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Review

Reverse genetics systems for SARS-CoV-2: Development and applications

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ABSTRACT

The recent emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) caused serious harm to human health and struck a blow to global economic development. Research on SARS-CoV-2 has greatly benefited from the use of reverse genetics systems, which have been established to artificially manipulate the viral genome, generating recombinant and reporter infectious viruses or biosafety level 2 (BSL-2)-adapted non-infectious replicons with desired modifications. These tools have been instrumental in studying the molecular biological characteristics of the virus, investigating antiviral therapeutics, and facilitating the development of attenuated vaccine candidates. Here, we review the construction strategies, development, and applications of reverse genetics systems for SARS-CoV-2, which may be applied to other CoVs as well.

1. Introduction

Coronaviruses (CoVs) are enveloped, positive-sense, single-stranded RNA viruses that can pose a great threat to public and animal health. To date, seven CoVs are known to cause disease in humans (V'Kovski et al., 2021), including four associated with the common cold (hCoVs 229E, OC43, NL63 and HKU1) and three highly pathogenic zoonotic CoVs that have resulted in significant morbidity and mortality over the past two decades, namely severe acute respiratory syndrome (SARS)-CoV, Middle East respiratory syndrome (MERS)-CoV and SARS-CoV-2. Recently, a canine CoV isolate (CCoV-HuPn-2018) and a porcine deltacoronavirus strain (Hu-PDCoV) were found to infect humans, suggesting their potential risk for cross-species transmission from animals to humans (Lednicky et al., 2021; Vlasova et al., 2022). Most famously, SARS-CoV-2 is the causative agent of the CoV disease 2019 (COVID-19) pandemic that started in December 2019 (Wu J. T. et al., 2020; Zhu et al., 2020). Thanks to the experience and lessons learned from the previously emerged CoVs, initial research into the clinical disease, pathogenesis and antiviral therapies for SARS-CoV-2 was carried out rapidly. Despite this, the great transmissibility and genetic variability of SARS-CoV-2 have resulted in more than 676 million infections and 6.8 million deaths as of March 2023 (https://coronavirus.jhu.edu/map.html).

SARS-CoV-2 belongs to the genus *Betacoronavirus* in the subfamily *Orthocoronavirinae* of family *Coronaviridae* with a genome of approximately 30 kb. The SARS-CoV-2 genomic RNA possesses a 5′ cap and a poly(A) tail at the 3′ end. The untranslated regions (UTRs) on both flanks contain *cis*-acting secondary RNA structures essential for RNA synthesis. The 5′ two-thirds of the genome is translated directly from genomic RNA and encompasses two large open reading frames (ORF1a and ORF1b) that encode two partially overlapping replicase polyproteins (pp1a and 1 ab). These polyproteins are proteolytically cleaved into 16 nonstructural proteins (nsp1–nsp16) by viral papain-like protease (PLpro) and 3C-like protease (3CLpro), and subsequently constitute the replication-transcription complex (RTC), posing some functions in viral replication and transcription (Gorbalenya et al., 2006; V'Kovski et al., 2021; Shi et al., 2022). One-third of the genome is transcribed via a discontinuous transcription mechanism to form a nested set of subgenomic RNAs (sgRNAs), which are then translated into four structural proteins (spike [S], envelope [E], membrane [M], and nucleocapsid [N]) and more than eight accessory proteins (3a, 3b, 6, 7a, 7b, 8b, 9b and 10) (Wu A. et al., 2020; Hu et al., 2021).

Reverse genetics is defined as the dissection of the structural and functional relationships of specific genes or non-coding nucleic acids by making necessary modifications, such as targeted mutations, deletions or insertions. Improvements to gene editing, recombination and sequencing methods have made reverse genetics a powerful research tool to effectively study molecular characteristics, test antiviral therapeutics and develop vaccine candidates *in vivo* and *in vitro* by directly manipulating the genomes of RNA viruses. CoVs were once considered to be the most difficult positive-sense RNA virus to rescue due to their large genome

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sizes, occasional instability and toxicity of some viral clones in bacterial systems (Almazan et al., 2014). Before the first full-length transmissible gastroenteritis virus (TGEV) infectious clone was obtained in 2000 (Almazán et al., 2000), strategies for devising CoV reverse genetics systems were mainly targeted RNA recombination based on the model of mouse hepatitis virus (MHV) (Makino et al., 1986; Baric et al., 1990). However, this technique didn't involve the assembly of full-length genomic infectious clones because of limitations to the length of template vectors for the donor RNA. Other major flaws also existed, such as the inability to manipulate genes close to the replicase region (Almazan et al., 2014).

With the ongoing evolution of reverse genetics techniques, several reverse genetics systems have been successfully established to manipulate the genomes of human and animal CoVs. The construction strategies used are basically the same for each of them: (i) Obtain all fragments of the viral genome from RT-PCR or chemically synthesized DNA; (ii) Assemble these fragments into a full-length complimentary DNA (cDNA) by *in vitro* ligation, transformation-associated recombination (TAR), or sequentially cloning into a bacterial artificial chromosome (BAC), vaccinia virus vectors or using other unconventional approaches like circular polymerase extension reaction (CPER) (Almazan et al., 2014; Aubry et al., 2015; Kurhade et al., 2023); (iii) Transfect the cDNA plasmid or electroporate *in vitro*-transcribed RNA transcripts into susceptible cells, initiating a viral life cycle including transcription, translation, replication, assembly and release, and then rescue infectious viruses (Fig. 1).

The aim of this review is to describe the role of reverse genetics systems in the construction of infectious clones and BSL-2-adapted SARS-

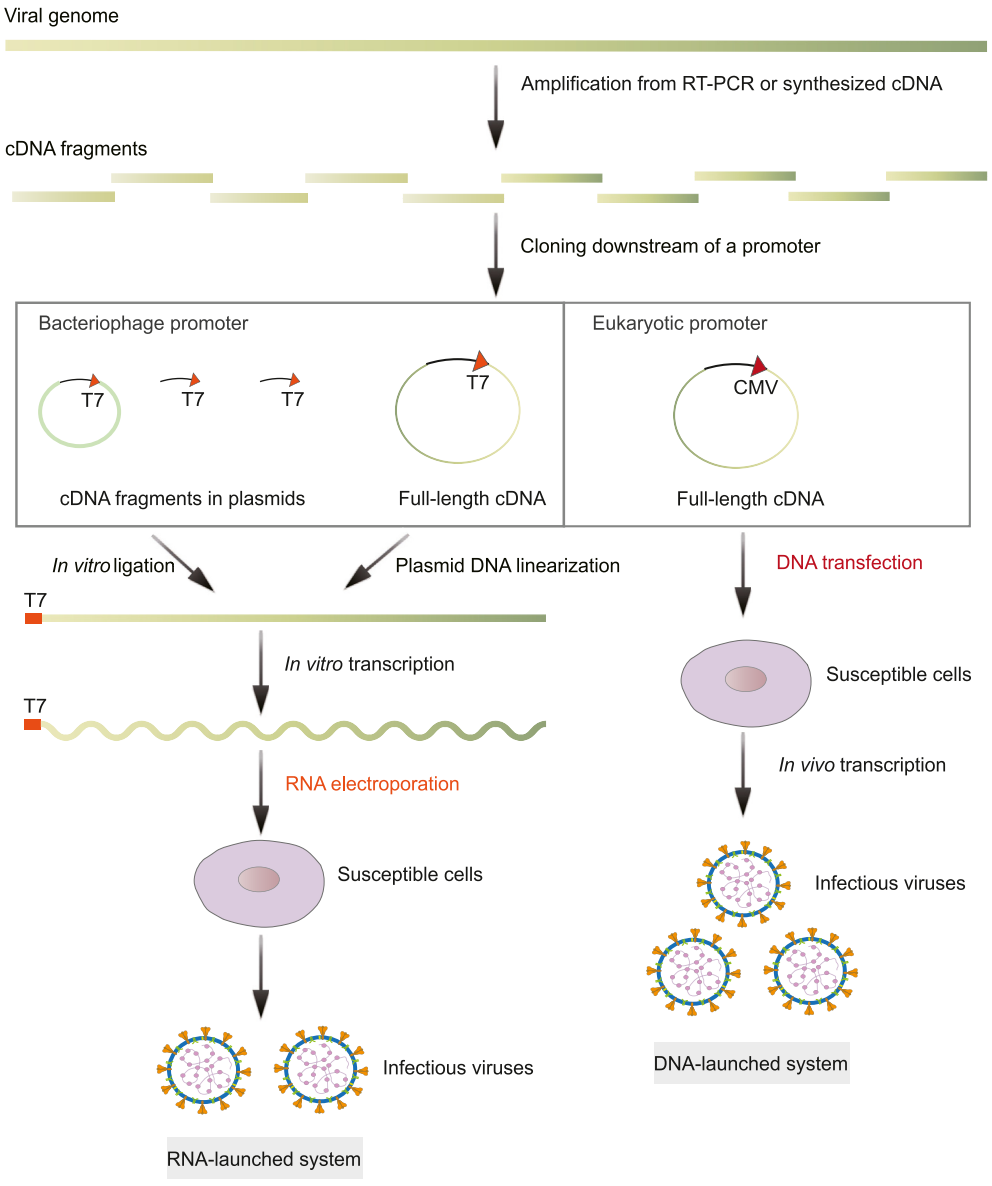

Fig. 1. Generic procedure for the construction of a reverse genetics system. The viral genome is divided into several fragments that are amplified from RT-PCR or chemically synthesized DNA. These cDNA fragments are stably incorporated into vectors with a bacteriophage promoter or eukaryotic promoter. In RNA-launched systems, cDNA fragments are cloned into plasmids with T7 promoters and used to assemble the full-length cDNA by *in vitro* ligation, or directly achieved by one-step assembly in plasmids. Then, the ligated products or linearized DNA serve as templates for *in vitro* transcription to obtain capped infectious genomic RNA, which will generate infectious viruses by electroporation of RNA transcripts into susceptible cells. In DNA-launched systems, direct transfection of cytomegalovirus (CMV)-driven plasmids containing the full-length cDNA allows initiation of RNA polymerase II-dependent *in vivo* transcription in host cells, leading to the successful rescue of the virus.

