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Construction of a Severe Acute Respiratory Syndrome Coronavirus Infectious cDNA Clone and a Replicon To Study Coronavirus RNA Synthesis[▽]

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The engineering of a full-length infectious cDNA clone and a functional replicon of the severe acute respiratory syndrome coronavirus (SARS-CoV) Urbani strain as bacterial artificial chromosomes (BACs) is described in this study. In this system, the viral RNA was expressed in the cell nucleus under the control of the cytomegalovirus promoter and further amplified in the cytoplasm by the viral replicase. Both the infectious clone and the replicon were fully stable in *Escherichia coli*. Using the SARS-CoV replicon, we have shown that the recently described RNA-processing enzymes exoribonuclease, endoribonuclease, and 2′-*O*-ribose methyltransferase were essential for efficient coronavirus RNA synthesis. The SARS reverse genetic system developed as a BAC constitutes a useful tool for the study of fundamental viral processes and also for developing genetically defined vaccines.

The etiologic agent causing severe acute respiratory syndrome (SARS) is a novel coronavirus (CoV) (8, 10, 16–18, 21). This virus causes a life-threatening respiratory disease for which no fully efficacious therapy is available. SARS-CoV is a member of group 2 of the *Coronaviridae* family within the order *Nidovirales* (13), which is composed of enveloped, single-stranded, positive-sense RNA viruses relevant in animal and human health (5, 9). Two-thirds of the 29.7-kb SARS-CoV genome carries the replicase gene, which comprises two overlapping open reading frames, ORF 1a and ORF 1b, the latter being translated by a ribosomal frameshift mechanism (29). Translation of both ORFs results in the synthesis of two polyproteins that are processed by viral proteinases to release the components of the replication-transcription complex (36, 37). Besides containing RNA-dependent RNA polymerase, RNA helicase, and proteases (4, 12, 15, 23, 37), which are all common to positive-strand RNA viruses, the CoV replicase was recently predicted to contain a variety of RNA-processing enzymes that are extremely rare or absent in other RNA viruses, including endoribonuclease (NendoU), 3′-to-5′ exoribonuclease (ExoN), 2′-*O*-ribose methyltransferase (2′-O-MT), ADP ribose 1′′-phosphatase, and, in a subset of group 2 coronaviruses, cyclic phosphodiesterase (25, 36). These enzymatic activities might be involved in the replication of the largest known RNA virus genome and in the production of an extensive set of 5′- and 3′-coterminal subgenomic RNAs (11, 14, 25, 36).

The study of CoV molecular biology has been profoundly advanced by the recent construction of full-length cDNA clones (3, 6, 26, 27, 32–34) and self-replicating RNAs, or replicons (2, 28, 30). Due to the large size of the CoV RNA genome and the instability of some CoV replicase gene sequences in bacteria, cDNA clones and replicons have been engineered using bacterial artificial chromosomes (BACs) (3), in vitro ligation of CoV cDNA fragments (32), and vaccinia virus as a vector for the propagation of CoV full-length cDNAs (27). Recently, a SARS-CoV full-length cDNA clone has been generated by the approach of using the in vitro ligation of cDNA fragments (33). This system has been shown to be efficient for the recovery of infectious virus and has been used for the generation of a large collection of deletion mutants of SARS-CoV accessory genes located in the 3′ end of the genome (35). However, the construction of a SARS-CoV replicon as a biosafe tool to study fundamental viral processes and to test antiviral drugs has not yet been reported.

This paper describes the construction of a full-length infectious cDNA clone and the first functional replicon of the SARS-CoV Urbani strain (GenBank accession number AY278741) as BACs. In addition, using the SARS-CoV replicon, we have shown that the RNA-processing enzymes ExoN, NendoU, and 2′-O-MT were essential for efficient CoV RNA synthesis.

