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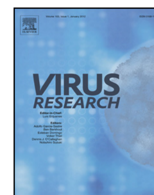

Reprint of: Coronavirus reverse genetic systems: Infectious clones and replicons☆

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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).

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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.

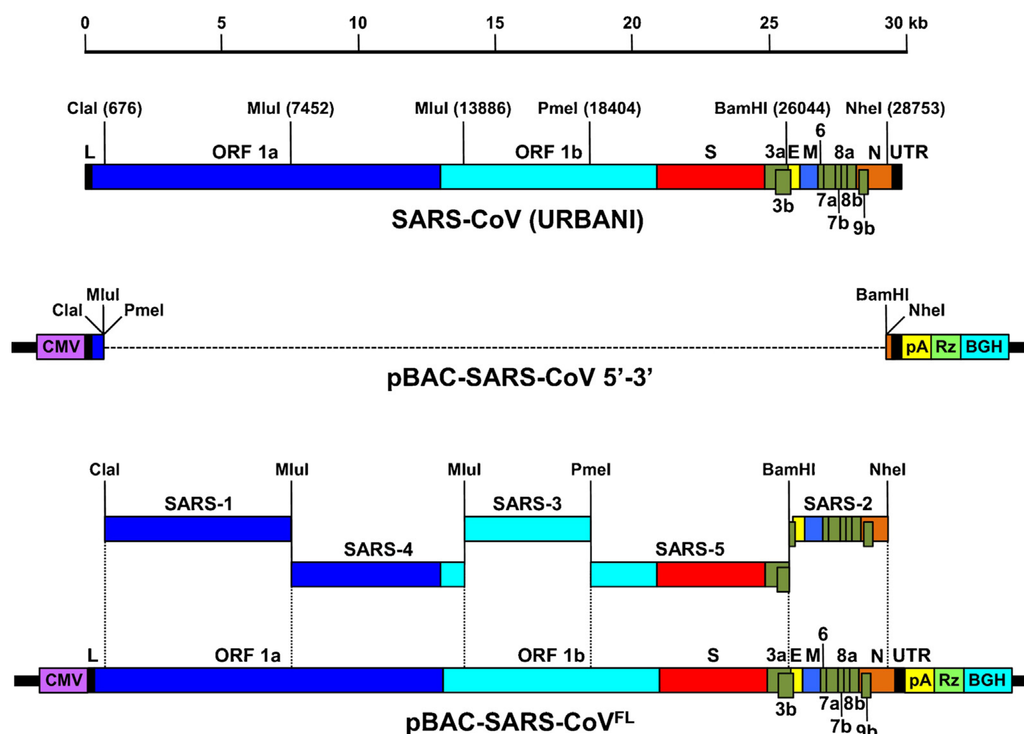

Fig. 1. Assembly of a SARS-CoV full-length cDNA clone as a BAC. After selection of appropriate restriction sites in the genome of the SARS-CoV Urbani strain (top of the figure), the intermediate plasmid pBAC-SARS-CoV 5′–3′ was generated and used as the backbone to assemble the full-length cDNA clone (pBAC-SARS-CoV^{FL}) by sequential cloning of five overlapping cDNA fragments (SARS-1 to SARS-5) covering the entire viral genome. The viral genes (ORF 1a, ORF 1b, S, 3a, 3b, E, M, 6, 7a, 7b, 8a, 8b, 9b and N), relevant restriction sites and their genomic position (in brackets), the CMV promoter, the poly(A) tail (pA), the HDV ribozyme (Rz), and the BGH termination and polyadenylation sequences (BGH) are indicated. L, leader; UTR, untranslated region.

Figure adapted from Almazan et al. (2006).

This reverse genetic approach has been successfully used to study the role of specific viral proteins in viral replication and pathogenesis and for the generation of genetically attenuated viruses that are potential vaccine candidates for SARS-CoV and other CoVs (Almazan et al., 2013; DeDiego et al., 2007; Enjuanes et al., 2008; Fett et al., 2013; Lamirande et al., 2008; Netland et al., 2010).