CoV-2, and to summarize their applications in studying viral protein functions, screening for antiviral drugs and developing live attenuated vaccine (LAV) candidates for further understanding and control.

2. SARS-CoV-2 reverse genetics systems and construction strategies

2.1. Infectious clones

2.1.1. In vitro ligation-based systems

In vitro ligation is a classical and widely used reverse genetics technique that uses unique type IIS restriction endonucleases (e.g. *Bsa*I, *Esp*3I and *Bbs*I) to recognize junction sequences of individual segmented fragments, systematically and sequentially assembling them into a full-length cDNA by *in vitro* enzymatic ligation. These enzymes (*Bsa*I site, GGTCTCN$^{\downarrow}$NNNN; *Esp*3I site, CGTCTCN$^{\downarrow}$NNNN) recognize asymmetrical sites and make a staggered cut 1 and 5 nucleotides downstream of the recognition sequences, usually generating 4-nucleotide variable overhangs. Based on this property, traditional cloning approaches would involve the insertion of restriction sites into the viral genome during the ligation of two fragments. When using "no-see-um" cloning technology, the junctions of each fragment, including the reverse-oriented recognition sequence of each restriction enzyme and a 4-nucleotide sequence derived from the viral genome, can be subtly introduced at virtually any position between two adjacent fragments without mutating the viral genome sequence. These restriction sites are then removed in the subsequent digestion and ligation, thus allowing for the systematic assembly of full-length cDNAs (Yount et al., 2000, 2002, 2003; Baric and Sims, 2005).

To efficiently generate viral RNA from cDNA *in vitro*, *in vivo* or *in cellulo*, the viral sequences need to be flanked by a suitable promoter. The most commonly used promoters for infectious clones are the bacteriophage promoter T7 and the eukaryotic promoter of the human cytomegalovirus (CMV), and each has its own advantages (Aubry et al., 2015). In combination with electroporation of RNA transcripts, the strategy of *in vitro* transcription using bacteriophage RNA polymerases, especially T7 RNA polymerase, is widely used for the rescue of various human and animal positive-sense RNA viruses, which is known as the RNA-launched system for virus recovery (Fig. 1). The presence of T7 promoter (5′ end) and a poly(A) tail (3′ end) in the linearized full-length cDNA are essential for *in vitro* transcription to generate infectious, 5′-capped and 3′-polyadenylated viral genomic RNAs, which resist shearing by host cell nucleases and thereby have improved stability. By splitting unstable regions and cloning the segmented genome into separate vectors, this approach avoids the probability of instability of cloning when constructing the recombinant DNA plasmid (Yount et al., 2000), and ensures straightforward manipulation of genes of interest without affecting other unrelated genomic regions. Nevertheless, RNA transcripts cannot be viewed as genetically homogeneous populations since RNA polymerases are error prone (Boyer et al., 1992; Sooknanan et al., 1994), and some technical challenges (e.g., low efficiency of RNA electroporation, poor stability of prepared RNA transcripts and the high cost of reagents) also limit the application of *in vitro* ligation (Table 1) (Nguyen et al., 2021).

Several research groups have already used this systematic assembly approach to construct SARS-CoV-2 infectious clones, each using a similar strategy (Fig. 2) (Hou et al., 2020; Xie et al., 2020b, 2021). The whole genome of SARS-CoV-2 was divided into seven contiguous fragments; the

Table 1
Characteristics of SARS-CoV-2 reverse genetics systems.

Construction strategies	Pros and cons	Gene deletions or insertions	Reporter genes
Infectious clones			
In vitro ligation	• Pros: avoids cloning instability, ensuring direct gene manipulations; • Cons: labor-intensive and time-consuming, some technical challenges.	ORF7	mNeonGreen (Xie et al., 2020b), Nluc (Xie et al., 2020a)
		partial ORF7	GFP, GFP-nLuc (Hou et al., 2020)
TAR cloning	• Pros: rapid and unrestricted one-step cDNA assembly by homologous recombination; • Cons: requires whole-genome sequencing.	partial ORF7	GFP (Thi Nhu Thao et al., 2020)
BAC	• Pros: stable and convenient with direct transfection of BAC plasmids, allowing insertion of large DNA sequences; • Cons: labor-intensive and time-consuming, does not completely eliminate cloning instability.	ORF7a	Venus, mCherry, Nluc (Chiem et al., 2021a)
		upstream of N	Venus-2A, mCherry-2A, Nluc-2A, mCherry-Nluc-2A (Chiem et al., 2021b; Ye et al., 2021)
		upstream of ORF7b	2A-mCherry, 2A-Nluc, 2A-ZsGreen (Rihn et al., 2021)
CPER	• Pros: rapid and simple by using PCR amplification; • Cons: low rescue efficiency and undesired mutations, needs additional passages to rescue viruses.	partial ORF7	ZsGreen (Amarilla et al., 2021), GFP (Torii et al., 2021)
		downstream of ORF6	high-affinity NanoLuc binary technology (HiBiT) (Torii et al., 2021)
ISA		partial ORF3a	mCherry (Melade et al., 2022)
BSL-2 adapted SARS-CoV-2			
Replicons	• Pros: allows manipulations in BSL-2 laboratories, providing an important alternative for SARS-CoV-2 research;	S-ORF8	Rluc (Zhang Q. Y. et al., 2021), EGFP (Feng et al., 2022), Rluc-2A-Neo (Xia et al., 2020), Gluc-2A-BSD (Zhang Y. et al., 2021), Rluc-Neo (Tanaka et al., 2022), Nluc-Neo (Nguyen et al., 2021), Nluc-BSD (Zhang et al., 2022), GFP-BSD (Wang B. et al., 2021)
	• Cons: difficulty of generating stable replicons and defects for research towards viral entry, assembly and release.	S	mNeonGreen, Puro, Luc (Jin et al., 2021), Luc-GFP (Malicoat et al., 2022)
		S, E, M	Luc-GFP, Neo (He et al., 2021), Nluc, Neo (Liu et al., 2022a)
Trans-complementary systems	• Pros: allows propagation of defective viruses in packaging cells, recapitulating an authentic infection process; • Cons: the possibility of viral recombination and reversion.	S	Neo-2A-mNeonGreen, Neo-2A-Gluc (Ricardo-Lax et al., 2021)
		N	GFP (Ju et al., 2021)
		ORF3-E	mNeonGreen (Zhang X. et al., 2021)
CPD	• Pros: genetically stable and valid attenuation with numerous mutations; • Cons: needs rational design for selection of recoding regions.		