Construction and functional analysis of a SARS-CoV infectious cDNA clone as a BAC. In this article, we use the BAC approach to assemble the SARS-CoV full-length cDNA under the control of the cytomegalovirus (CMV) immediate-early promoter to allow the expression of the viral RNA in the nucleus by cellular RNA polymerase II. This approach was used in our laboratory for the construction of the first CoV infectious cDNA clone (3) and, more recently, for the successful construction of other coronavirus (26) and arterivirus (7) cDNA clones. This system permits the stable maintenance in

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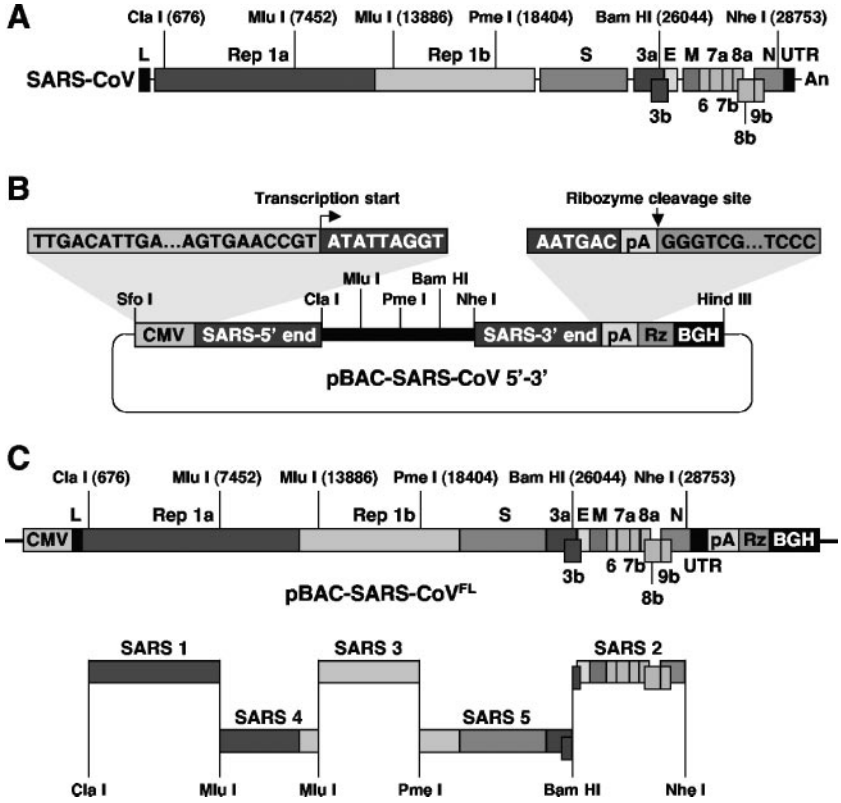

FIG. 1. Strategy to assemble a SARS-CoV infectious cDNA clone as a BAC. (A) Genetic structure of the SARS-CoV Urbani strain genome. Relevant restriction sites used for the assembly of the full-length cDNA clone are indicated. Numbers in parentheses indicate the genomic positions of the first nucleotide of the restriction endonuclease recognition sequence. Letters and numbers indicate the viral genes. L, leader sequence; UTR, untranslated region; An, poly(A) tail. (B) Construction of pBAC-SARS-CoV 5′-3′. After the selection of appropriate restriction sites, the intermediate plasmid pBAC-SARS-CoV 5′-3′ was constructed as the backbone for assembling the infectious cDNA clone. This plasmid includes the first 681 nt of the genome under the control of the CMV promoter, a multiple-cloning site containing the restriction sites selected for the final assembly of the infectious clone, and the last 975 nt of the genome, followed by a synthetic poly(A) tail (pA), the hepatitis delta virus ribozyme (Rz), and the bovine growth hormone termination and polyadenylation sequences (BGH). All these elements were precisely joined by overlapping PCR. The CMV promoter transcription start and the ribozyme cleavage site are shown. (C) Schematic diagram showing the five-step cloning strategy used for the assembly of the SARS-CoV full-length cDNA clone. The five overlapping cDNA fragments, named SARS 1 to SARS 5, were sequentially cloned into the plasmid pBAC-SARS-CoV 5′-3′ to generate the plasmid pBAC-SARS-CoV^{FL}. Relevant restriction sites are indicated. The labels are as described for panel A.

bacteria of large DNA fragments from a variety of complex genomic sources (1, 24). Furthermore, the manipulation of the cDNA clone is similar to that of a conventional plasmid and directly allows the recovery of infectious virus from the cDNA clone without the need for in vitro ligation and transcription steps.