3. Reverse genetic system using in vitro ligation

This method, originally applied to TGEV (Yount et al., 2000), involves the assembly of full-length cDNAs from a panel of contiguous cDNA fragments that span the entire viral genome, flanked by native or engineered specific restriction sites with characteristics that allow the systematic and precise assembly of a full-length cDNA by in vitro ligation. The assembled full-length cDNA, containing a T7 RNA polymerase promoter at the 5′-end and a poly(A) tail at the 3′-end, is in vitro transcribed to generate capped full-length transcripts that are used together with capped N gene transcripts to efficiently recover infectious virus after transfection of susceptible cells. In this approach, some fragment boundaries were arranged in such a way that interrupt genomic regions that were unstable when propagated as cloned cDNA in bacteria.

Following this strategy, a genome-length cDNA of the SARS-CoV Urbani strain was generated by in vitro ligation (Fig. 2) (Yount et al., 2003). Initially, a panel of six contiguous cDNA fragments spanning the entire SARS-CoV genome was generated by standard RT-PCR using specific primers that introduced unique BglI restriction sites at the 5′ and 3′-ends of each fragment without altering the amino acid coding sequences of the virus. BglI is a class II restriction enzyme that cleaves a symmetrical palindromic sequence (GCCNNNN↓NGGC) but leaves different asymmetric 3-nt overhangs that do not randomly self-assemble and only anneal with the complementary 3-nt overhang generated at an identical BglI site. These cDNA fragments were systematically and unidirectionally assembled into a full-length cDNA by in vitro ligation and, after in vitro transcription, genome-length transcripts were used to recover infectious virus. This in vitro assembly technique combined with synthetic biology was further used successfully to engineer infectious clones of a bat SARS-like CoV (Becker et al., 2008) and the recently identified MERS-CoV (Scobey et al., 2013).

One potential problem of this approach is that several silent mutations have to be inserted in the genome to avoid potential T7 transcription termination signals and to introduce the unique BglI sites used to assemble the full-length cDNA clones. To overcome this problem, a variation of the approach was used to engineer the MHV infectious cDNA (Yount et al., 2002). The strategy is based on the incorporation of type IIS restriction enzymes (Esp31, SapI, BsaI and BsmI, among others) at the ends of the cDNA fragments. These enzymes recognize asymmetrical sites and cleave external to the recognition sequences, leaving 1–4-nt variable ends that can be only anneal with the complementary overhang generated at an identical site (Esp31 site, CGTCTCN↓NNNN). Using these type IIS restriction enzyme sites, two contiguous cDNA fragments could be ligated in vitro with traditional cloning approaches, leaving the restriction site within the viral genome sequence. However, due to the asymmetrical nature of these recognition sites, a simple reverse orientation allows for the insertion of these sites on the ends of two adjacent cDNA fragments with the variable overhang generated from the virus sequence (No See'm cloning strategy). Upon cleavage and ligation, both restriction sites are removed, leaving the exact viral sequence at the junction. This approach allows the generation of cDNA clones without mutating the viral genome sequence. Following this modified approach, the full-length cDNA clone of