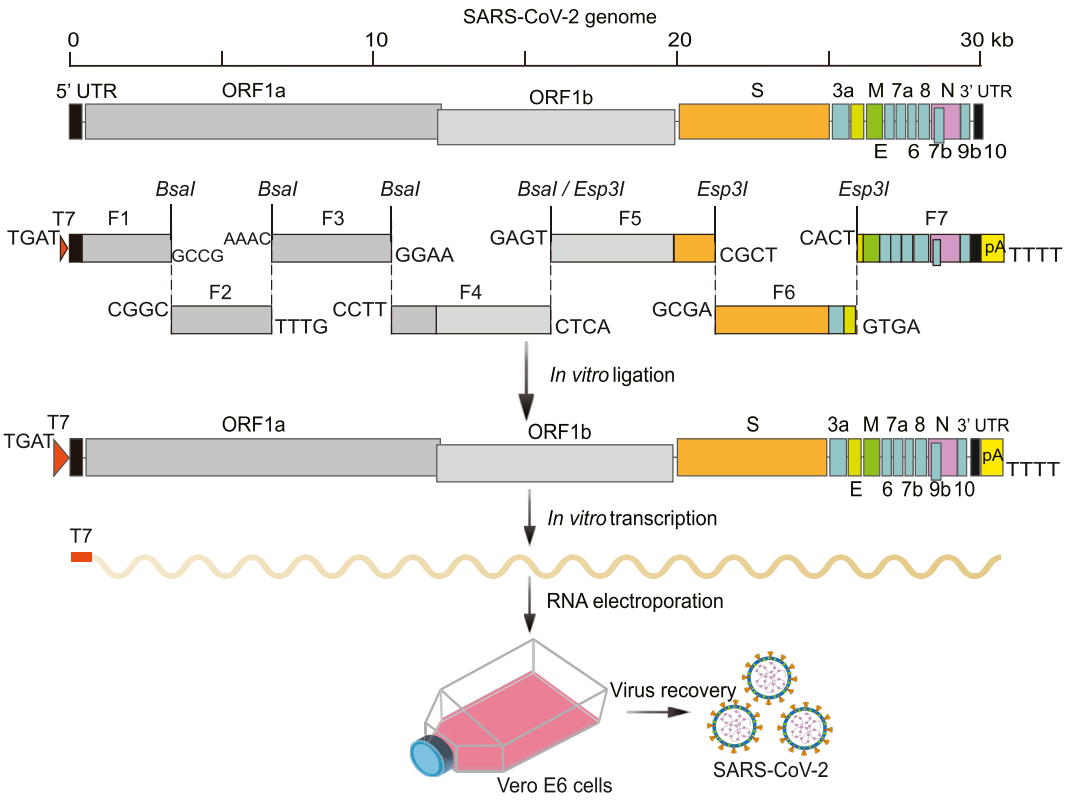

Fig. 2. Schematic representation of *in vitro* ligation–based reverse genetics systems for constructing a SARS-CoV-2 cDNA clone. The viral genome of SARS-CoV-2 strain USA-WA1/2020 is split into seven contiguous fragments (F1 to F7). A T7 promoter and poly(A) tail are added at the 5′ and 3′ ends, respectively. Each fragment is flanked by unique class IIS restriction endonuclease sites (*Bsa*I or *Esp3*I) to generate unique cohesive ends and used to assemble into full-length cDNA by *in vitro* ligation. The obtained full-length cDNA allows synthesis of full-length SARS-CoV-2 RNA transcripts by *in vitro* transcription, which are electroporated into Vero E6 cells together with SARS-CoV-2 N mRNA to rescue infectious viruses.

unstable fragments (prone to mutations) that failed to propagate in *E. coli* were amplified by RT-PCR and cloned into a single-copy vector (pCC1BAC) to increase the stability of the cDNA plasmids, whereas the more stable fragments were chemically synthesized and cloned into a high-copy plasmid (pUC57). During PCR or gene synthesis, silent mutations were introduced into conserved regions as a genetic marker, and a class IIS restriction endonuclease site (*Bsa*I or *Esp*3I) was added to flanks of each cDNA fragment to recognize asymmetric DNA sequences, cleave outside of their recognition sequence and generate unique cohesive ends. After digestion with enzyme *Bsa*I or *Esp*3I, all fragments were sequentially ligated using T4 DNA ligase to obtain a full-length cDNA, which served as template for *in vitro* transcription to synthesize full-length SARS-CoV-2 RNA transcripts. Previous studies have suggested that the presence of N transcripts may function in the recovery of CoVs (Curtis et al., 2002; Yount et al., 2002, 2003; Almazan et al., 2004). To improve rescue efficiency, the *in vitro*-transcribed RNA was electroporated into Vero E6 cells together with an mRNA encoding SARS-CoV-2 N protein. Thus, the recovery of recombinant SARS-CoV-2 (rSARS-CoV-2) or reporter viruses expressing green fluorescent protein (GFP) and mNeonGreen, was confirmed by monitoring the presence of cytopathic effect (CPE) after transfection, detecting viral antigens by immunofluorescence, or directly observing fluorescence signals in cells.

2.1.2. TAR cloning-based systems

Recently, the yeast *Saccharomyces cerevisiae* has become popular in eukaryotic biology and genetics as a useful vector for the design, synthesis and assembly of entire genomes from scratch, depending on the technique of transformation-associated recombination (TAR) cloning (Kouprina and Larionov, 2008; Zhao N. et al., 2023). Taking advantage of the yeast's homologous recombination capability, this method only requires corresponding overlapping upstream and downstream sequences across multiple fragments to realize one-step assembly of genomes larger than 30 kb in a yeast artificial chromosome (YAC), avoiding the time-consuming and laborious experimental scheme of segmental cloning followed by sequential splicing, and greatly shortening the operation time and improving the construction efficiency of infectious clones (Table 1).

At the beginning of the COVID-19 outbreak, a YAC-based synthetic genomics platform was used to reconstruct infectious clones of CoVs including MHV, MERS-CoV and SARS-CoV-2 (Thi Nhu Thao et al., 2020). The specific method used to successfully generate rSARS-CoV-2 is summarized in Fig. 3. First, several overlapping DNA fragments were amplified from RT-PCR or chemical synthesis; the first fragment contained overlapping sequences of the TAR vector pCC1BAC-His3 and a T7 promoter at the 5' end, while the last fragment included overlapping sequences of the TAR vector and a restriction enzyme cleavage site (*Eag*I) downstream of the 3′ poly(A) tail. Secondly, the mixture of genome fragments was simultaneously transformed into *S. cerevisiae* together with the linearized TAR vector, and assembled the SARS-CoV-2 full-length genomic cDNA into a YAC by homologous recombination in yeast. After purifying the yeast plasmid and digesting it with *Eag*I, the resulting linearized YAC DNA was used to generate capped viral genomic RNA by *in vitro* transcription using T7 RNA polymerase. Finally, the RNA was electroporated into BHK-21 cells together with SARS-CoV-2 sgRNA-N. Electroporated cells were subsequently seeded on Vero E6 cells to realize virus propagation and isolation through plaque purification. Although this RNA-launched system simplified the step of directional assembly, a sequencing step was required to confirm that the yeast plasmid did not introduce any undesired mutations during homologous recombination and propagation in yeast. Some technical challenges may still persist such as acquisition of high-quality full-length RNA by *in vitro* transcription used for electroporation (Table 1).

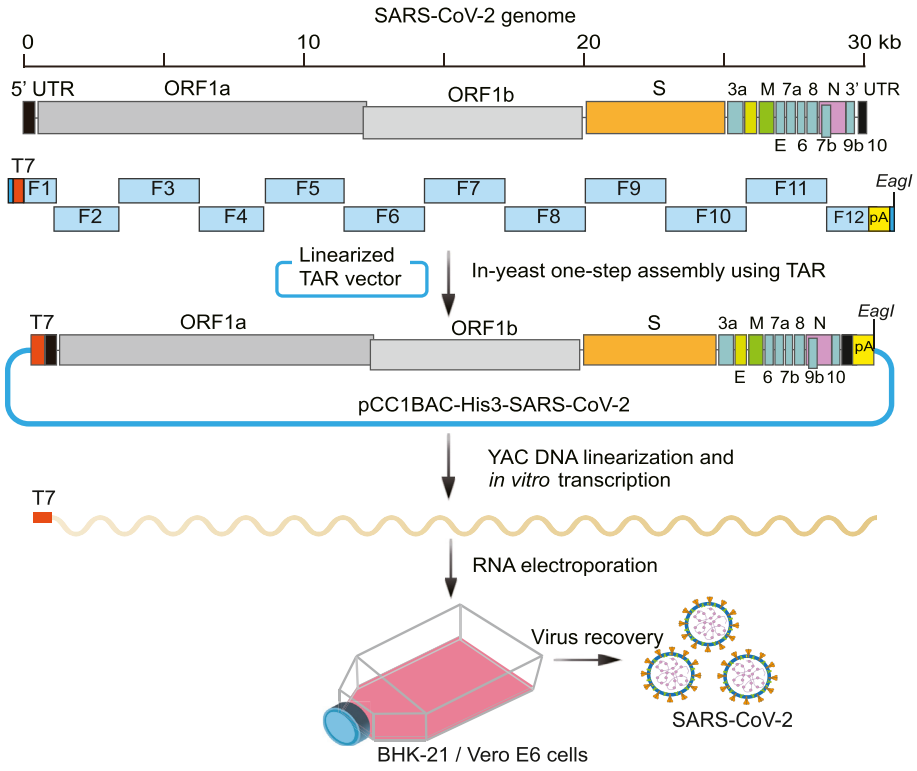

Fig. 3. Schematic representation of TAR cloning–based reverse genetics systems for constructing a SARS-CoV-2 cDNA clone. The SARS-CoV-2 genome is divided into several overlapping cDNA fragments (F1 to F12). The first fragment contains overlapping sequences of the TAR vector pCC1BAC-His3 and a T7 promoter at the 5′ end, while the last fragment includes overlapping sequences of the TAR vector and a restriction enzyme cleavage site (*Eag*I) after the poly(A) tail downstream of the 3′ end. Afterwards, the amplified fragments are simultaneously transformed into *S. cerevisiae* together with the linearized TAR vector to achieve one-step assembly in yeast. After the purification and linearization of yeast DNA, infectious viruses are generated using *in vitro* transcription followed by RNA electroporation.