The BAC clone carrying an infectious genome of the SARS-CoV Urbani strain was generated in three steps. The first was selection of appropriate restriction sites in the viral genome (Fig. 1A). The second was construction of the intermediate plasmid pBAC-SARS-CoV 5′–3′ as the backbone for assembling the full-length cDNA clone (Fig. 1B). This plasmid includes the first 681 nucleotides (nt) of the genome under the control of the CMV promoter and the last 975 nt of the genome, followed by a 25-nt synthetic poly(A), the hepatitis delta virus ribozyme, and the bovine growth hormone termination and polyadenylation sequences to produce synthetic RNAs bearing authentic 5′ and 3′ ends of the viral genome. In addition, a multiple-cloning site containing the restriction sites ClaI, MluI, PmeI, BamHI, and NheI, selected in the first step, was cloned between the viral sequences to allow the assembly of the full-length clone. After transfection of baby hamster kidney (BHK) cells with the intermediate plasmid, the sequence of the 5′ and 3′ ends of the synthetic RNA generated was confirmed by 5′ and 3′ rapid amplification of cDNA ends analysis (data not shown). Finally, the third step was assembly of the full-length cDNA clone (pBAC-SARS-CoV^{FL}) by sequential cloning of five overlapping cDNA fragments into the plasmid pBAC-SARS-CoV 5′–3′ (Fig. 1C). These cDNAs were generated by standard reverse transcriptase PCR (RT-PCR) with specific oligonucleotides using total RNA from cells infected with the SARS-CoV Urbani strain, kindly provided by the Centers for Diseases Control and Prevention (Atlanta, Ga.), as the template. The cDNA clone sequence was identical to the reported Urbani sequence (21), with the exception of two silent point mutations at positions 10338 (C to T) and 11163 (T to A) that were introduced as genetic markers to distinguish between the virus recovered from the cDNA and the wild-type virus. The assembled SARS-CoV infectious cDNA clone was fully stable during its propagation in *E. coli*

