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Recovery of a Neurovirulent Human Coronavirus OC43 from an Infectious cDNA Clone†

Julien R. St-Jean,[1] Marc Desforges,[1] Fernando Almazán,[2] Hélène Jacomy,[1] Luis Enjuanes,[2] and Pierre J. Talbot[1]*

Laboratory of Neuroimmunovirology, INRS-Institut Armand-Frappier, 531 Boulevard des Prairies, Laval, Québec H7V 1B7, Canada,[1] and Department of Molecular and Cell Biology, Centro Nacional de Biotecnología, CSIC, Campus Universidad Autónoma, Darwin 3, 28049 Madrid, Spain[2]

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This study describes the assembly of a full-length cDNA clone of human coronavirus (HCoV)-OC43 in a bacterial artificial chromosome (BAC). The BAC containing the full-length infectious cDNA (pBAC-OC43^{FL}) was assembled using a two-part strategy. The first step consisted in the introduction of each end of the viral genome into the BAC with accessory sequences allowing proper transcription. The second step consisted in the insertion of the whole HCoV-OC43 cDNA genome into the BAC. To produce recombinant viral particles, pBAC-OC43^{FL} was transfected into BHK-21 cells. Recombinant virus displayed the same phenotypic properties as the wild-type virus, including infectious virus titers produced in cell culture and neurovirulence in mice.

Human coronaviruses (HCoVs) are positive-stranded enveloped RNA viruses that are mainly associated with respiratory infections and are responsible for up to one-third of common colds (10) and possibly neurologic disease (3, 8). Like SARS-CoV, which was identified as the causative agent of severe acute respiratory syndrome (SARS) (11), HCoV-OC43 (ATCC VR-759 from the mid 1960s; GenBank accession number AY585228) (17) belongs to the second genetic group of the *Coronaviridae* (7, 14).

Several coronavirus infectious clones have been assembled, although this has proven difficult given the length of the viral genome (9), as well as the presence of unstable viral cDNA regions in bacteria (2, 6, 21). Uncommon strategies were therefore necessary to circumvent technical limitations engendered by the viral genome. Among the strategies available for the construction (2, 4, 5, 18, 19, 20, 21), the bacterial artificial chromosome (BAC) approach was selected given the availability of the technology, as well as the number of possibilities it offers for downstream applications (1, 2, 15, 16, 22). This approach uses a two-step amplification system that couples viral RNA expression in the nucleus from the cytomegalovirus (CMV) immediate-early promoter, with a second amplification step in the cytoplasm driven by the viral polymerase.

Construction of the full-length HCoV-OC43 cDNA clone. Prior to introducing the full-length viral cDNA into the pBeloBAC11 (13), pBAC-OC43-5′-3′ was first assembled. This plasmid includes the first 720 nucleotides (nt) of the genome under the control of the CMV promoter, and the last 1,651 nt of the viral RNA, followed by a 25-bp synthetic poly(A) tail, the hepatitis delta virus ribozyme (HDV) and the bovine growth hormone termination and polyadenylation sequences (BGH) to lead to an accurate 3′ end. In order to join the CMV promoter with the viral 5′ end and the viral 3′ end with the HDV-BGH sequences in a contiguous and very precise way, overlapping PCRs were performed.

Primers used to perform the overlapping PCRs are listed in Table 1. The vector pBAC-TGEV-5′-3′, which contains the accessory sequences CMV, HDV, and BGH, was used as a template to amplify these latter sequences. After two rounds of amplification, an overlapping PCR was performed for each end of the viral genome (Fig. 1A). Both overlapping PCR amplicons were directly introduced into the pBeloBAC11 plasmid in order to create the pBAC-OC43-5′-3′ (Fig. 1B). After each cloning step, the new BAC vector was sequenced to assess that no undesired mutations were introduced into the new construct.