2.1.3. *BAC-based systems*

As mentioned above, the viral genome size of CoV is too large for conventional plasmid vectors to accommodate as a full-length cDNA clone. Therefore, researchers cloned the full-length cDNA into a BAC, which are low-copy vectors that can maintain very large DNA insertions (Almazán et al., 2000). Moreover, it can reduce the potential toxicity of certain expressed sequences on the full-length CoV cDNA clone. This BAC-based approach has been already used to construct CoV infectious clones, including TGEV (Almazán et al., 2000), SARS-CoV (Almazan et al., 2006), HCoV-OC43 (St-Jean et al., 2006), MERS-CoV (Almazan et al., 2013), swine acute diarrhea syndrome (SADS)-CoV (Yang et al., 2019), and most recently SARS-CoV-2 (Ye et al., 2020; Rihn et al., 2021). In order to recover rSARS-CoV-2 from a DNA-launched system, a full-length cDNA clone was assembled under the control of a eukaryotic CMV promoter, and contained a poly(A) stretch, a hepatitis delta virus ribozyme (HDVRz), and bovine growth hormone (BGH) or simian virus 40 (SV40) termination and polyadenylation sequences downstream of the 3′ end. The incorporation of the CMV promoter drives the transcription of the capped mRNA from cDNA by host cell RNA polymerase II, while the other elements ensure homogenous 3' end processing during the rescue of recombinant viruses (Dubensky et al., 1996). Unlike the T7-driven approach, direct transfection of CMV-driven plasmids containing the full-length cDNA allows initiation of *in vivo* transcription in host cells, which appears to be more stable and convenient than the *in vitro* transcription strategy followed by RNA electroporation (Table 1).

In this approach, all fragments were sequentially cloned to the BAC plasmid pBeloBAC11 or pCC1-4k either by ligation with restriction enzymes (Fig. 4) or by in-fusion PCR between overlapping fragments (Ye et al., 2020; Rihn et al., 2021; Fahnøe et al., 2022). The former required existing unique restriction enzymes sites in the SARS-CoV-2 genome, or the introduction of new ones via silent mutations, which also served as genetic tags to distinguish the rescued virus from the natural parent isolate. In this way, junction sequences corresponding to unique enzyme sites were added to the flanks of each chemically synthesized fragment, which were used for sequential assembly to generate the full-length cDNA. After electroporating into DH10B electrocompetent *E. coli* cells, the BAC plasmid containing the entire viral genome was sequenced and directly transfected into Vero E6 cells using Lipofectamine reagents for recovery of rSARS-CoV-2.

Since the high variability of SARS-CoV-2, research towards a variety of SARS-CoV-2 mutants becomes more attractive. In addition to standard protocols, another rapid and reliable cloning method for the construction of emerging SARS-CoV-2 variants is Golden Gate assembly, which needs ligation with type IIS restriction enzymes and digestion with temperature cycling (Taha et al., 2023). To quickly and efficiently introduce almost any type of genetic modification into already existing CoV genomic clones, BAC clones integrated with a Lambda-based Red recombination have also provided a supplementary tool. First, a resistance cassette containing the desired modification and a unique endonuclease recognition site is synthesized, then the purified product is electroporated into BAC clone-containing *E. coli* strain such as GS1783 or SW105, which could be inserted into the target region using positive selection via Red recombination. Next, the clones are induced to remove the resistance cassette and introduce the targeted one using negative selection by a second intracellular Red recombination. Finally, validated clones are purified again and applied to virus recovery (Herrmann et al., 2021; Ye et al., 2022).

2.1.4. *PCR amplicon-based systems*

As an alternative, circular polymerase extension reaction (CPER) has been developed for rapid generation of SARS-CoV-2 infectious clones. It completely eliminates the need for cDNA assembly in bacteria or yeast

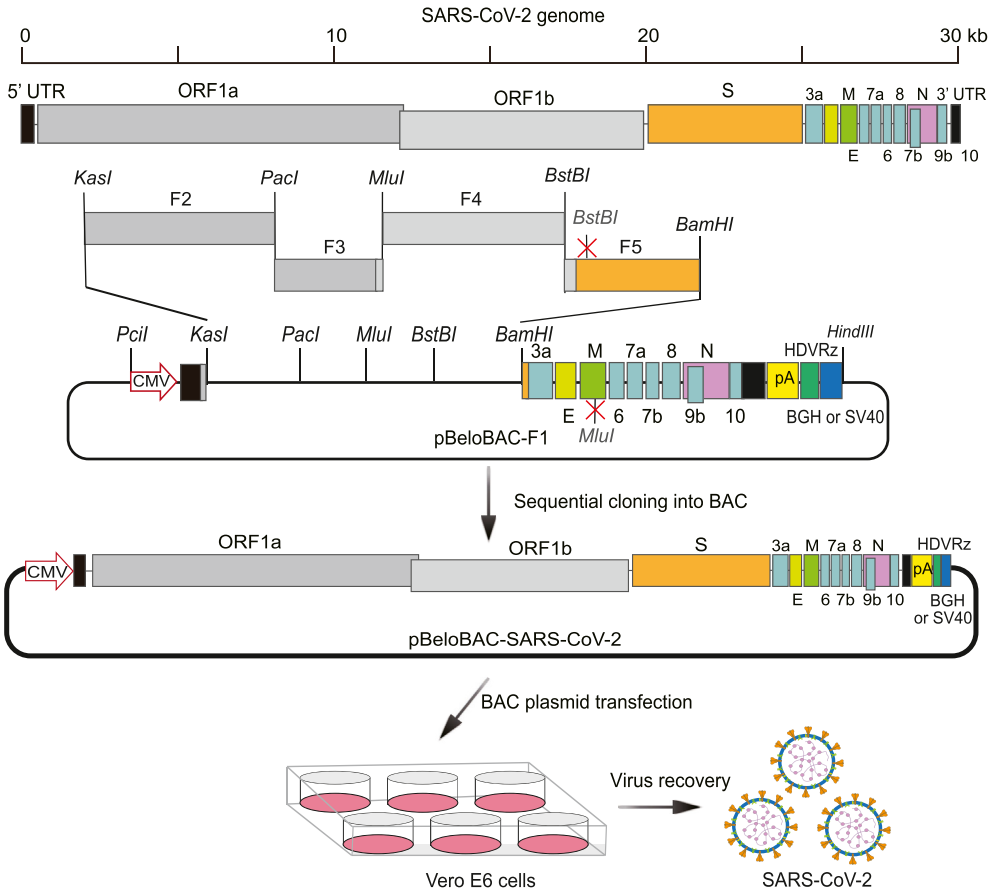

Fig. 4. Schematic representation of BAC-based reverse genetics systems for constructing a SARS-CoV-2 cDNA clone. The entire viral genome of SARS-CoV-2 strain USA-WA1/2020 is cloned into the pBeloBAC11 plasmid. Repetitive restriction sites *BstB*I and *Mlu*I within the genome are removed by artificially introducing silent mutations into the *S* and *M* genes, which also serve as genetic markers to distinguish rescued viruses from the natural isolate. The intermediate plasmid pBeloBAC-F1 is constructed under the control of the eukaryotic cytomegalovirus (CMV) promoter and is flanked at the 3′ end by a poly(A) tail, the hepatitis delta virus ribozyme (HDVRz), the bovine growth hormone (BGH) or simian virus 40 (SV40) termination and polyadenylation sequences. The full-length cDNA clone is assembled by sequential cloning of other chemically synthesized fragments (F2 to F5) into the intermediate plasmids using the indicated restriction enzyme sites. The BAC plasmid containing the entire viral genome is directly transfected into Vero E6 cells for recovery of recombinant SARS-CoV-2.

and *in vitro* RNA transcription steps, enabling site-directed mutagenesis by PCR amplification (Edmonds et al., 2013). In this approach, several cDNA fragments spanning the entire SARS-CoV-2 genome were amplified with high-fidelity DNA polymerase using similar annealing temperatures. To facilitate DNA circularization, all cDNA fragments containing overlapping ends were used to generate a circular full-length cDNA in a single CPER reaction, together with a linker fragment carrying a HDVRz/BGH or SV40 poly(A) signal sequences, a spacer sequence separating functional elements and a CMV promoter, respectively. The CPER reaction products were then transfected into HEK293T cells or HEK293T expressing angiotensin converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2) without any purification. The CPER viruses were amplified by serial passages of the culture supernatants of transfected cells in the highly permissive Vero E6 or Vero E6/TMPRSS2 cells to enable the recovery of infectious viruses (Fig. 5), which exhibited properties comparable to the parental virus, providing a feasible and robust tool for generating reporter and mutant viruses (Amarilla et al., 2021; Torii et al., 2021; Liu and Gack, 2023). Alternatively, in an optimized circular polymerase extension cloning (CPEC) methodology, the utilization of a plasmid vector pYES1L combined circular polymerase extension with sequence-independent cloning, simplifying the cloning steps and improving efficiency better than TAR cloning in yeast (Kim et al., 2023).