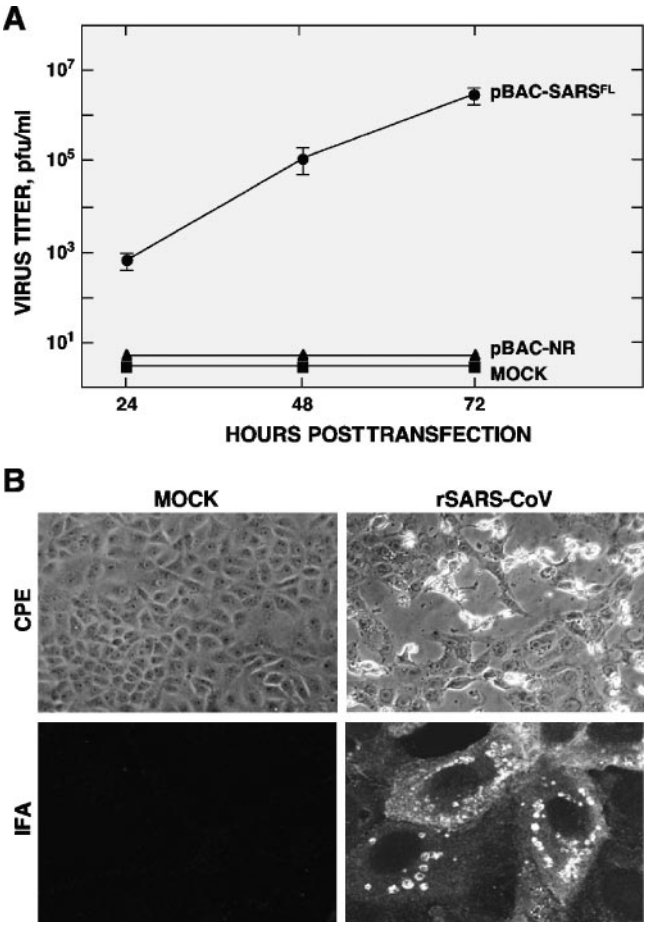

FIG. 2. Recovery of infectious rSARS-CoV from the full-length cDNA clone. (A) Virus rescue. BHK cells were mock transfected or transfected with either the full-length cDNA clone (pBAC-SARS^{FL}) or a nonreplicative cDNA clone (pBAC-NR) with a deletion in the replicase gene. Cells were removed with trypsin at 6 hpt and plated over a confluent monolayer of VeroE6 cells, and at the indicated times posttransfection, virus titers were determined by plaque assay on VeroE6 cells. Error bars represent standard deviations of the means from three experiments. (B) VeroE6 cells were mock infected or infected with rSARS-CoV and subsequently analyzed for the induction of cytopathic effect (CPE) by light microscopy. Viral protein expression was analyzed by indirect immunofluorescence (IFA) with a human anti-SARS-CoV polyclonal serum, kindly provided by A. Xu (Sun Yat-sen University, Guangzhou, People's Republic of China), followed by fluorescein isothiocyanate-labeled goat anti-human antibody.

DH10B cells for more than 200 generations, considerably facilitating the genetic manipulation of the viral genome (data not shown). The detailed cloning strategy, plasmid maps, and sequences are available upon request.

To recover infectious virus, BHK cells were grown to 95% confluence in a 25-cm^2 flask and transfected with the cDNA clone by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications. At 6 h posttransfection (hpt), cells were trypsinized, plated over a confluent monolayer of VeroE6 cells grown in a 25-cm^2 flask, and incubated at 37°C for 72 h. Virus titers quickly increased to around 2×10^6 PFU/ml at 72 hpt (Fig. 2A). After two passages in VeroE6 cells, the recovered virus was cloned by three rounds of plaque purification, and the genotypic and phenotypic properties were determined. The rescued virus (rSARS-CoV) induced a clear cytopathic effect in VeroE6 cells, and its identity was confirmed by indirect immunofluorescence using SARS-CoV-specific antibodies (Fig. 2B). Furthermore, it conserved the genetic markers introduced and was identical to the parental virus in terms of plaque morphology, growth kinetics, and mRNA and protein patterns (data not shown).

All work involving infectious SARS-CoV was performed in a biosafety level 3 laboratory at the National Health Institute Carlos III (Madrid, Spain), following the guidelines of the European Commission and the National Institutes of Health. Personnel were double-gloved and wore powered air-purifying respirators (HEPA AirMate; 3M, Saint Paul, MN) to provide a positive-pressure environment within the hoods.