Before performing the assembly of the full-length cDNA clone, 5′ rapid amplification of cDNA ends was carried out with RNA extracted from BHK-21 cells transfected with pBAC-OC43-5′-3′ in order to check that the transcription machinery was functional (data not shown). The HDV cleavage efficiency was also determined by using a reverse transcription-PCR approach and, as expected, it was estimated at approximately 60% (data not shown) (12).

The HCoV-OC43 infectious cDNA clone was assembled using a five-step cloning approach, starting with the pBAC-OC43-5′-3′ as a backbone. Given the low availability of unique restriction sites in the HCoV-OC43 genome, three additional restriction sites (MluI, AsiSI, and SacII) were introduced into the sequence of the cDNA clone. This modification required the introduction of six silent point mutations (T6479C, T6482G, C12143T, T12146C, A24068G, and T24071G). These sequence changes were used as genetic markers to identify the recombinant virus (2, 19). For cDNA assembly, we took advantage of the presence of the unique SfoI restriction site in both the BAC vector and the HCoV-OC43 genome (Fig. 1B and 2A).

The genome of HCoV-OC43 was therefore amplified in five distinct overlapping regions, named A to E, which spanned the entire viral genome, except for the 5′ and 3′ ends (Fig. 2A). Each

* Corresponding author. Mailing address: Laboratory of Neuroimmunovirology, INRS-Institut Armand-Frappier, 531 Boulevard des Prairies, Laval H7V 1B7, Québec, Canada. Phone: (450) 686-5515. Fax: (450) 686-5566. E-mail: pierre.talbot@iaf.inrs.ca.

† Supplemental material for this article may be found at http://jvi.asm.org/.

TABLE 1. Primers used for the construction of the HCoV-OC43 cDNA clone

Primer pairs and location	Sequence (5′→3′)
Primers for pBAC-OC43-5′-3′	
CMV5′ (7716–7736 of pBAC-TGEV-5′-3′)[a,d]	**GCGGAATTCGTGCAC**TTGACATTGATTATTGACTAG[b]
CMV3′ (8297–8316 of pBAC-TGEV-5′-3′)	**GCACGCAAATCGCTCACAAT**ACGGTTCACTAAACGAGCTC
OC43L-5′ (1–41 of the OC43 genome)	ATTGTGAGCGATTTGCGTGCGTGCATCCCGCTTCACTGATC
OC43L-3′ (699–719 of the OC43 genome)[d]	**GCGGAATTCGTGCAC**CCCAACCCACGAATTGACCTG
OC43R-5′ (29064–29083 of the OC43 genome)[d]	**GCGCAAGCTT**ATCTAAATTTTAAGGATGTC
OC43R-3′ (30683–30713 of the OC43 genome)	GTGATTCTTCCAATTGGCCATAATTAACTTC
HDV5′ (30697–30746 of the OC43 genome and 217–227 of pBAC-TGEV-5′-3′, respectively)	CCAATTGGAAGAATCACAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA*GGGTCGGCATG*
BGH3′ (506–525 of pBAC-TGEV-5′-3′)[d]	**GCGCAAGCTTG**CTCTCCCCAGCATGCCTGC
Primers for pBAC-OC43^{FL}	
JUB1 (28–47 of the OC43 genome)	CCCGCTTCACTGATCTCTTG
JUBMlu reverse (6469–6491 of the OC43 genome)	**TCGTCCGGCGCC**TCTGCTCAAC<u>G</u>C<u>G</u>TTAGCAGTTC[c]
JUBMlu forward (6469–6492 of the OC43 genome)	GAACTGCTAAC<u>G</u>C<u>G</u>TTGAGCAGAG
JUBAslSI reverse (12127–12154 of the OC43 genome)	**AGGTTGGGCGC**CGCTACAGC<u>G</u>CG<u>A</u>TCGCGTTCATAAGCAG
JUBAslSI forward (12127–12154 of the OC43 genome)	CTGCTTATGAACGCGA<u>T</u>CG<u>C</u>GCTGTAGC
JUB105 (18973–18993 of the OC43 genome)	TACAAAAGAGTCTTAACAGAC
JUB94 (18562–18582 of the OC43 genome)	TAGAACTGGTTACTATGGTTG
JUBSac2 reverse (24056–24082 of the OC43 genome)	GAATTGATTGT<u>C</u>CG<u>C</u>GGTTGTACTACC
JUBSac2 forward (24058–24082 of the OC43 genome)	**TAAGGTGGCGCC**TAGTACAACC<u>G</u>CG<u>G</u>ACAATCAATTC
JUO2 (30495–30514 of the OC43 genome)	GCAGCAAGACATCCATTCTG

