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Reprint of: Coronavirus reverse genetic systems: Infectious clones and replicons☆

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ABSTRACT

Coronaviruses (CoVs) infect humans and many animal species, and are associated with respiratory, enteric, hepatic, and central nervous system diseases. The large size of the CoV genome and the instability of some CoV replicase gene sequences during its propagation in bacteria, represent serious obstacles for the development of reverse genetic systems similar to those used for smaller positive sense RNA viruses. To overcome these limitations, several alternatives to more conventional plasmid-based approaches have been established in the last 13 years. In this report, we briefly review and discuss the different reverse genetic systems developed for CoVs, paying special attention to the severe acute respiratory syndrome CoV (SARS-CoV).

1. Introduction

Coronaviruses (CoVs) are enveloped RNA viruses mainly responsible for respiratory and enteric infections in animals and humans (Lai et al., 2007; Masters, 2006). Historically, CoV infection in humans has been associated with mild upper respiratory tract diseases caused by the human CoVs (HCoVs) HCoV-229E and HCoV-OC43 (Masters, 2006). However, the identification in 2003 of a novel life-threatening CoV causing the severe acute respiratory syndrome (SARS-CoV) redefined historic perceptions (Stadler et al., 2003). More recently, three novel HCoVs associated with respiratory diseases have been identified, including HCoV-HKU1 associated with chronic pulmonary disease (Woo et al., 2005), HCoV-NL63 that causes upper and lower respiratory tract disease in children and adults worldwide (van der Hoek et al., 2004), and the recently emerged (April 2012) Middle East respiratory syndrome CoV (MERS-CoV), which has been associated with acute pneumonia and occasional renal failure (Zaki et al., 2012). These findings have potentiated the relevance of CoVs as important human pathogens and highlight the need of reverse genetic systems to facilitate the genetic manipulation of the viral genome to study fundamental viral processes, to develop vaccine candidates and to test antiviral drugs.

CoVs belong to the *Coronaviridae* family within the order *Nidovirales* (de Groot et al., 2012). They contain the largest known RNA genome among RNA viruses, consisting in a plus-sense, 5′-capped and polyadenylated RNA molecule of 27–31 kb in length. The first two-thirds of the genome encode the replicase gene, which comprise two overlapping open reading frames (ORFs), ORF 1a and ORF 1b, the latter being translated by a ribosomal frameshift mechanism. Translation of both ORFs results in the synthesis of two polyproteins that are processed by viral proteinases to release the replication–transcription complex components. The final one-third of the genome includes the genes encoding the structural proteins S, E, M, and N, as well as the genus specific proteins characteristic of each CoV, which are expressed from a nested set of 3′ coterminal subgenomic mRNAs (Enjuanes et al., 2006; Masters, 2006; Ziebuhr, 2005).

Until recently, the study of CoV genetics was broadly restricted to the analysis of temperature-sensitive (ts) mutants (Fu and Baric, 1992, 1994; Lai and Cavanagh, 1997; Schaad and Baric, 1994; Stalcup et al., 1998), defective RNA templates which depend on replicase proteins provided in trans by a helper virus (Izeta et al., 1999; Narayanan and Makino, 2001; Repass and Makino, 1998; Williams et al., 1999), and recombinant viruses generated by targeted recombination (Masters, 1999; Masters and Rottier, 2005). Among these methods, targeted RNA recombination was the first

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reverse genetic system devised for CoVs at a time when it was not clear whether the construction of full-length infectious cDNA clones would ever be technically feasible. Targeted RNA recombination, originally developed for mouse hepatitis virus (MHV), takes advantage of the high rate of homologous RNA recombination in CoVs (Baric et al., 1990; Kusters et al., 1990; Makino et al., 1986). In this system, a synthetic donor RNA expanding the last 10 kb of the genome is transfected into cells infected with a recipient parental virus presenting some characteristics that can be selected against (ts phenotype or host range-based selection). Mutant recombinant viruses are then identified by counterselection of the recipient parental virus and purified.

Despite its value, targeted RNA recombination presents clear limitations, such as the inability to easily manipulate the replicase gene and to study lethal mutations due to the requirement for virus passage. Therefore, the development of reverse genetic approaches based on full-length cDNAs, which do not have these limitations, should provide a tremendous encouragement to the study of CoV biology. However, the large size of the genome (around 30 kb), the instability of some CoV replicase gene sequences when they were propagated as cloned cDNA in bacteria, and the difficulty to synthesize full-length transcripts in vitro have hampered the generation of CoV full-length infectious cDNA clones. Recently, these problems were overcome employing three creative nontraditional approaches based on the use of bacterial artificial chromosomes (BACs) (Almazan et al., 2000), in vitro ligation of cDNA fragments (Yount et al., 2000), and vaccinia virus as a vector for the propagation of CoV full-length cDNAs (Thiel et al., 2001a). In this report, we review and discuss these three different approaches developed for building CoV infectious cDNAs by using SARS-CoV as a model, and how these CoV reverse genetic systems have now been extended to the generation of CoV replicon RNAs.