Generation of a functional SARS-CoV replicon as a BAC. The availability of a SARS-CoV replicon provides an important tool for the study of fundamental viral processes and for the selection of antiviral drugs against this human pathogen, without the need for growing infectious virus.

To engineer the SARS-CoV-derived replicon, the BAC system was used because it presents several advantages over other approaches, such as easy manipulation, unlimited production of the cDNA, high efficiency of transfection, and intracellular expression of the replicon RNA. The generation of the SARS-CoV replicon was based on the full-length cDNA clone described above and on published data showing that the N protein is required for efficient CoV replication (2, 22). Accordingly, a cDNA containing the untranslated 5′ and 3′ ends of the Urbani strain genome, the replicase gene, and the N gene preceded by the upstream 143 nt including its natural transcription-regulating sequence was cloned as a BAC under the control of the CMV promoter by following the same approach described for the generation of the full-length cDNA clone (Fig. 3A). In addition, a multiple-cloning site containing the unique restriction sites PacI, AscI, and BamHI was cloned downstream of the replicase gene to allow the cloning and expression of heterologous genes.

To study SARS-CoV replicon activity, the synthesis of N gene mRNA was analyzed in human 293T and BHK cells by RT-PCR (Fig. 3B). Cells were grown to 95% confluence on 35-mm-diameter plates and transfected with 4 μg of SARS-CoV replicon by using 12 μg of Lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications. Total intracellular RNA was extracted at 24 hpt by using the RNeasy mini kit (QIAGEN) and was used as a template for RT-PCR analysis of N gene mRNA synthesis. Amplifications were performed with the reverse primer URB-28630RS (5′-TGCTTC CCTCTGCGTAGAAGCC-3′), complementary to nucleotides 511 to 532 of the N gene, and the forward primer URB-29VS (5′-GCCAACCAACCTCGATCTCTTG-3′), spanning nucleotides 29 to 50 of the Urbani leader sequence. High levels of transcript were detected in both 293T and BHK cells transfected with the replicon (Fig. 3B), indicating that the SARS-CoV replicon was functional, at least at the transcriptional level. To confirm whether the SARS-CoV replicon was also functional at the replication level, the synthesis of genomic positive-strand RNA was analyzed on DNase I-treated samples by real-time RT-PCR using the reverse primer URB-1995RS (5′-ATGGCGTCGACAAGACGTAAT-3′), complementary to nucleotides 1995 to 2015 of the genome, and the forward