[a] The sequence of pBAC-TGEV-5′-3′ was provided by Luis Enjuanes.
[b] Nucleotides in boldface contain restriction sites and are not included in the primer location.
[c] Underlined nucleotides indicate mutated bases with regard to the genome sequence of the HCoV-OC43 ATCC strain (GenBank accession number AY585228).
[d] External primer used for the generation of overlapping PCRs.

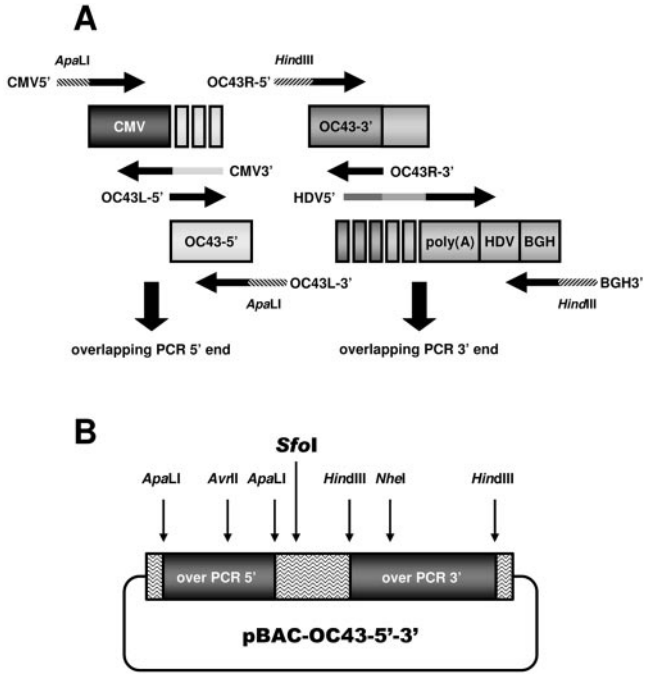

FIG. 1. Assembly of the pBAC-OC43-5′-3′. (A) Schematic representation of the overlapping PCR method, used to juxtapose each viral end to accessory sequences. The viral 5′ end was joined to the CMV promoter in order to generate the overlapping PCR 5′ end, whereas the viral 3′ end was joined to the HDV-BGH sequences to generate the overlapping PCR 3′ end. In the first PCR round, two products were generated for each end using specific primers. In the second round of amplification, the overlapping extension, CMV and OC43-5′ PCR products were pooled and fused since they slightly overlap each other, generating overlapping PCR 5′ products. The same method was used for the generation of overlapping PCR 3′. The primers used for the generation of the final products were the external ones (Table 1), which contained restriction sites for subsequent cloning into the BAC vector. (B) Schematic representation of pBAC-OC43-5′-3′. The overlapping PCR 5′ end was introduced in the ApaLI restriction site, whereas the overlapping PCR 3′ end was introduced using the HindIII site. The vector contains the central SfoI restriction site, as well as the NheI and AvrII sites, which were used to start the assembly with regions A and B, respectively. Supplemental material is available at http://jvi.asm.org.