Another novel method based on infectious subgenomic amplicons (ISA) was also applied to generate recombinant infectious CoVs including SARS-CoV-2 and feline enteric CoV (FeCoV). This procedure does not require cloning or additional steps beyond cDNA amplification. First, entire viral genomes were amplified by PCR in eight overlapping fragments, with CMV promoter and HDVRz/SV40pA sequences added to the first and last fragments during amplification, respectively. These subgenomic fragments were then mixed and directly transfected into BHK-21 cells with the plasmid encoding SARS-CoV-2 N. After transfection, extensive CPE was observed when the supernatants were passaged twice on Vero E6 cells, with the ISA strain exhibiting similar replication kinetics and viral RNA loads post-infection compared with the clinical strain, suggesting that this method achieved DNA recombination and virus production in cells (Fig. 5) (Melade et al., 2022).

Although the two PCR-based methods seem very easy to achieve, we need to pay close attention to their relatively low rescue efficiency and undesired mutations during preparation of stock viruses. Compared to the BAC approach, the use of DNA polymerase for PCR in CPER was reported to be less accurate, with the introduction of unwanted substitution in rescued viruses (Furusawa et al., 2023). Without the intermediate step of full-length cDNA clone assembly, low probability of exact *in cellulo* recombination is expected, and the transfected supernatants must be passaged several times to propagate viruses. Therefore, an external

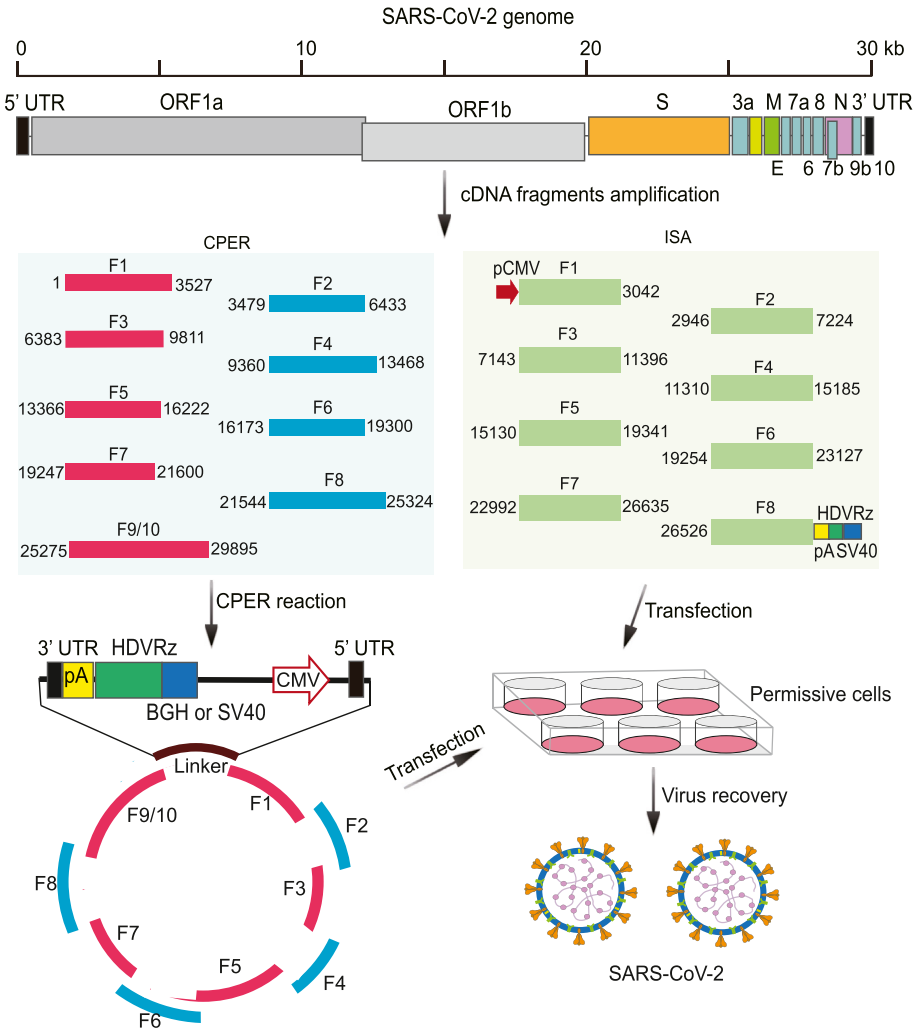

Fig. 5. Schematic representation of PCR amplicon-based reverse genetics systems for constructing a SARS-CoV-2 cDNA clone. Several cDNA fragments encompassing the entire SARS-CoV-2 genome are amplified with a high-fidelity DNA polymerase. *Left side:* In a circular polymerase extension reaction (CPER) approach, ten overlapping cDNA fragments of SARS-CoV-2 strain Hu/DP/Kng/19–020 are used to generate a circular full-length cDNA in a single CPER reaction, together with a linker fragment harboring the hepatitis delta virus ribozyme (HDVRz), the bovine growth hormone (BGH) or simian virus 40 (SV40) termination and polyadenylation sequences, and the cytomegalovirus (CMV) promoter. The CPER reaction products are then transfected into permissive cells for recovery of infectious viruses. Right side: Alternatively, using the infectious subgenomic amplicon (ISA) technology, infectious viruses are rescued after direct transfection of eight overlapping cDNA fragments of SARS-CoV-2 European strain with the CMV promoter and HDVRz/SV40pA sequences added to the first and last fragment during PCR amplification, respectively.

operation to verify the true recovery of infectious virus is required (Table 1).

2.2. *BSL-2-adapted SARS-CoV-2*

Since SARS-CoV-2 is a biosafety level-3 (BSL-3) pathogen, experiments on infectious viruses must proceed under high containment levels, greatly limiting the basic research; this necessitates the development of subgenomic replicons and *trans*-complementary systems capable of being used in BSL-2 laboratories.

2.2.1. *Non-infectious replicons*

Replicons are self-replicating RNAs that possess all genetic elements competent for autonomous replication and discontinuous transcription of sgRNAs, but lack complete structural genes sufficient to produce progeny virions (Kummerer, 2018; Hannemann, 2020). By replacing one or more structural genes with reporter genes and selection markers, RNA replication and transcription of these replicons can be monitored by the expression of reporter genes and stably maintained in cell lines by antibiotic selection for further drug screening. As a favorable substitute for reverse genetics systems, replicon systems have been widely used in several RNA viruses, especially for hepatitis C virus (HCV) (Lohmann et al., 1999; Bartenschlager, 2002) and some BSL-3 agents (Lo et al., 2003; Xie et al., 2016; Kummerer, 2018; Fernandes et al., 2020).

SARS-CoV-2 replicons have been generated using the same basic reverse genetics approaches presented above. In the *in vitro* transcription strategy, the full-length replicon cDNA was assembled under the control of a T7 promoter by *in vitro* ligation (Xia et al., 2020; Kotaki et al., 2021), TAR cloning (Ricardo-Lax et al., 2021) or BAC-based methods (He et al., 2021; Zhang Y. et al., 2021), and served as a template for *in vitro* transcription to synthesize replicon RNAs using T7 RNA polymerase. RNA transcripts were electroporated into a range of cell lines together with sgRNA-N, leading to the production of a transient replicon. Similarly, a CMV-driven replicon plasmid constructed via BAC (Jin et al., 2021; Nguyen et al., 2021; Malicoat et al., 2022; Tian et al., 2022), yeast-BAC shuttle vectors (Wang B. et al., 2021; Feng et al., 2022) or a CPER-based

approach (Tanaka et al., 2022) could transcribe replicon RNAs in the host cell nucleus after transfection into cells. Additionally, an unconventional replicon system consisting of four ordinary plasmids carrying necessary viral genes and segments was reported to realize replicon RNA replication and transcription under the control of CMV promoters after co-transfection into cells (Luo et al., 2021).

In principle, the genome of SARS-CoV-2 replicons maintains the minimal viral gene set necessary for replication, which includes nonstructural ORF1a, ORF1b, N, and the 5′ and 3′ UTRs. Various design strategies have been used for the replacement of specific regions with reporter and resistance genes (Fig. 6). One deletion region encompasses S, E, M, ORF3, 6, 7 and 8 (ΔS-ORF8) (Xia et al., 2020; Nguyen et al., 2021; Wang B. et al., 2021; Zhang Q. Y. et al., 2021; Zhang Y. et al., 2021; Feng et al., 2022; Zhang et al., 2022). Reporter genes could be introduced separately or in combination with selection markers such as neomycin phosphotransferase (Neo), blasticidin (BSD) or puromycin (Puro) via a fusion cassette, which can be separated by a foot-and-mouth disease virus (FMDV) 2A or a porcine teschovirus 1 (PTV-1) 2A self-cleaving peptide. The engineered cassette is placed under the control of transcription regulatory sequence (TRS) of the deleted S gene. Alternatively, the introduction of reporter genes and selection markers has been described to take the place of individual S gene (ΔS) (Jin et al., 2021; Ricardo-Lax et al., 2021; Malicoat et al., 2022), or simultaneously replace the *S*, *M* and *E* genes (ΔS/M/E) (He et al., 2021; Liu et al., 2022a).