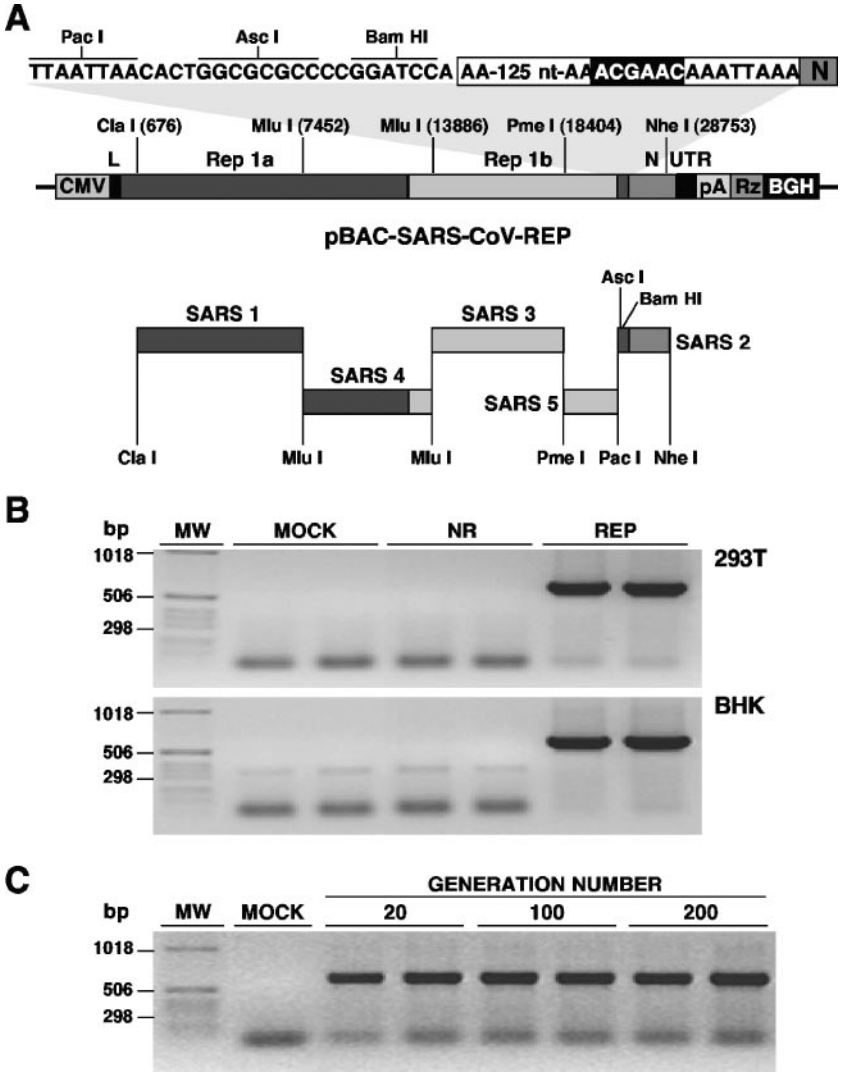

FIG. 3. Assembly and functional analysis of a SARS-CoV-derived replicon. (A) Strategy for the construction of the SARS-CoV replicon. A five-step cloning strategy in which the overlapping cDNA fragments, named SARS 1 to SARS 5, were sequentially cloned into the plasmid pBAC-SARS-CoV 5′–3′ to generate plasmid pBAC-SARS-CoV-REP was used. The genetic structure of the replicon and the positions of relevant restriction sites are illustrated. The sequences containing the multiple-cloning site (PacI, AscI, and BamHI) downstream of the replicase gene, and the N gene transcription-regulating sequence (white box) and core sequence (black box) are indicated at the top. Abbreviations are as described in the legend to Fig. 1. (B) Functional analysis of SARS-CoV replicon by RT-PCR. Human 293T and BHK cells were mock transfected or transfected with the replicon (REP) or a nonreplicative construct (NR) by using Lipofectamine 2000 (Invitrogen). Total RNA was isolated at 24 hpt and analyzed by RT-PCR with specific oligonucleotides to detect N gene mRNA. Duplicate RT-PCR products amplified in parallel were resolved by electrophoresis in 1% agarose gels. MW, molecular weight markers. (C) Stability of SARS-CoV replicon in *E. coli* cells. Plasmid pBAC-SARS-CoV-REP extracted from *E. coli* DH10B cells that were grown for the indicated number of generations was transfected in 293T cells and the replicon activity analyzed by RT-PCR as described above. Duplicate RT-PCR products amplified in parallel were resolved by electrophoresis in 1% agarose gels. MW, molecular weight markers.

primer URB-1931VS (5′-ACCACTCAATTCCTGATTTGC A-3′), spanning nucleotides 1931 to 1952 of the genome. In our system, the amount of replicon RNA in the cytoplasm of transfected cells is the sum of the RNA transcribed from the CMV promoter in the nucleus plus the amount of RNA self amplified by the viral replicase in the cytoplasm. To determine the amplification exclusively due to the viral replicase, the synthesis of genomic RNA was analyzed in cells transfected with either the wild-type replicon or a nonreplicative construct in which most of the polymerase domain has been deleted. An increase in RNA synthesis of at least 10-fold over the RNA background level due to the CMV promoter was detected in cells transfected with the wild-type replicon, indicating RNA self-amplification of the SARS-CoV replicon (data not shown).

Similar to the full-length cDNA clone, the replicon was fully stable during its propagation in bacteria for more than 200 generations, as determined by restriction endonuclease analysis. To confirm this result, the replicon activities of cDNAs purified after amplification in bacteria during 20, 100, and 200 generations were analyzed in 293T cells following the procedure described above. No differences in the replicon activities after 200 generations were detected (Fig.