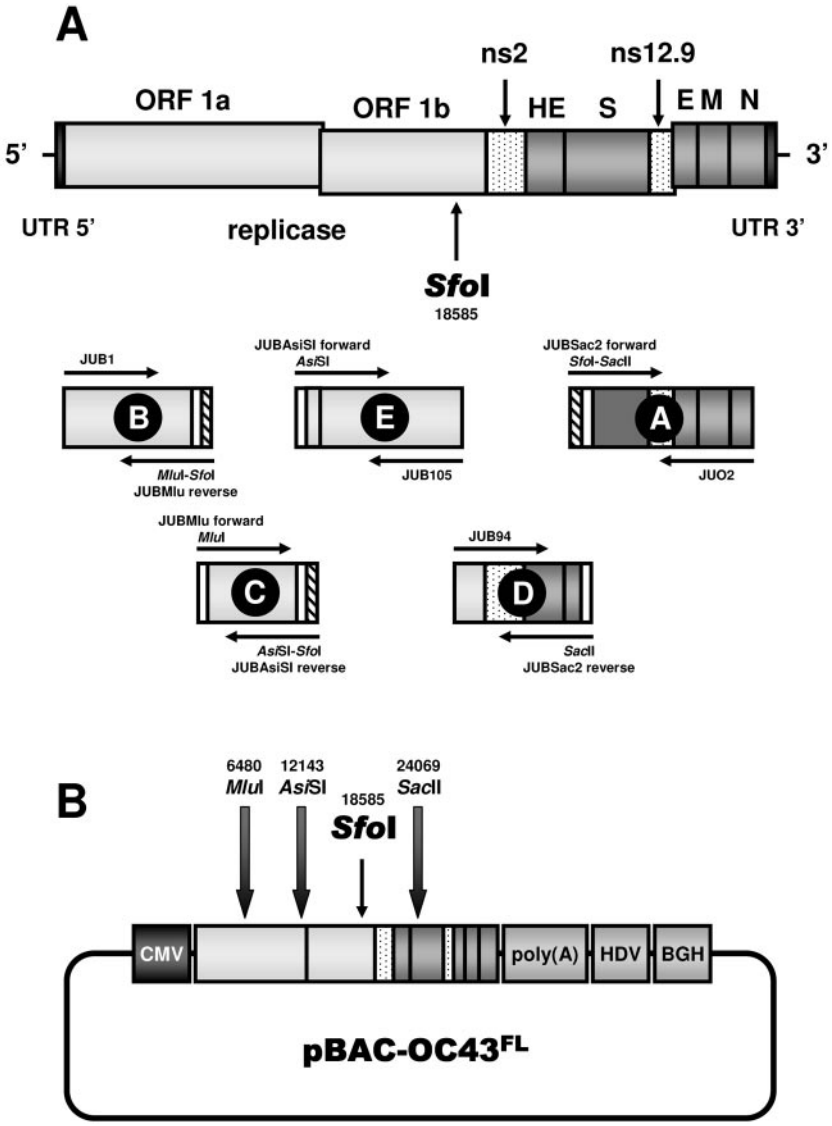

FIG. 2. Assembly of the pBAC-OC43^{FL}. (A) Schematic representation of the five-step cloning strategy used for the construction of pBAC-OC43^{FL}. Primers used for the amplification of regions A to E are shown (Table 1). Regions A, B, and C allowed the introduction of the SacII, MluI, and AsiSI restriction sites, respectively. These same regions also contained a mutagenized SfoI site (▨), whereas regions D and E contained the original SfoI site, located at position 18585 of the viral genome. Other specific restriction sites, introduced by site-directed mutagenesis using primers JUBSac2 forward and reverse, JUBMlu forward and reverse and JUBAsiSI forward and reverse, are shown in white. (B) Schematic representation of the full-length infectious cDNA clone. Restrictions sites introduced by mutagenesis are represented by large arrows. The SfoI restriction site is also indicated by a small arrow. ORF, open reading frame; ns, accessory genes encoding nonstructural proteins; HE, hemagglutinin-esterase protein gene; S, spike protein gene; E, envelope protein gene; M, membrane protein gene; N, nucleocapsid protein gene; UTR, untranslated region; CMV, cytomegalovirus promoter; HDV, hepatitis delta virus ribozyme; BGH, bovine growth hormone termination and polyadenylation sequences. Supplemental material is available at http://jvi.asm.org.

region was generated in such a way that it contained a given cloning site at one end and a SfoI site at the other end. Primers used for the generation of region A to E are described in Table 1. Each amplicon was first inserted into the pSMART HCKan vector in order to generate plasmids pSMART-A to -E (Fig. 2A) and positive clones with the appropriate sequence were obtained.

