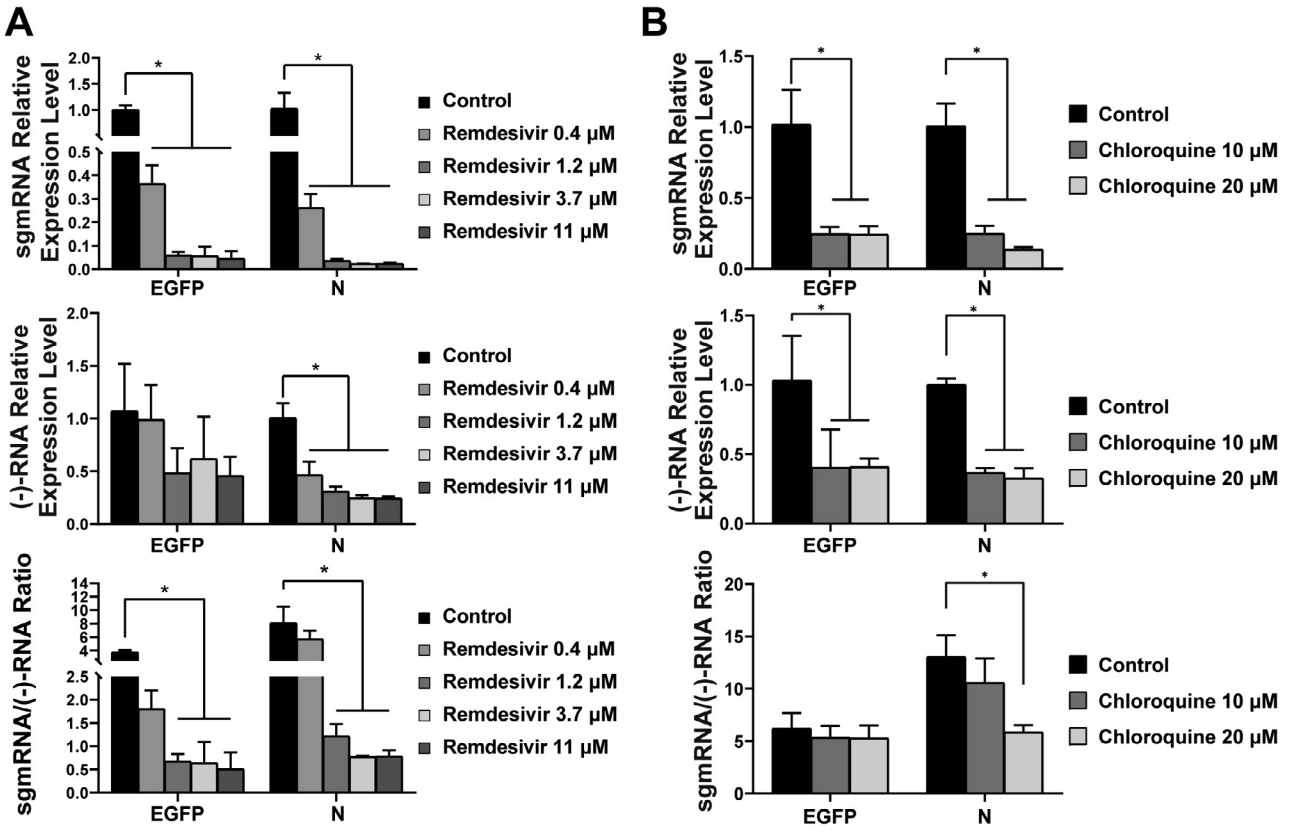


Fig. 4. Sensitivity of the replicon to antiviral drugs. (A, B) Vero E6 cells were preliminarily treated with remdesivir at the concentrations of 0.4 μM, 1.2 μM, 3.7 μM, and 11 μM or chloroquine at the concentrations of 10 μM and 20 μM for 2 h, respectively, followed by co-transfection with the replicon and pcDNA3.1-mCherry-N for 12 h. Then, the plasmid-containing medium was removed and cells were cultured in drug-containing fresh whole medium for 24 h. Finally, cells were collected for quantitative RT-PCR analysis to determine the expression levels of EGFP and N. The level of ORF1ab gRNA was used as control. *, P<0.05, significance was assessed by Mann-Whitney *U* test.

for 12 h. Finally, the medium was replaced by a drug-containing one at the corresponding concentration, in which the cells were cultured for another 24 h.