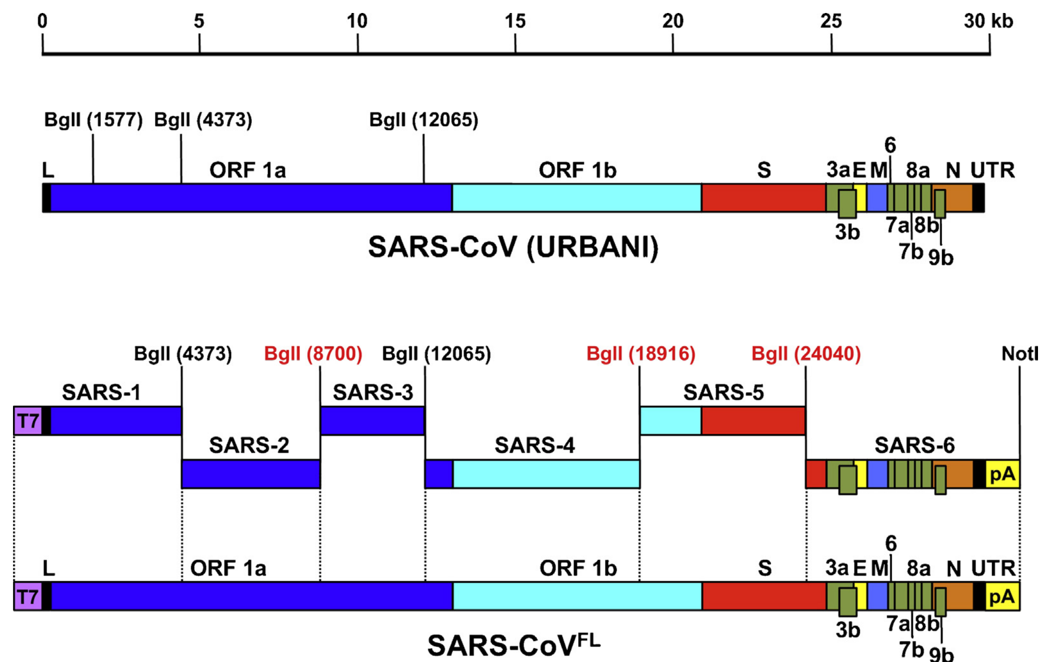

Fig. 2. Systematic assembly of a SARS-CoV full-length cDNA clone by in vitro ligation. A full-length cDNA of the SARS-CoV Urbani strain (SARS-CoV^{FL}) was assembled by in vitro ligation of six contiguous cDNA fragments (SARS-1 to SARS-6) spanning the entire viral genome, which were flanked by native (in black) or engineered (in red) unique BglI restriction endonuclease sites. The assembled full-length cDNA contained a T7 RNA polymerase promoter (T7) at the 5′-end and a poly(A) tail (pA) at the 3′-end, allowing for in vitro transcription of full-length, capped polyadenylated transcripts. The viral genes and relevant restriction sites are indicated.

Figure adapted from Yount et al. (2003).

MHV was generated from either the sequential (in several steps) or simultaneous in vitro ligation of seven cDNA fragments covering the entire MHV genome (Yount et al., 2002). The feasibility of this method has been demonstrated later by the successful assembly of full-length infectious clones of infectious bronchitis virus (IBV) (Fang et al., 2007; Tan et al., 2006; Youn et al., 2005) and HCoV-NL63 (Donaldson et al., 2008).

In this reverse genetic system, inclusion of N transcripts during the transfection process has been shown to enhance virus rescue in all analyzed cases, although its mechanism of action is largely unknown.

The in vitro cDNA assembly approach is simple and straightforward, allows the rapid mutagenesis of independent cDNA fragments in parallel using conventional techniques, and is compatible with BAC or vaccinia vectors. Furthermore, using the No See'm technology it is possible to mutate any given nucleotide in the viral genome by designing primers that incorporate a type IIS restriction site and the mutation of interest in the variable domain of the recognition site. Adding the type IIS restriction site in the proper orientation, the site is removed during reassembly, leaving only the mutation of interest in the final DNA product (Donaldson et al., 2007; Yount et al., 2002).

This reverse genetic approach has been successfully used to study the role of specific viral proteins in SARS-CoV replication and pathogenesis, to identify zoonotic vaccine candidate strains (Deming et al., 2006), and to develop a recombination safe vaccine platform using a novel strategy that prevents recombination between wild-type and recombinant viruses by rewiring the viral transcription regulating sequences (TRSs) (Yount et al., 2006).

4. Reverse genetic system using vaccinia virus vectors

An alternative reverse genetic system to study the biology and pathogenesis of CoVs is based on the cloning and propagation of CoV genomic cDNAs in vaccinia virus vectors (Eriksson et al., 2008; Thiel and Siddell, 2005). This approach was first described for the generation of recombinant HCoV-229E (Thiel et al., 2001a), and subsequently applied to successfully engineer full-length infectious clones of MHV (Coley et al., 2005), SARS-CoV (van den Worm et al., 2012), IBV (Casais et al., 2001), and FIPV (Tekes et al., 2008, 2012).