Viral DNA regions contained in clones pSMART-A to -E were used to fill the pBAC-OC43-5′-3′ and to generate the pBAC-OC43^{FL} (Fig. 2B). Regions A to E were respectively introduced into the BAC vector using the restriction sites they were sharing with the construct (Fig. 2A). Region E, which contained the disrupted unstable segment of ORF1a, was introduced at the last cloning step to minimize the toxicity problem usually observed with this region of the genome (6, 21). Junction sites created for each new construct were sequenced to make sure no unexpected mutations were introduced into the genome.

Recovery, replication, and neurovirulence of recombinant HCoV-OC43. One of the major advantage provided by the BAC system is that the recovery of recombinant viral particles is very efficient and simple. This was especially true for pBAC-OC43^{FL} given that the cell line BHK-21 provided a good transfection rate and was also susceptible to infection by HCoV-

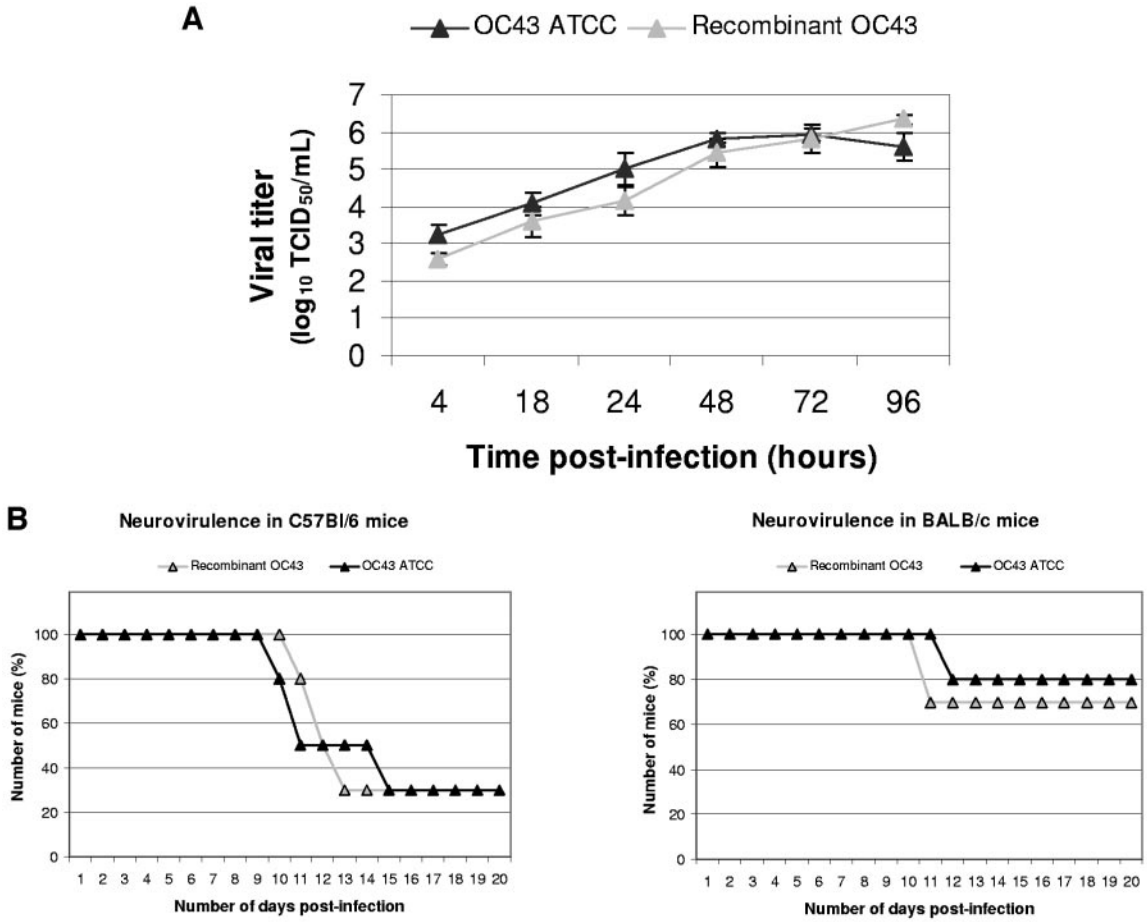

FIG. 3. In vitro and in vivo properties of the recombinant virus. (A) Viral growth kinetics of both the HCoV-OC43 ATCC strain and the recombinant HCoV-OC43 virus. Culture supernatants from HRT-18-infected cells were harvested at the indicated times postinfection. Infectious titers were determined using the immunoperoxidase assay and are expressed in log $TCID_{50}$/ml. Both viruses displayed similar patterns of replication. (B) Neurovirulence of the recombinant HCoV-OC43 in mice. Two different strains of MHV-seronegative female mice, BALB/c and C57BL/6, were inoculated with the same dose of virus.

OC43. For rescue of recombinant virus, 5 μg of pBAC-OC43^{FL} were directly transfected into BHK-21 cells using Lipofectamine 2000 (Invitrogen). Virus titers obtained for the recombinant virus reached $10^{6.5}$ 50% tissue culture infective doses ($TCID_{50}$)/ml, which are comparable to those obtained after infection with the HCoV-OC43 laboratory strain. To make sure rescued viruses were indeed recombinant particles, reverse transcription-PCR were performed and the presence of the newly introduced restriction sites, which are exclusive to the recombinant virus, was assessed (data not shown).