The RT-qPCR results illustrated that remdesivir effectively inhibited the replicon transcription in a dose-dependent manner (Fig. 4**A**). The abundance of sgmRNAs was suppressed to <10% in the presence of 1.2 μM and higher concentration of remdesivir. The level of minus-strand sgRNAs was also decreased, suggesting that remdesivir partially inhibited the discontinuous synthesis process of transcription by limiting quantities of minus-strand sgRNAs. Also the ratio of sgmRNAs to minus-strand sgRNAs was reduced, implying that remdesivir mainly impaired the continuous synthesis of minus-strand sgRNAs to sgmRNAs (Fig. 4**A**). In addition, chloroquine also exhibited dose-dependent inhibitory effects on viral transcription (Fig. 4**B**), demonstrating that the pBAC-SARS-CoV-2 replicon was sensitive to the two drugs against SARS-CoV-2. In conclusion, we designed and constructed a DNA-based pBAC-SARS-CoV-2 replicon that may provide a safe and convenient tool for discovering novel antiviral drugs against SARS-CoV-2 through HTS.

4. Discussion

Since 2019, the outbreak of COVID-19 has posed a huge threat to global public health, yet there remains few effective antiviral agents against it. At present, two drugs called remdesivir and molnupiravir and the Comirnaty, Spikevax, and Novavax vaccine are approved by Food and Drug Administration (FDA) or the Medicines and Healthcare products Regulatory Agency (MHRA) to treat COVID-19. The Paxlovid and Janssen COVID-19 vaccines and four kinds of monoclonal antibodies, casirivimab, imdevimab, bamlanivimab and etesevimab, are issued Emergency Use Authorization (EUA) by the FDA. The BSL-3 requirement is indispensable to SARS-CoV-2 studies, which greatly confines the progress of research on pathogenesis and development of specific treatment. Considering the large size of the CoV genome, many CoV replicons have been constructed into the BAC backbone, which is also commonly used for researches on other families of virus [28–31]. To date, several studies have developed the SARS-CoV-2 replicon systems by different methods, but most of them relied heavily on the transfection of *in vitro* transcribed RNA [55–57]. Here, we designed and validated a DNA-based SARS-CoV-2 replicon that can be directly transfected into cells without *in vitro* RNA transcription. The system can be used more readily for SARS-CoV-2 RNA synthesis studies in cells. Furthermore, we explored several applications of the pBAC-SARS-CoV-2 replicon, including studying the functions of N protein in viral replication and transcription and the feasibility for high-throughput drug screening in a BSL-2 laboratory.

According to previous studies, the N protein is thought to be required for the template switch and viral replication only in the early stage [18,49]. In our DNA-based replicon, we found that the N protein regulates sgRNAs synthesis in both discontinuous and continuous synthesis steps of transcription. When adding extra N protein, our replicon exhibited transcriptional enhancement without any obvious effect on viral replication. In addition, we co-transfected the SARS-CoV-2 replicon with six N mutants into Vero E6 cells and found that three of six N mutants (R203K, S194L and S202N) greatly facilitate viral transcription while one of them (S188L) compromises this process. Moreover, five of the six muta-

tions occurred within the SR region, which is considered as a potential phosphorylation region involved in N multimerization, translation inhibitory activity, and cellular localization in SARS-CoV [50]. Therefore, we speculate that the mutants that influence the transcriptional activity of the pBAC-SARS-CoV-2 replicon likely associates with the phosphorylation state of N, though further verification is needed [50]. In addition, the most frequent mutation, R203K, displayed the highest activity of N protein to promote viral transcription, implying that this mutation may facilitate proliferation and transmission of the SARS-CoV-2 variant in humans. In turn, the stronger viral amplification competency further stabilized this SARS-CoV-2 mutation. Our research explored the underlying mechanisms of N protein polymorphisms that may involve in viral transcription, indicating that our system is a useful tool for studying the mechanisms of SARS-CoV-2 replication and transcription.