This approach presents some advantages. The cloning capacity of poxvirus vectors, and vaccinia virus in particular, exceeds 26 kb of foreign sequences (Smith and Moss, 1983). Recombinant vaccinia virus genomes are stable, infectious and replicate efficiently in tissue culture. Vaccinia virus vectors designed for the insertion of foreign DNA by in vitro ligation (Merchlinsky and Moss, 1992) have been developed, thus obviating the need for plasmid intermediates containing the entire cDNA insert. Finally, a vaccinia virus-mediated homologous recombination strategy is available to make accessible for site directed mutagenesis the CoV cDNA cloned in the vaccinia vector (Britton et al., 2005; Thiel and Siddell, 2005).

The generation of a reverse genetic system for SARS-CoV (isolate HKU-39849) (van den Worm et al., 2012) will be described to illustrate the vaccinia virus-based system. Methodologically, this reverse genetic system can be divided into three phases (Fig. 3).

The first step involves the generation of subgenomic cDNA fragments that are prepared in large amounts either by amplification as bacterial plasmid DNA or by preparative RT-PCR. The cDNAs are subsequently ligated in vitro to produce a small number of cDNAs, which encompass the entire genome. The specific ligation strategy is determined by the sequence of the particular CoV, and mainly consists on naturally occurring or engineered restriction sites. For SARS-CoV strain HKU-39849, five cDNA fragments encompassing the whole genome were initially in vitro ligated with each other and with synthetic oligonucleotide linkers, resulting in the generation of two cDNAs spanning nucleotides 1–20,288 and 20,272–29,727, respectively (van den Worm et al., 2012) (Fig. 3). A crucial step in this process is to modify the cDNAs containing the 5′ and 3′-ends of the CoV genome in order to finally produce infectious RNA molecules. To this end, a transcription promoter sequence