Despite the use of antibiotic resistance genes, establishing a stable cell line to maintain replicon RNAs remains a challenge (Table 1), perhaps due to the cytotoxicity of SARS-CoV-2 proteins or their removal by intracellular innate immunity (He et al., 2021). To overcome this problem, nsp1 mutations K164A/H165A were introduced to diminish cellular toxicity associated with viral replication, successfully generating stable cell clones harboring non-cytopathic replicons from BHK-21 cell lines (Liu et al., 2022a). Another stable replicon cell line derived from Vero E6 cells, which are incapable of producing type I interferon (IFN–I) (Osada et al., 2014). In this case, the system was established by antibiotic selection, and identified by the expression of reporter genes and sgRNAs, which is better suited to high-throughput screening more than transient replicon systems (Tanaka et al., 2022).

2.2.2. *Trans*-complementary systems

Apart from replicon systems, generation of single-cycle infectious SARS-CoV-2 can be achieved by the removal of key proteins such as spike (ΔS) (Ricardo-Lax et al., 2021), nucleocapsid (ΔN) (Ju et al., 2021), or accessory protein ORF3a and envelope (ΔORF3-E) (Zhang X. et al., 2021). Deficient proteins are genetically *trans*--complemented by ectopic expression or lentiviral transduction to continuously produce replication-competent, propagation-defective viruses, recapitulating an authentic infection process in packaging cell lines. Only a single round of infection takes place in normal cells, which allows for manipulation and application to a wide range of basic pathogenicity studies in BSL-2 laboratories. In order to eliminate the possibility of wild-type (WT) SARS-CoV-2 production, several measures have been adopted in the construction and recovery of deficient SARS-CoV-2 with specific deletions. The first approach is to modify both leader and body TRS sequences of the SARS-CoV-2 genome to reduce the risk of reversion (Yount et al., 2006; Graham et al., 2018). For the packaging cell lines, Zhang et al. constructed a bicistronic lentivirus plasmid to simultaneously provide the deleted ORF3 and E proteins, thus minimizing homologous recombination during *trans*-complementation (Zhang X. et al., 2021). Ju et al. utilized an intein-mediated protein *trans*-splicing approach to separately express halves of the N protein, which would stably form a functional N protein for the recovery of N-deficient virions (Ju et al., 2021). Nevertheless, the risk of virulence reversion of the BSL2-adapted SARS-CoV-2 still needs much attention (Table 1).

In some cases, S-deleted SARS-CoV-2 replicons could be *trans*-complemented with viral spike protein to generate single-cycle infectious virus replicon particles (VRPs) in Huh7.5 cells expressing ACE2 and TMPRSS2, or be packaged to yield ΔS-VRP(G) virions from cells expressing vesicular stomatitis virus glycoprotein (VSV-G). In contrast to the spike protein delivery strategy, the ΔS-VRP(G) system can efficiently infect various cell types and provided an ACE2-independent means of replicon delivery, as viral entry of ΔS-VRPs is mediated by the VSV-G protein (Ricardo-Lax et al., 2021; Malicoat et al., 2022). Moreover, overexpression of the N protein *in trans* enhanced replication of the replicon in a dose-dependent manner (Nguyen et al., 2021).

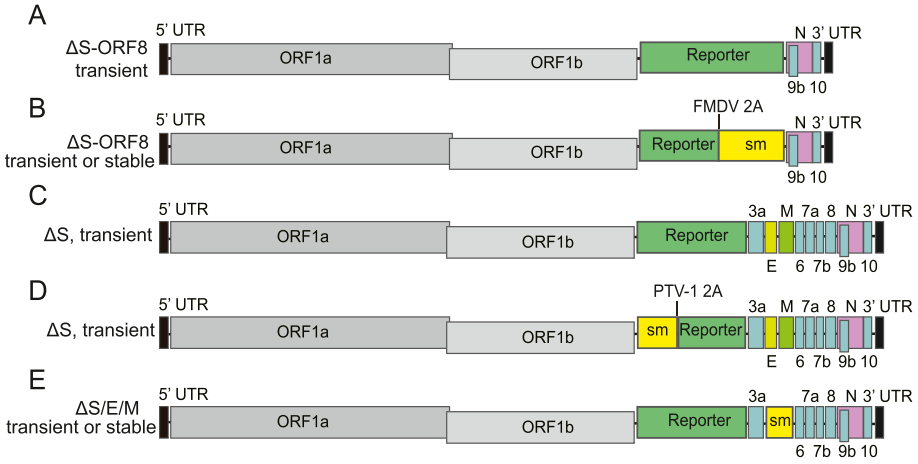

Fig. 6. Schematic representation of SARS-CoV-2 replicons with different deletion and insertion strategies. (**A**) A SARS-CoV-2 replicon is established by deleting the genomic region spanning from the spike (*S*) gene to ORF8 (generating ΔS-ORF8), and replacing it with reporter genes to produce transient reporter replicons under the control of transcription regulatory sequence (TRS) of the deleted *S*. (**B**) The ΔS-ORF8 region is replaced by a fusion cassette of reporter genes and selection markers (*sms*), which are separated by cleavage of the foot-and-mouth disease virus (FMDV) 2A autoprotease sequence. Expression of *sms* allows for selection of stable cell lines harboring reporter replicons. (**C**) Reporter genes are engineered to replace the *S* gene (ΔS). (**D**) A replicon is established by replacement of the *S* gene with reporter genes fused to *sms* via a porcine teschovirus 1 (PTV-1) 2A proteolytic cleavage site. (**E**) To generate transient or stable replicons, the *S* gene is replaced by reporter genes, and the envelope (*E*) and membrane (*M*) genes are replaced with *sms*.

2.2.3. Codon pair-deoptimized SARS-CoV-2

Another strategy to construct live attenuated viruses by rational design is codon pair deoptimization (CPD), also known as synthetic attenuation virus engineering (SAVE), which exploits the natural codon pair bias in human cells and has been used to attenuate several viruses including poliovirus (Coleman et al., 2008), influenza A virus (IAV) (Mueller et al., 2010) and more recently SARS-CoV-2. In this approach, the SARS-CoV-2 genome is partially recoded by synonymous suboptimal codon pairs without changing the amino acid composition of encoded proteins. Thus, the codon pair-deoptimized SARS-CoV-2 containing hundreds of mutations could be attenuated *in vitro* and *in vivo* by reducing mRNA stability and translation efficiency of the recoded genes. Due to its genetic stability and valid attenuation, the SARS-CoV-2 mutant sCPD9 carrying ~1000 bp recoded in a region spanning part of nsp15 and nsp16 was reclassified as a BSL-2 pathogen in Germany, providing a valuable tool for attenuated SARS-CoV-2 research (Table 1) (Trimpert et al., 2021b; Wang Y. et al., 2021; Kunec et al., 2022).

3. Applications

3.1. Research on the functions of viral proteins

Utilizing reverse genetics systems, the viral genome can be artificially manipulated to introduce point changes, deletions or insertions at the cDNA level (Almazan et al., 2014). Phenotypic differences between the rescued modified strains and the parent virus can then be characterized *in vitro* and *in vivo* to bring insight into the functional analysis of relevant genes in terms of viral replication, pathogenesis, and antiviral activity of experimental drugs and vaccines (Table 2).

3.1.1. Spike (S) protein

The spike (S) protein is a class I fusion glycoprotein that mediates receptor binding and entry. The precursor of the SARS-CoV-2 S protein is cleaved by furin at the S1–S2 junction in infected cells to yield S1 and S2 subunits; the S1 subunit contains the receptor-binding domain (RBD) that binds to the host cell receptor ACE2, thereby determining host and cellular tropism as well as inducing the production of neutralizing antibodies. The S2′ site (within the S2 subunit) is cleaved by host protease TMPRSS2 or cathepsins, which triggers the fusion between viral and cellular membranes (Hoffmann et al., 2020; V'Kovski et al., 2021).