2. Reverse genetic system using BACs

The first CoV full-length infectious cDNA clone was generated for transmissible gastroenteritis virus (TGEV) using the BAC approach (Almazan et al., 2000; Gonzalez et al., 2002). In this system, the full-length cDNA copy of the viral genome is assembled in the BAC plasmid pBeloBAC11 (Wang et al., 1997), a synthetic low-copy-number plasmid based on the *Escherichia coli* F-factor (Shizuya et al., 1992) that presents a strictly controlled replication leading to one or two plasmid copies per cell. This plasmid allows the stable maintenance in bacteria of large DNA fragments from a variety of complex genomic sources (Adler et al., 2003; Shizuya et al., 1992) and minimizes the toxicity associated with several CoV sequences when amplified in high-copy-number plasmids. The full-length cDNA is assembled under the control of the cytomegalovirus (CMV) immediate-early promoter that allows the expression of the viral RNA in the nucleus by the cellular RNA polymerase II (Dubensky et al., 1996), and it is flanked at the 3′-end by a poly(A) tail, the hepatitis delta virus (HDV) ribozyme and the bovine growth hormone (BGH) termination and polyadenylation sequences to produce synthetic RNAs bearing authentic 3′-ends of the viral genome. This DNA-launched system ensures capping of the viral RNA and allows the recovery of infectious virus from the cDNA clone without the need of an in vitro transcription step.

Using this approach, a BAC clone carrying an infectious genome of the SARS-CoV Urbani strain was generated in three steps (Fig. 1) (Almazan et al., 2006). The first step was the selection of appropriate restriction sites in the viral genome that were absent in the BAC plasmid. In case that no adequate restriction sites were available in the viral genome, new restriction sites could be generated by the introduction of silent mutations. In the second step, the intermediate BAC plasmid pBAC-SARS-CoV 5′–3′ was generated as the backbone to assemble the full-length cDNA clone. This plasmid contained the 5′-end of the genome under the control of the CMV promoter, a multicloning site containing the restriction sites selected in the first step, and the 3′-end of the genome followed by a 25-nt synthetic poly(A), the HDV ribozyme and the BGH termination and polyadenylation sequences. Finally, the full-length cDNA clone (pBAC-SARS-CoV^{FL}) was assembled by sequential cloning of five overlapping cDNA fragments (SARS-1 to SARS-5) into the multicloning site of the intermediate BAC plasmid (Fig. 1). The overlapping cDNA fragments flanked by the appropriate restriction sites were generated by standard reverse transcription PCR (RT-PCR). The assembled SARS-CoV BAC clone was fully stable in *E. coli* and infectious virus was rescued after transfection of susceptible cells (Almazan et al., 2006).

The BAC approach presents several advantages, such as the high stability of exogenous sequences, unlimited production of the cDNA clone, high efficiency of cDNA transfection into mammalian cells, and intracellular expression of the viral RNA. Furthermore, the manipulation of BAC clones is relatively easy and essentially similar to that of a conventional plasmid with slight modifications owing to the large size of the BAC clones and the presence of this plasmid in only one or two copies per cell (Shizuya et al., 1992). Besides standard protocols for the manipulation of conventional plasmids, the BAC clones could be easily and efficiently modified into *E. coli* by homologous recombination using a two-step procedure that combines the Red recombination system and counterselection with the homing endonuclease I-SceI (Jamsai et al., 2003; Lee et al., 2001; Tischer et al., 2006; Zhang et al., 1998). In a first step, a linear marker construct containing the desired modification and I-SceI recognition site is inserted via Red recombination into the target site using positive selection. In a second step, the induced I-SceI cleaves at its recognition site creating DNA double strand breaks. Then, the adjoining duplicate sequence previously introduced is used as the substrate for a second intramolecular Red recombination, resulting in the loss of the previously introduced marker. This novel approach results in an accurate and highly efficient method to introduce insertions, deletions or point mutations in BAC clones without retention of unwanted foreign sequences.

In addition to SARS-CoV and TGEV, the BAC approach has been successfully used to engineer infectious clones of HCoV-OC43 (St-Jean et al., 2006), feline infectious peritonitis virus (FIPV) (Balint et al., 2012), and the recently emerged MERS-CoV (Almazan et al., 2013). In the last case, a combination of synthetic biology and the use of BACs allowed the generation of a MERS-CoV infectious clone only four months after the first MERS-CoV outbreak, illustrating the power of the BAC approach. Recently, modified BAC approaches have been used to generate full-length cDNA clones of the SARS-CoV strains Frankfurt-1 (Pfefferle et al., 2009) and TOR2 related clinical isolate CV7 (Tylor et al., 2009), assembled in a BAC under the control of the T7 RNA polymerase promoter. In the case of the Frankfurt-1 strain, infectious virus was rescued after transfection of the full-length transcripts derived from the in vitro transcription of the linearized BAC construct. This approach combines plasmid-based handling of the infectious clone with direct delivery of genome-like RNA into the cytoplasm, circumventing transcription of the infectious clone in the nucleus driven by the CMV promoter, and avoiding the possibility of splicing. However, although some splicing could occur during the nuclear expression of the viral genome, the efficiency of this phenomenon is very low and does not affect the recovery of infectious virus (Almazan et al., 2000). In contrast, in the case of the CV7 isolate, infectious virus was recovered in situ from cells transfected with the BAC clone and infected with a modified vaccinia Ankara expressing T7 RNA polymerase. In this system an in vitro transcription step is also avoided.