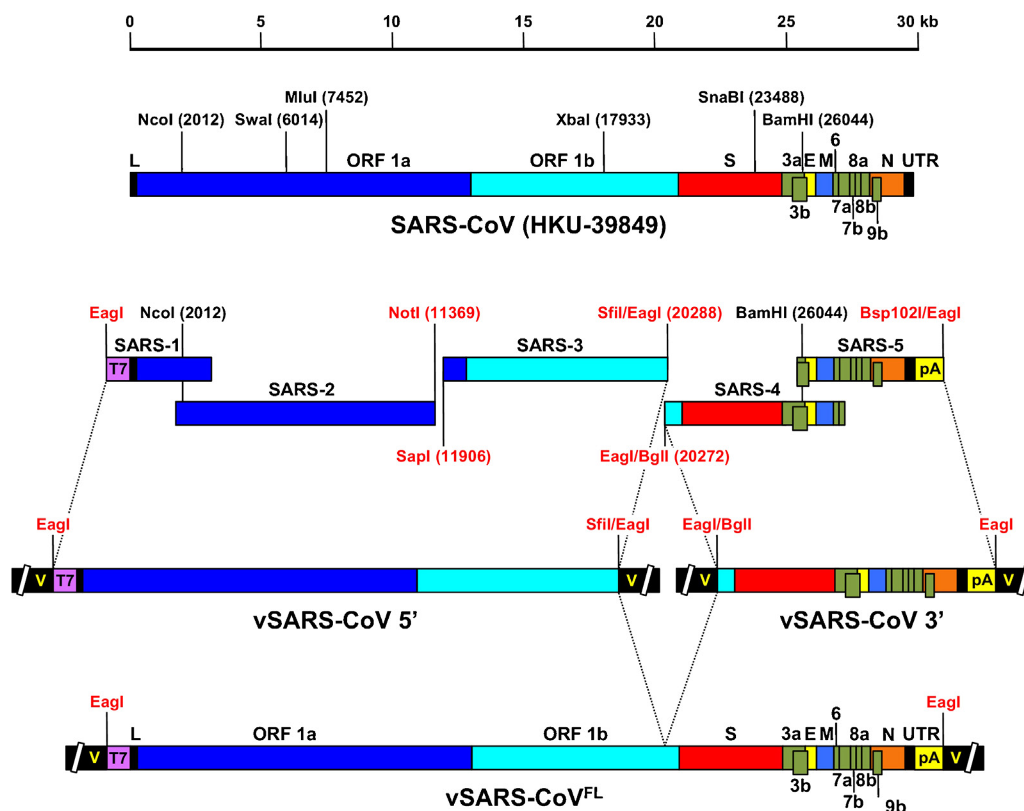

Fig. 3. Assembly of a SARS-CoV full-length cDNA clone in the vaccinia virus genome. A panel of five contiguous cDNA fragments (SARS-1 to SARS-5) spanning the entire viral genome of the SARS-CoV HKU-39849 strain was cloned in vaccinia virus DNA (V) by in vitro ligation to produce two recombinant vaccinia viruses containing nucleotides 1–20,288 (vSARS-CoV 5′) and 20,272–29,727 (vSARS-CoV 3′) of the SARS-CoV genome, respectively. SARS-1 fragment contained a T7 RNA polymerase promoter (T7) at the 5′-end and SARS-5 fragment a poly(A) tail (pA) at the 3′-end. DNAs from both recombinant vaccinia viruses were then cleaved with SfiI and BglI, and in vitro ligated to create a full-length SARS-CoV cDNA (vSARS-CoV^{FL}) template for in vitro transcription of SARS-CoV infectious RNA transcripts. The viral genes and relevant restriction sites (native in black and engineered in red) used for the assembly of the infectious clone are indicated.

Figure adapted from van den Worm et al. (2012).

for the bacteriophage T7 RNA polymerase is located upstream the CoV genome and a synthetic poly(A) at the 3′-end of the genome, followed by a unique restriction site that can be used to generate run-off transcripts (Fig. 3). Finally, to insert the CoV cDNA into a single NotI site present in the genomic DNA of the vaccinia virus vector vNotI/tk, the 5′ and 3′-genomic termini of cDNA fragments have to contain the restriction sites EagI or Bsp120I, which lead to DNA ends compatible with NotI-cleaved vaccinia virus vector DNA. During the cloning steps of SARS-CoV cDNA sequences, some problems arose such as the instability in bacterial plasmids of viral cDNA sequences (nt 11,370–11,905) and unexpected RT-PCR introduced mutations and deletions. The incorrect cDNA regions were replaced by appropriate SARS-CoV sequences using vaccinia virus-mediated homologous recombination, as it will be described below.

In the second step, in vitro assembled CoV cDNA fragments representing the full-length genome are cleaved with EagI and then ligated in vitro to the long and short arms of NotI-cleaved vNotI/tk vaccinia virus DNA (Merchlinsky and Moss, 1992). Subsequently, the ligation reaction is transfected into CV-1 mammalian cells infected with fowlpox virus as a helper (Scheiflinger et al., 1992) to rescue recombinant vaccinia viruses including the cDNA of CoV genome sequences. Because fowlpox virus infection of mammalian cells is abortive, and possible recombination events between both poxviruses are extremely rare, the resulting virus stocks collected from cells contain exclusively recombinant vaccinia viruses (Thiel et al., 2001a, 2001b). In the case of SARS-CoV, two recombinant vaccinia viruses spanning nucleotides 1–20,288 (SARS-CoV 5′) and 20,272–29,727 (SARS-CoV 3′) were generated (Fig. 3). DNA derived from these vaccinia viruses was cleaved with SfiI and BglI restriction enzymes producing compatible ends and then ligated to create a cDNA containing a genome-length SARS-CoV cDNA (van den Worm et al., 2012).