To explore the availability of our replicon in the identification of anti-SARS-CoV-2 inhibitors by HTS, we tested the sensitivity of the replicon to two well-known compounds, remdesivir and chloroquine [52]. Both of them effectively impeded viral transcription in a dose-dependent manner, demonstrating that the replicon may be used to perform antiviral screening in the discovery of drugs against SARS-CoV-2.

However, as the replicon could generate RNAs bearing authentic 5′ and 3′-ends after transcription whose genome structure is similar to the RNAs synthesized by the virus, it is difficult for us to distinguish between the products of CMV and viral replication by RT-qPCR detection. The gRNA produced by CMV could account for the result that there was no significant change in the level of replicon gRNA after Vero E6 cells were co-transfected with additional wild type or mutant N plasmid or treated with the two drugs (**Fig. S4**). In our work, flow cytometry was used to determine the transfection efficiency of the replicon in HEK293T cell line, the proportion of EGFP-positive cells was about 13% when the BAC plasmid was transfected alone and 40% when the BAC and N plasmid were transfected simultaneously, which further confirmed the promoting effect of N on replicon expression. However, the transfection efficiency of the replicon in Vero E6 cells determined by the flow cytometer was not inspiring. We think the possible reasons are as follows: firstly, considering the large genome of the replicon, the transfection of the replicon in Vero E6 cells may be more difficult than HEK293T. Secondly, the sequence elements around EGFP in the replicon may have some defects in the protein expression, thus making the EGFP fluorescence intensity insufficient to be detected by flow cytometer. Now, we are actively conducting the elements optimization of the replicon, and hope to enhance the EGFP expression. At the same time, we also expect to improve the transfection efficiency of Vero E6 cells by replicon electroporation or introducing a resistance gene to enrich more positive cells. Nonetheless, our results confirmed that the BAC system is a feasible alternative to SARS-CoV-2 related studies, and our DNA-based replicon provides a fundamental framework for future studies.

5. Conclusion

In this study, we designed and constructed a DNA-based SARS-CoV-2 replicon system incapable of producing viruses to mimic the replication and transcription process of virus in Vero E6 cells, which are crucial to the survival of virus. Preliminary experiments showed that the replicon could carry out efficient amplification in Vero E6 cells. The N protein can not only promote the overall transcription of replicon RNA, but also facilitate the transformation from minus-strand sgRNAs to sgmRNAs. Moreover, the SNPs that occurred in N protein, like R203K and S194L, could significantly up-regulate the expression of sgRNAs. Furthermore, the inhibitory effect of two well-known drugs on transcription level of the replicon suggested that the system could be used for the detection of antiviral effect of candidate drugs against SARS-CoV-2. Thus, our replicon system is bio-safe and convenient for studying SARS-CoV-2 pathogenesis, and also has potential applications in SARS-CoV-2 antiviral discovery, which will greatly accelerate basic research on SARS-CoV-2 and help prevent the global spread of COVID-19.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

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Author's contributions

Xiaolong Feng, Xiaofan Zhang, Dan Zhao and Jianyang Zeng conceived the study. Junbiao Dai, Dan Zhao, Tian Xia, and Jianyang Zeng supervised the project. Xiaolong Feng and Xiaofan Zhang performed the experiments. Xiaolong Feng and Xiaofan Zhang analyzed the data. Shuangying Jiang and Yuanwei Tang contributed to the plasmid construction. Xiaoting Wang assisted the reagent. Xiaolong Feng, Xiaofan Zhang, Shuangying Jiang, Chao Cheng, Parthasarathy Abinand Krishna, Tian Xia, Dan Zhao and Jianyang Zeng wrote the paper with support from all authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2022.08.044.

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