To test whether the recombinant virus displayed the same growth kinetics as the parental strain in HRT-18 cells, replication was assessed at different times postinfection in a multicycle growth curve. Wild-type and recombinant virus shared very similar overall patterns of replication, reaching virus titers of approximately $10^{6.0}$ $TCID_{50}$/ml after 4 days of infection, although the replication of the recombinant virus was slightly delayed during the first 2 days (Fig. 3A). As expected and like the wild-type HCoV-OC43 strain, recombinant virus did not form any plaques (data not shown). Taken together, these data confirm that the recombinant virus shares phenotypic properties with the wild type HCoV-OC43 strain in cell culture.

To determine whether the HCoV-OC43 strain, as well as the recombinant virus, displayed the same phenotype in vivo, their neurovirulence was assessed in mice. C57BL/6 and BALB/c mice were infected intracerebrally with either virus (8). In C57BL/6 mice, a survival rate of 30% was achieved for both viruses, whereas 70 and 80% of BALB/c mice survived the infection with the recombinant virus and the parent strain, respectively (Fig. 3B). Both recombinant and wild-type virus displayed very similar levels of virulence following injection into the brain. Furthermore, both viruses showed very similar patterns of replication in mouse brain (data not shown).

Overall, the virus recovered from the infectious cDNA clone exhibited the same genotypic and phenotypic properties as the HCoV-OC43 from ATCC, both in vitro and in vivo.

The cDNA infectious clones represent very interesting tools for the study of potential vaccine candidates, like genetically attenuated viruses, but also allow multiple possibilities for the characterization of any viral functions and mechanisms by re-

verse genetics. The pBAC-OC43^{FL} will be invaluable in the characterization of the adaptation of the HCoV-OC43 virus in a neural environment and will provide an opportunity to better understand the biology of the HCoV-OC43, as well as other related coronaviruses, such as SARS-CoV.

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