Finally, recombinant CoVs are rescued by generating genomic-length RNA transcripts from the CoV cDNA insert of the recombinant vaccinia virus. Purified recombinant vaccinia virus DNA containing the full-length cDNA of CoV genome is cleaved at a unique site located downstream of the poly(A) sequence, representing the 3′-end of the CoV genome. The resulting DNA is used as template for in vitro transcription with bacteriophage T7 RNA polymerase to generate a capped RNA corresponding to the CoV genome. These transcripts are then transfected into permissive cells, leading to the rescue of the corresponding CoV from the tissue culture supernatant.

A limiting step of this procedure is the variable amount and purity of full-length synthetic CoV RNA from in vitro transcription, leading sometimes to failure to rescue recombinant CoVs. Therefore, an alternative strategy is the transcription of template DNA in the permissive cell itself by expressing the bacteriophage T7 RNA polymerase from a recombinant fowlpox virus rFPV-T7 (Britton et al., 1996; Casais et al., 2001).

Previous experience in the vaccinia virus reverse genetic system has shown that the N transcript may become essential for the recue of some CoVs such as IBV (Casais et al., 2001). Therefore, to contribute positively to the recovery of CoVs, different methods involving N protein expression were used. Initially, N transcripts were cotransfected with the infectious RNA, as described for MHV (Coley et al., 2005). More recently, full-length SARS-CoV RNA

synthesized in vitro with the T7 RNA polymerase was transfected into BHK cells inducibly expressing the SARS-CoV N protein (Chang et al., 2010) and the transfected cells were then co-cultivated with susceptible cells to rescue eventually the recombinant SARS-CoV (van den Worm et al., 2012). The requirement of the N protein to establish a productive CoV infection from infectious RNA or DNA has been shown to be variable. Several CoVs such as TGEV, SARS-CoV and MERS-CoV were efficiently rescued from infectious cDNAs engineered as BACs, independently of N protein coexpression (Almazan et al., 2000, 2006, 2013). Similarly, HCoV-229E was rescued from vaccinia virus in the absence of N protein expressed in trans (Thiel et al., 2001a). In contrast, N transcripts were essential to rescue IBV from recombinant vaccinia virus (Casais et al., 2001). In an intermediate situation, the rescue of MHV and TGEV from full-length cDNA constructs assembled in vitro was significantly enhanced by the inclusion of N-coding transcripts (Baric and Sims, 2005; Yount et al., 2002). These results suggest that CoV cDNAs engineered as BACs and transcribed by the cellular pol II to produce infectious RNAs represent a more efficient method for virus recovery as compared to the other systems relying on infectious RNAs transcribed by T7 polymerase, since these reverse genetic systems depend at different extents on N transcripts. At this moment, it is not clear whether N transcripts, N protein, or both are essential for increased virus recovery yields. Nevertheless, previous results have shown that the N protein is involved in CoV RNA transcription and increases RNA synthesis (Almazan et al., 2004; Schelle et al., 2005; Zuñiga et al., 2010).

One important advantage of the vaccinia virus reverse genetic system is that vaccinia virus-mediated homologous recombination (Ball, 1987) can be used to modify the CoV genomic cDNA during its propagation in vaccinia virus. This site-directed mutagenesis strategy has been extensively applied to introduce heterologous sequences in the genome of recombinant CoVs (van den Worm et al., 2012), for gene deletion or modification (Casais et al., 2005; Lei et al., 2013; Roth-Cross et al., 2009; Stokes et al., 2010) or even to repair error sequences (Coley et al., 2005; van den Worm et al., 2012). Two recombination methods based on the sequential use of the *E. coli* guanine-phosphoribosyl transferase gene (*gpt*) as both a positive and a negative selection marker (Armesto et al., 2008; Britton et al., 2005; Eriksson et al., 2008) have been developed. In the first method (Falkner and Moss, 1988; Thiel and Siddell, 2005) the CoV region of interest in the recombinant vaccinia virus is replaced by the *E. coli gpt* gene. This gene is encoded in a plasmid DNA and flanked by CoV sequences to facilitate a double recombination event. Vaccinia viruses containing the *gpt* gene at the expected CoV genome position are isolated under gpt-positive selection on mammalian CV-1 cells. In a second step, the introduced *gpt* gene is replaced by a gene of interest encoded in a plasmid DNA and flanked by CoV sequences mediating double homologous recombination events. Isolation of recombinant viruses with the gene of interest requires gpt-negative selection in HeLa-D980R cells.