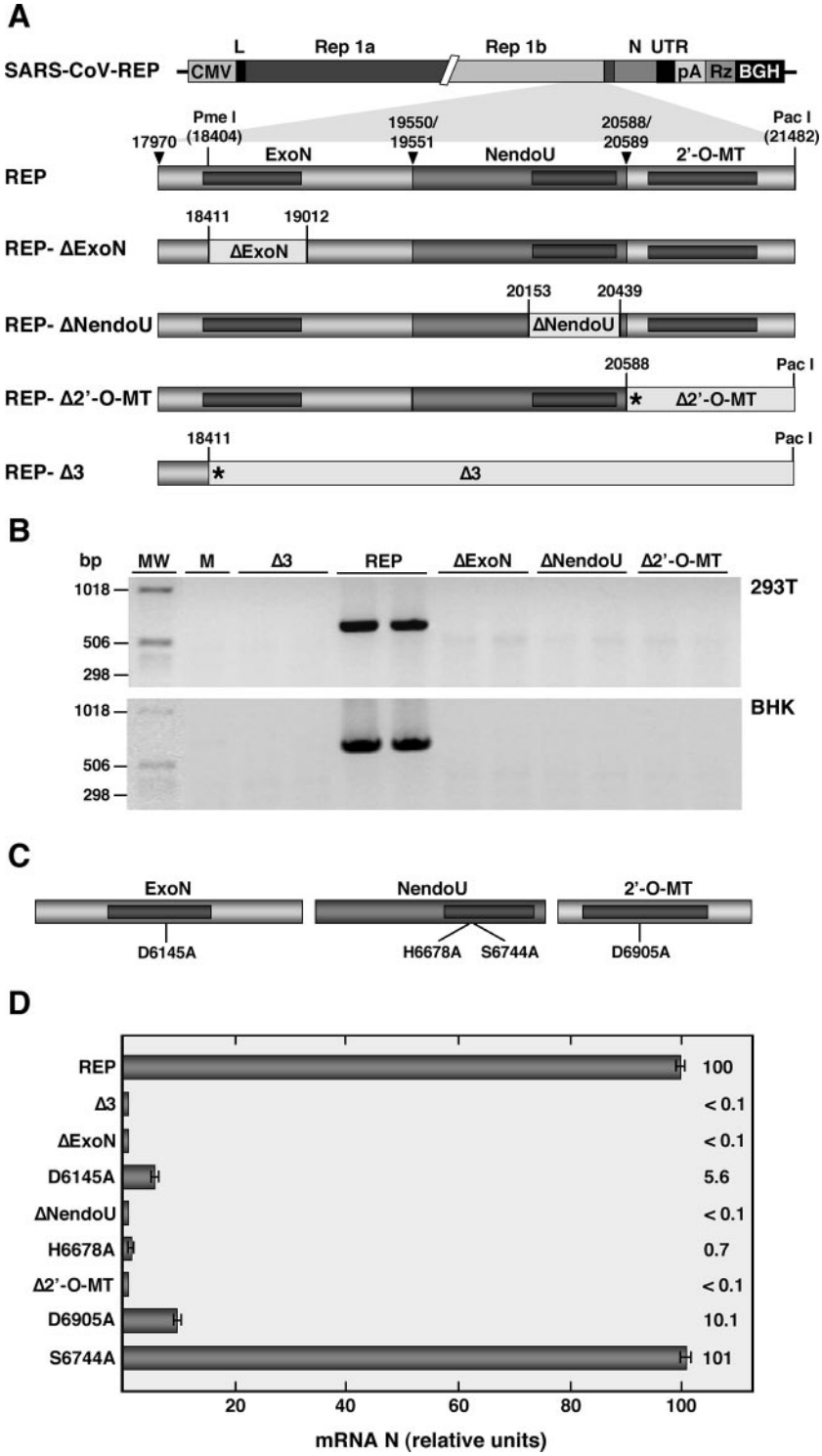

FIG. 4. Construction and functional analysis of a set of SARS-CoV replicons defective in ExoN, NendoU, and 2′-O-MT activities. (A) Schematic diagram showing the genetic structures of the deletion mutant replicons. Relevant restriction sites and the nucleotide positions flanking the deletions from the replicons are indicated. Stop codons are indicated by asterisks. The conserved domains described by Snijder and colleagues (25) are illustrated by the darker bars. Arrowheads indicate the predicted main proteinase (3CL) cleavage sites. Abbreviations above the top bar are as described in the legend to Fig. 1. (B) Functional analysis of mutant replicons by RT-PCR. Human 293T and BHK cells were mock transfected (M) or transfected with the wild-type replicon (REP), the single-deletion mutants (ΔExoN, ΔNendoU, and Δ2′-O-MT), or the deletion mutant lacking the three conserved domains (Δ3). Replicon activity was analyzed by RT-PCR with specific oligonucleotides to detect N gene mRNA. Duplicate RT-PCR products amplified in parallel were resolved by electrophoresis in 1% agarose gels. MW, molecular weight markers. (C) Schematic diagram showing the positions of the point mutations introduced into the ExoN (D6145A), NendoU (H6678A or S6744A), and 2′-O-MT (D6905A) domains. Amino acid numbers refer to their positions in the pp1ab polyprotein. (D) Functional analysis of mutant replicons by real-time RT-PCR. The amount of N gene mRNA, expressed as relative units, was determined by real-time RT-PCR in RNA samples isolated at 24 hpt from BHK cells transfected with the wild-type replicon (REP), the deletion mutant lacking the three conserved domains (Δ3), the single-deletion mutants (ΔExoN, ΔNendoU, and Δ2′-O-MT), or the point mutants (D6145A, H6678A, S6744A, and D6905A mutants). Mean values from three experiments are represented, with standard deviations shown as error bars.

3C), demonstrating that the generated replicon was fully stable in bacteria.

Generation of a panel of SARS-CoV replicons defective in ExoN, NendoU, and 2′-O-MT activities. These RNA-processing enzymes are encoded at the 3′ end of ORF 1b, downstream of the helicase domain (25). The identification of a stable processing intermediate comprising these three domains in infectious bronchitis virus-infected cells (31) suggests that their activities might cooperate in the same metabolic pathway.