During the early pandemic, SARS-CoV-2 variants carrying spike substitution D614G became more common for research due to its fitness advantage (Korber et al., 2020; Yurkovetskiy et al., 2020). Using an engineered SARS-CoV-2 D614G mutant, it was found that the spike D614G substitution enhanced viral replication in the human respiratory cell line Calu-3 and human airway epithelial (HAE) cells, which was associated with increased infectivity and stability of virions, but was not caused by a difference in spike cleavage. In a Syrian golden hamster infection model, viral loads in the nasal wash and trachea were higher in hamsters infected with G614 virus compared with D614 virus, suggesting its role in enhanced transmissibility in the upper respiratory tract (Plante et al., 2021; Melade et al., 2022). The Alpha variant (B.1.1.7 lineage) that originated in the United Kingdom in September 2020 contained 8 mutations or deletions in the S protein (including the D614G substitution), and spread worldwide with high transmissibility (Leung et al., 2021). To identify the determinant mutations of this variant, Liu et al. introduced individual or combined point mutations into the genome of a SARS-CoV-2 D614G mutant using a reverse genetics system. Among the mutants tested, both N501Y and Δ69-70 showed fitness advantages for replication in HAE cells and the upper airway of hamsters, but N501Y was the major contributor responsible for increased binding affinity between the S RBD and ACE2 (Wan et al., 2020; Liu et al., 2022d).

Research on the functions of the furin cleavage motif (PRRAR) at the site of S1/S2 cleavage was performed using engineered SARS-CoV-2 mutants. Compared to WT SARS-CoV-2, the deletion of the furin cleavage site (ΔPRRA) or the QTQTN motif upstream of the furin cleavage site (ΔQTQTN) reduced spike processing and improved fitness in Vero E6 cells, which could be reverted by ectopic expression of TMPRSS2. In contrast, both mutants impaired viral replication in Calu-3 cells, and were attenuated both in hamsters and the K18-hACE2 transgenic mouse model (Johnson et al., 2021; Sasaki et al., 2021; Vu et al., 2022). In the subsequent wave of the Delta variant (B.1.617.2 lineage), the P681R mutation in the furin cleavage site drove improved fitness, enhanced fusogenicity and pathogenicity over the Alpha variant through an increase in spike cleavage efficiency (Liu et al., 2022c; Saito et al., 2022a).

In November 2021, Omicron variants (B.1.1.529 and BA lineages) accumulated several adapted mutations to become the globally dominant strains owing to high transmissibility and immune evasion capability (Yamasoba et al., 2022a). To characterize Omicron BA.1 spike-mediated attenuation, chimeric SARS-CoV-2 viruses with the S gene of the ancestral virus or Delta variant replaced by BA.1 S were generated, indicating its limited roles in reduced infection efficiency *in vitro* and attenuated pathogenicity *in vivo*. Moreover, BA.1 S increased replication propensity in the bronchiolar epithelium of K18-hACE2 mice, suggesting spike-mediated alterations in viral tropism. Mutations (including E484A, Q493R, G496S, Q498R, N501Y and Y505H) in the receptor-binding motif (RBM), a portion of the RBD that makes contact with ACE2,

Table 2
Applications of SARS-CoV-2 reverse genetics systems in functional viral protein research.

Protein	Modifications	Evaluation
Spike	D614G	• Enhanced viral replication and infectivity, transmissibility (Plante et al., 2021);
	N501Y	• Improved fitness advantages by increasing binding affinity (Liu et al., 2022d);
	ΔPRRA, ΔQTQTN	• Reduced spike processing and attenuated (Johnson et al., 2021; Sasaki et al., 2021; Vu et al., 2022);
	P681R	• Enhanced fusogenicity and pathogenicity through increasing the spike cleavage efficiency (Liu et al., 2022c; Saito et al., 2022a);
	BA.1 S	• Reduced infection efficiency and attenuated (Barut et al., 2022; Suzuki et al., 2022; Chen et al., 2023).
	BA.2 S	• More pathogenic than BA.1 S (Yamasoba et al., 2022b);
	L452R/Q (BA.4/5)	• Increased viral infectivity (Kimura et al., 2022; Wang et al., 2022);
	F486V (BA.4/5)	• Increased immune evasion and reduced ACE2 binding affinity (Ong et al., 2023);
	D339H/N460K (BA.2.75)	• Enhanced pathogenicity over BA.2 (Saito et al., 2022b);
	Q498Y/P499T, N501Y	• Mouse-adapted viruses (Dinnon et al., 2020; Muruato et al., 2021).
Nucleocapsid	S188A/S206A	• Abolished N function (Ju et al., 2021);
	S202R, R203 M, R203K/G204R	• Increased viral replication and pathogenesis with enhanced fitness and augmented N protein phosphorylation (Syed et al., 2021; Johnson et al., 2022).
Nonstructural proteins	nsp1: Δ500-532	• Reduced IFN-I response (Lin et al., 2021);
	nsp6 in Omicron	• Reduced replication (Chen et al., 2023; Taha et al., 2023);
	nsp12/nsp14: mutations	• Decreased replication and transcription in replicons (Jin et al., 2021; Zhang Q. Y. et al., 2021);
	nsp15: H235A/H250A and Δnsp15	• Reduced replication in replicons (Nguyen et al., 2021);
	nsp16: D130A	• Attenuated with higher sensitivity to IFN-I (Ye et al., 2022; Schindewolf et al., 2023).
Accessory proteins	ORF 3 6 7 8	• Attenuated (Silvas et al., 2021; Liu et al., 2022e; Lin et al., 2023).

contributed to vaccine resistance in combination with other spike substitutions (Barut et al., 2022; Suzuki et al., 2022; Chen et al., 2023). However, BA.2 S chimeric virus was more pathogenic than that of BA.1 S, exhibiting higher fusogenicity without increased S cleavage efficiency (Yamasoba et al., 2022b). The chimeric BA.2 subvariant rBA.4/5 showed higher fusogenicity and pathogenicity than rBA.2, while the reverted substitutions L452R/Q contributed to increased viral infectivity, and the F486V mutation accounted for the immune evasion and reduced hACE2 binding affinity in combination with other spike mutations (Kimura et al., 2022; Wang et al., 2022; Ong et al., 2023). In addition, the Omicron variant BA.2.75, which contained the critical substitutions D339H/N460K and evolved independently of BA.5, also exhibited enhanced pathogenicity compared with BA.2 (Saito et al., 2022b).

The spike mutations Q498Y/P499T or N501Y could be introduced by reverse genetics systems to generate mouse-adapted viruses that were capable of utilizing mACE2 to mediate cell entry, thus providing an alternative mouse infection model for further study of SARS-CoV-2 infection and disease (Table 2) (Dinnon et al., 2020; Zhou et al., 2020; Muruato et al., 2021).

3.1.2. Nucleocapsid (N) protein

The CoV nucleocapsid (N) protein is a relatively conserved and extensively phosphorylated structural protein, playing a vital role in genomic RNA package and virus particle release (McBride et al., 2014). Ju et al. used N-based *trans*-complementation systems to investigate the function of SARS-CoV-2 N protein. The N protein from SARS-CoV (but not MERS-CoV) had high identity with SARS-CoV-2 N and could rescue N-deficient virus. Among the phosphorylation-null mutants in the Ser-Arg (SR)-rich motif, S188A/S206A mutations completely abolished the function of N protein (Ju et al., 2021). Furthermore, SARS-CoV-2 mutants containing substitutions like those present in the Alpha, Gamma and Omicron variants (e.g., S202R, R203 M or R203K/G204R) showed increased viral replication and pathogenesis *in vitro* and *in vivo*, with enhanced fitness and augmented N protein phosphorylation compared to WT SARS-CoV-2, which may provide a molecular basis for virus adaption to human infection (Table 2) (Syed et al., 2021; Johnson et al., 2022).

3.1.3. Nonstructural proteins (Nsps)

CoV nsp1 is a potential virulence factor that mediates host mRNA degradation and translation inhibition (Schubert et al., 2020). Lin et al. identified deletion of the 500–532 locus in SARS-CoV-2 nsp1 associated with lower viral load and serum IFN-β levels in clinical samples. An rSARS-CoV-2 containing nsp1 Δ500-532 exhibited a smaller plaque phenotype and led to a reduced IFN-I response compared to the WT virus, demonstrating the biological and clinical importance of nsp1 (Lin et al., 2021). Nsp6 plays a main role in formation of double-membrane vesicles (DMVs); two groups recently found the SARS-CoV-2 nsp6 was the critical non-spike protein involved in attenuation of the Omicron BA.1 variant. Mutations in Omicron nsp6 reduced replication by impairing the lipid droplet channeling function of the protein (Chen et al., 2023; Taha et al., 2023). SARS-CoV-2 nsp1 and nsp6 also suppress IFN-I production; Xia et al. evaluated the biological relevance of this inhibition among three CoVs via reporter replicon systems. Upon IFN-I treatment, a SARS-CoV-2 WT replicon inhibited IFN-I signaling more efficiently than chimeric replicons containing SARS-CoV nsp1/nsp6 or MERS-CoV nsp6 (Xia et al., 2020).