In the second method, known as transient dominant selection (Britton et al., 2005; Falkner and Moss, 1990; Kerr and Smith, 1991), the modified CoV cDNA region is inserted into a plasmid containing the *gpt* selective marker under the control of a vaccinia virus promoter. Then, the complete sequence of this plasmid is transiently integrated into the vaccinia virus including the CoV genome by homologous recombination involving a single crossover event. The recombinant vaccinia viruses expressing the *gpt* gene are positively selected. Finally, the *gpt* marker is lost in the absence of the selective pressure, by a single homologous recombination event between duplicated sequences. Two recombination events can occur with equal frequency leading either to the generation of the unmodified CoV sequence or to the generation of a CoV cDNA with the desired modification.

5. Generation of CoV replicons

Replicons have been developed for several RNA viruses as an important complementary resource of virus reverse genetic systems (Bartenschlager, 2002; Brass et al., 2007; Frolov et al., 1996; Khromykh, 2000; Nakamura et al., 2008; Zimmer, 2010). Replicons are self-amplifying nucleic acids that contain all viral proteins and RNA signals required for viral RNA synthesis, and in some cases reporter genes to facilitate the analyses. As replicons lack structural proteins and no infectious viral particles are produced, they constitute a safe alternative to full-length infectious cDNAs, especially for BSL3 viruses. Moreover, stable cell lines containing non-cytopathic selectable virus replicons have also been obtained (Bartenschlager, 2002). Therefore, replicons represent a safe tool for virus replication studies and antiviral compound screening, and even for the setup of semi-automated high-throughput screening systems.

CoV replicons have been generated for many CoVs using all the reverse genetic approaches described above (Almazan et al., 2004, 2006; Curtis et al., 2002; Ge et al., 2007; Hertzig et al., 2004; Thiel et al., 2001b). These replicons were very useful for the identification of viral and cellular factors involved in CoV RNA synthesis (Almazan et al., 2004; Galan et al., 2009; Moreno et al., 2008), and for antiviral drug testing (Chen et al., 2005). In the case of SARS-CoV, a collection of replicons has been engineered, either using the BAC system (Almazan et al., 2006) or by in vitro ligation (Ge et al., 2007). Different replicon versions include a variety of reporter genes, such as green fluorescent protein (GFP), firefly luciferase (FLuc), or *Renilla* luciferase (RLuc), alone or in combination with antibiotic selection genes (Ahn et al., 2011; Ge et al., 2007; Pan et al., 2008; Tanaka et al., 2012).

The genomic structure of the RNA encoded by CoV replicons is very similar in all cases. They contain the 5′ and 3′ cis acting signals required for viral replication, the large ORFs 1a and 1b that encode the replicase non-structural proteins (nsps), and the N gene, which is essential for efficient CoV RNA synthesis (Almazan et al., 2004). The SARS-CoV replicon obtained by in vitro ligation also includes a GFP-*Bla*R fusion gene, located downstream of the ORF 1b, under the transcriptional control of S gene TRS (TRS-S). This heterologous reporter gene, which confers resistance to blasticidin selection, allowed the generation of the first selectable SARS-CoV replicon cell line (Ge et al., 2007). Several BAC-based SARS-CoV replicons also included heterologous genes located downstream of the ORF 1b. RLuc was expressed under the control of TRS-N (Tanaka et al., 2012), although eventually, the duplication of TRS-N sequences could lead to reporter gene loss after several replication cycles. Alternatively, FLuc-*Neo*R fusion gene (conferring neomycin resistance) was expressed under the control of TRS-N (Ahn et al., 2011) or TRS-M (Pan et al., 2008). In this case, antibiotic selection would avoid reporter gene loss and allows the future development of selectable cell lines.