To study the role of the enzymes ExoN, NendoU, and 2′-O-MT in CoV RNA synthesis, in-frame single-deletion mutants in which the conserved domain of each enzyme (25) was deleted and a mutant replicon lacking the three conserved enzymes were generated by using the SARS-CoV replicon (Fig. 4A). For the generation of these mutants, PmeI-PacI fragments with the desired in-frame-deletions were generated by PCR mutagenesis and cloned into the SARS-CoV replicon, replacing the wild-type PmeI-PacI fragment. The functionality of these constructs was analyzed in comparison with that of the wild-type replicon in 293T and BHK cells by RT-PCR as described above. Interestingly, high levels of N gene mRNA were detected only for cells transfected with the parental replicon (Fig. 4B), suggesting that the RNA-processing enzymes ExoN, NendoU, and 2′-O-MT are essential for SARS-CoV RNA synthesis. Formally, it is not possible to exclude the possibility of a lethal effect of these deletions at the level of RNA structure or pp1ab processing, even though their N- and C-terminal borders were relatively distant from the proteolytic cleavage sites. To overcome this potential limitation, three point mutants, in which putative catalytic and highly conserved residues of ExoN (Asp-6145), NendoU (His-6678), and 2′-O-MT (Asp-6905) (14, 19, 25) were replaced by Ala (D6145A, H6678A, and D6905A, respectively), were generated. In addition, since the Ser-6744 residue has been described to be nonessential for NendoU activity (14), an additional mutant containing an Ala instead of a Ser at this position (S6744A) was generated as a control to confirm that single amino acid substitutions could be tolerated in this domain of the polyprotein (Fig. 4C). The activity of these replicons was compared to that of the deletion mutants and the wild-type replicon. To this end, a quantitative analysis of N gene mRNA expression was performed by real-time RT-PCR using the reverse primer URB-28163RS (5′-TGGGTCCACCAAATGTAATGC-3′), complementary to nucleotides 44 to 64 of the N gene, and the forward primer URB-27VS (5′-AAGCCAACCAACCTCGATCTC-3′), spanning nucleotides 27 to 47 of the Urbani leader sequence. As expected, high levels of N gene mRNA were detected in cells transfected with the wild-type replicon or with the control S6744A mutant (Fig. 4D). However, a reduction of more than 90% was observed in the case of both the deletion and point mutants, confirming that the effect on CoV RNA synthesis is most probably due to the abrogation of ExoN, NendoU, and 2′-O-MT activities and not to major changes affecting the RNA structure or polyprotein processing (Fig. 4D). In addition, a 10-fold reduction in the amount of genomic positive-strand RNA was detected for all mutant replicons (data not shown), suggesting that ExoN, NendoU, and 2′-O-MT are essential for efficient CoV replication and transcription. Although it was previously reported that NendoU and ExoN were essential for CoV RNA synthesis (14, 19), this is the first evidence that 2′-O-MT activity is also essential. Further genetic and biochemical studies will be required to address the specific role of these enzymes in CoV transcription and replication.

Within the *Nidovirales* order, these three RNA-processing enzymes are highly conserved in the families *Coronaviridae* (coronavirus and torovirus) and *Roniviridae*. It has been postulated that these enzymes are involved in RNA processing, possibly in the synthesis of the 5′- and 3′-coterminal subgenomic RNAs (11, 14, 25, 36). Interestingly, only the NendoU domain is conserved in the family *Arteviridae* (20, 25), suggesting that the RNA synthesis mechanisms of coronavirus and arterivirus might present some differences at the molecular level or that the extra RNA-processing activities (ExoN and 2′-O-MT) present in coronaviruses and roniviruses may be required to synthesize and maintain the large RNA genome (~30 kb) of these virus families. In this context, the ExoN domain, which is predicted to contain a 3′→5′ exonuclease activity, may have been acquired to operate in proof-reading mechanisms to improve the fidelity of the RNA-dependent RNA polymerase.

The SARS-CoV replicon assembled as a BAC provides a useful tool for basic studies on CoV transcription and replication, for the safe screening of new antiviral drugs, and for the development of SARS-CoV vaccines.

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