SARS-CoV-2 RNA-dependent RNA polymerase (RdRp) is encoded by nsp12, and the exonuclease and cap N7 methyltransferase (MTase) are encoded by nsp14. By introducing key mutations or deletions to impair nsp12 or nsp14 activities, mutant replicons showed decreased replication and transcription capability (Jin et al., 2021; Zhang Q. Y. et al., 2021). CoV nsp15 is a conserved nidoviral RNA uridylate-specific endoribonuclease (NendoU) that contains a C-terminal catalytic domain belonging to the EndoU family (Kim et al., 2020). Replicons mutants with the nsp15 deletion (Δnsp15) or H235A/H250A mutations reduced the replication of SARS-CoV-2 replicon by removing or diminishing the NendoU enzymatic activity (Nguyen et al., 2021). Nsp16 is a ribonucleoside 2′-*O*-MTase that binds to nsp10 and catalyzes methylation to form the viral RNA cap structure together with nsp14. Although nsp14-deficient mutants and Δnsp16 virus are nonviable (Ogando et al., 2020), rSARS-CoV-2 bearing nsp16 D130A in the conserved KDKE motif were successfully obtained for detailed research. The nsp16 mutant ablating MTase activity presented no distinct replication defect in Vero E6 cells, but was attenuated in Calu-3 cells and animal models, with higher sensitivity to IFN-I than parental SARS-CoV-2, causing reduced lung disease and decreased viral replication *in vivo*. The IFN-stimulated genes IFIT1 and IFIT3 mediated the nsp16 attenuation, suggesting the importance of nsps in viral replication (Table 2) (Ye et al., 2022; Schindewolf et al., 2023).

3.1.4. Accessory proteins

CoV accessory proteins are mostly non-conserved proteins that are principally thought to contribute to the regulation of host immune responses and determination of viral pathogenicity. Studies of rSARS-CoV-2 carrying deletions of individual or multiple accessory proteins (ORFs 3–8) revealed smaller plaque phenotypes than WT SARS-CoV-2, and a major role for ORF3a and ORF6 in virulence and disease incidence was emphasized (Silvas et al., 2021; Zhang X. et al., 2021; Liu et al., 2022e). Lin et al. identified that SARS-CoV-2 ORF8 was also a secretory protein that acts as a messenger for inter-cellular communication between alveolar epithelial cells and macrophages during SARS-CoV-2 infection. ORF8-deficient virus had alleviated inflammatory responses and cytokine release in hamsters compared with the WT virus, indicating a functional role for ORF8 in the development of cytokine storm (Table 2) (Lin et al., 2023).

3.2. Screening antiviral drugs

Reporter genes such as GFP, mNeonGreen, Venus, mCherry, ZsGreen or bioluminescent dinucleotide-optimized firefly luciferase (Fluc) and nanoluciferase (Nluc) were genetically engineered to partially or entirely replace ORF7a and generate reporter-expressing rSARS-CoV-2. To retain the viral ORF7a protein and enhance the expression levels of reporter genes, two alternative introduction strategies were used, linking the reporter genes to an FMDV 2A or PTV-1 2A cleavage site that was inserted upstream of the N gene or downstream of the ORF7a region (Table 1) (Chiem et al., 2021b; Rihn et al., 2021; Ye et al., 2021). These rSARS-CoV-2 carrying reporter genes of interest exhibited plaque phenotypes, replication and growth kinetics comparable to the WT virus *in vitro*, and stably maintained their reporter signals through several passages. The feasibility of these reporter viruses to track viral infection and pathogenesis in cultured cells and validate animal models was then confirmed, making it a robust platform for neutralization assays and high-throughput antiviral screening (Xie et al., 2020a; Chiem et al., 2021a; Chiem et al., 2021b; Ye et al., 2021; Chiem et al., 2022).

Apart from some already known antiviral drugs like remdesivir (RdRp inhibitor), GC376 (3CLpro inhibitor) and chloroquine, researchers have evaluated many potential SARS-CoV-2 inhibitors by exploiting reporter virus or replicon systems. Among a set of clinically approved antivirals against other viruses, Xie et al. identified three potential inhibitors for the treatment of COVID-19, which included nelfinavir, rupintrivir, and cobicistat (Xie et al., 2020a). He et al. used replicon systems to demonstrate differences in the potency of inhibitors targeting RdRp, 3CLpro, TMPRSS2 and cathepsin L in different cell lines (He et al., 2021). Three compounds (Darapladib, targeting 3CLpro; JNJ-5207852, targeting nsp15; and Genz-123,346, targeting nsp16) were validated to inhibit viral replication, both in stable cells harboring replicons and other cell lines infected by live viruses (Liu et al., 2022a). Schindewolf et al. measured a novel therapeutic approach by combining IFN-I treatment with an nsp16 MTase-targeted inhibitor sinefungin, which enhanced the antiviral effect by reducing toxicity on cell viability at lower treatment

doses in Vero E6 and Calu-3 cells (Schindewolf et al., 2023). Using SARS-CoV-2 ΔN/GFP *trans*-complementary system in BSL-2 laboratories, Zhao et al. showed that 17-DMAG (targeting heat shock protein 90) could inhibit viral infection in both N-packaging cells and adenovirus-N-transduced hamsters (Zhao Z. et al., 2023).

3.3. Developing LAV candidates

Vaccination is the most important and effective means for combating viral infections. To date, there are multiple vaccine candidates in development, including live attenuated, inactivated, viral vector, protein subunit, DNA, mRNA and virus-like particle (VLP) vaccines (Li et al., 2022). Of these, LAVs are considered valuable due to their comprehensive immune response. Attenuated by reverse genetics or adaptation, these vaccines elicit both humoral and cellular immune responses *in vivo*. In addition, intranasally administered LAVs may be more effective at inducing mucosal immunity to protect the upper respiratory tract.

Currently, there are two main methods for the construction of SARS-CoV-2 LAVs. The first approach introduces deletions or mutations in virulence genes such as spike, the furin cleavage site, nsps and accessory genes. Abdoli et al. generated a cold-adapted attenuated rSARS-CoV-2 by removing both the furin cleavage site (PRRA) and spike GTNGTKR motifs and examined its immunogenic potential as a vaccine candidate (Abdoli et al., 2022). Liu et al. deleted the PRRA motif and ORFs 6–8 to reduce pathogenicity while weakening IFN antagonism, and introduced mutations K164A/H165A in nsp1 to alleviate nsp1-mediated toxicity (Liu et al., 2022b). Attenuated viruses with ORF 3, 6, 7, and 8 deletions (Δ3678) (Liu et al., 2022e) or nsp16 D130A (Ye et al., 2022) have also been proven to elicit potent immune responses and protect against SARS-CoV-2-induced weight loss and pneumonia in hamsters and the K18-hACE2 mouse model, providing alternative LAV candidates.

Another attenuation strategy is achieved by CPD; Trimpert et al. generated a deoptimized SARS-CoV-2 mutant (sCPD9) that recoded the region of nsp15 and nsp16, and reported its enhanced mucosal and systemic immunity against the ancestral virus and variants (Trimpert et al., 2021a, 2021b; Nouailles et al., 2023). Wang et al. combined the recoding of S protein with a loss of the furin cleavage site to produce the LAV candidate COVI-VAC, which is now in phase III clinical trials (ISRCTN15779782) (Wang Y. et al., 2021).

4. Concluding remarks

The COVID-19 pandemic stimulated an enormous effort to conduct research of SARS-CoV-2 at a molecular level. Based on the established reverse genetics systems and burgeoning molecular biology technologies, SARS-CoV-2 full-length infectious clones and recombinant viruses expressing reporter genes were rapidly obtained by *in vitro* ligation of cDNA fragments, TAR cloning, BACs, or bacteria-free approaches (CPER and ISA). In combination with animal infection models, reverse genetics systems facilitate the characterization of recombinant and reporter viruses, enabling mechanistic studies on genes relevant to viral infection, transmission and pathogenesis. Due to naturally emerging mutations and deletions identified in clinical isolates and its major role in transmissibility and pathogenicity, the SARS-CoV-2 spike protein has been an extremely popular target. Other structural proteins, nsps and accessory proteins have also gained attention for their important roles in virulence. With a greater understanding of the spike and other proteins, a rational design of live attenuated viruses has expedited the generation of LAV candidates. However, the existing reverse genetics systems have great technical and facility requirements and have only been mastered by a limited number of laboratories. In addition to infectious clones, BSL-2-adapted SARS-CoV-2 replicons, *trans*-complemented single-round infectious viruses and live attenuated viruses have broadened the research platform to BSL-2 laboratories, providing alternative and powerful tools to expand the global effort against COVID-19.

At present, Omicron is still the dominant variant worldwide, with constantly arising subvariants. Despite our already extraordinary understanding of SARS-CoV-2 biology, the constant threat of the emergence of SARS-CoV-2 variants containing mutations, deletions or even recombination between two existing subvariants has prompted in-depth research to better explore viral fitness and virus-host interactions. Therapeutic strategies and vaccines remain the main weapons against SARS-CoV-2, which need to be constantly optimized and kept up to date. Taking full advantage of reverse genetics systems to elucidate the similarities and differences between SARS-CoV-2 and other viruses will inspire better preparation for any future pandemics.

Conflict of interest

The authors declare that there are no conflicts of interest.

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