CoV replicons could also be derived from replication-competent propagation-defective viruses, such as those lacking E gene engineered for TGEV (Curtis et al., 2002; Ortego et al., 2002) or MERS-CoV (Almazan et al., 2013). In this case, additional safety-guards must be introduced to work in a BSL2 containment facility. In this line, a SARS-CoV replicon was constructed based on an attenuated SARS-CoV-ΔE infectious cDNA, replacing the structural S gene by GFP reporter gene (Wang et al., 2008). This replicon was useful for antiviral drug testing, although a more comprehensive evaluation of replicon safety should be performed.

SARS-CoV replicons have been used to analyze the molecular basis of viral RNA synthesis and viral or cellular factors involved. In this sense, the function of some nsps, such as nsp1, nsp14, nsp15, or nsp16, in SARS-CoV replication was analyzed avoiding the use of infectious virus (Almazan et al., 2006; Chen et al., 2009; Tanaka et al., 2012). Moreover, a genome-wide analysis of viral proteins

involved in SARS-CoV RNA synthesis (Pan et al., 2008) and a quantitative proteomic analysis to determine host cell factors involved in SARS-CoV replication (Zhang et al., 2010) were also performed using replicons.

To date, despite the increased efforts since SARS-CoV outbreak, no antiviral drug approved by FDA exists against human CoVs (Barnard and Kumaki, 2011; Kilianski and Baker, 2014). One of the most promising applications of SARS-CoV replicons is their use for antiviral drug screening. Since SARS-CoV replicons lack structural proteins, compounds inhibiting virus entry or morphogenesis could not be tested. Nevertheless, with some exceptions (Kilianski and Baker, 2014), these virus life cycle steps are poor antiviral targets, as virus escape mutants could be easily recovered, especially when virus structural proteins are targeted. On the other hand, replicase proteins or viral RNA motifs involved in CoV replication are in general more conserved, and their mutation rate could be lower than that of the structural proteins due to higher fitness pressure. In fact, several key enzymatic activities, such as main protease or papain-like protease, have been analyzed as targets for CoV antiviral drugs (Kilianski and Baker, 2014).

Additionally, SARS-CoV replicons have been used for further validation of antiviral compounds or nucleic acids that were previously identified using other in vitro screening methods, such as helicase (Adedeji et al., 2012) or frameshifting (Ahn et al., 2011) inhibitors. Moreover, a selectable SARS-CoV replicon cell line was used for the development of a semi-automated high-throughput antiviral compound screening system, with minimum manipulation after assay set-up, low compound volume, and automated detection of end-point fluorescence (Ge et al., 2008). Interestingly, after a screening of a 7,035 compound library, and selection of 7 drugs for further testing, a good correlation was observed between fluorescence levels and SARS-CoV RNA or protein synthesis, highlighting the system suitability. A limitation of this kind of cell-based screening systems is that the viral or cell drug target is not identified, and further analyses using other experimental approaches are required.

6. Concluding remarks and future prospects

The potential risk to public health posed by SARS-CoV and other CoVs, and the lack of specific antiviral agents and vaccines, have triggered a global effort to study this family of viruses at the molecular level in order to develop effective strategies to prevent and control CoV infections. Molecular genetic analysis of the structure and function of RNA virus genomes has been profoundly advanced by the availability of full-length cDNA clones. In the case of CoV, the huge genome size and the instability of specific CoV cDNA sequences in bacterial systems hindered the development of infectious cDNA clones until recently. A tremendous amount of creativity in the CoV field resulted in the development of four independent and unique reverse genetic systems that overcame these problems. These reverse genetic systems have been established using non-traditional approaches, which are based on the use of targeted recombination, BACs, in vitro ligation of CoV cDNA fragments, and vaccinia virus as a vector for the propagation of CoV genomic cDNAs. The availability of CoV full-length infectious clones and recombinant viruses expressing reporter genes constitute important tools for the study of CoV replication and transcription mechanisms, virus-host interaction and pathogenesis, and also for the rapid and rational development and testing of genetically defined vaccines. In addition to full-length cDNA clones, the generation of CoV replicons has greatly facilitated the functional analysis of viral replication and transcription, as well as the analysis of antiviral drugs without the need of manipulating infectious viruses.

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