

Engineering a Replication-Competent, Propagation-Defective Middle East Respiratory Syndrome Coronavirus as a Vaccine Candidate

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ABSTRACT Middle East respiratory syndrome coronavirus (MERS-CoV) is an emerging coronavirus infecting humans that is associated with acute pneumonia, occasional renal failure, and a high mortality rate and is considered a threat to public health. The construction of a full-length infectious cDNA clone of the MERS-CoV genome in a bacterial artificial chromosome is reported here, providing a reverse genetics system to study the molecular biology of the virus and to develop attenuated viruses as vaccine candidates. Following transfection with the cDNA clone, infectious virus was rescued in both Vero A66 and Huh-7 cells. Recombinant MERS-CoVs (rMERS-CoVs) lacking the accessory genes 3, 4a, 4b, and 5 were successfully rescued from cDNA clones with these genes deleted. The mutant viruses presented growth kinetics similar to those of the wild-type virus, indicating that accessory genes were not essential for MERS-CoV replication in cell cultures. In contrast, an engineered mutant virus lacking the structural E protein (rMERS-CoV- Δ E) was not successfully rescued, since viral infectivity was lost at early passages. Interestingly, the rMERS-CoV- Δ E genome replicated after cDNA clone was transfected into cells. The infectious virus was rescued and propagated in cells expressing the E protein *in trans*, indicating that this virus was replication competent and propagation defective. Therefore, the rMERS-CoV- Δ E mutant virus is potentially a safe and promising vaccine candidate to prevent MERS-CoV infection.

IMPORTANCE Since the emergence of MERS-CoV in the Arabian Peninsula during the summer of 2012, it has already spread to 10 different countries, infecting around 94 persons and showing a mortality rate higher than 50%. This article describes the development of the first reverse genetics system for MERS-CoV, based on the construction of an infectious cDNA clone inserted into a bacterial artificial chromosome. Using this system, a collection of rMERS-CoV deletion mutants has been generated. Interestingly, one of the mutants with the E gene deleted was a replication-competent, propagation-defective virus that could only be grown in the laboratory by providing E protein *in trans*, whereas it would only survive a single virus infection cycle *in vivo*. This virus constitutes a vaccine candidate that may represent a balance between safety and efficacy for the induction of mucosal immunity, which is needed to prevent MERS-CoV infection.

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A new coronavirus (CoV) emerged during the summer of 2012 in Saudi Arabia (1–3). This virus has been named the Middle East respiratory syndrome coronavirus (MERS-CoV) by the International Coronavirus Study Group of the International Committee on Taxonomy of Viruses (ICTV) (4). Based on its genome sequence, the virus has been classified within lineage C of the genus *Betacoronavirus*. MERS-CoV is closely related to *Tylonycteris* bat CoV HKU4 and *Pipistrellus* bat CoV HKU5, the two prototype species in lineage C *Betacoronavirus*. MERS-CoV is even closer in sequence to two CoVs found in bats circulating in the Netherlands and Spain (5–7). Therefore, reemergence of the virus in these and other European and Middle Eastern countries is a realistic possibility. Using a nonhuman primate disease model

(rhesus macaques), it has been shown that Koch's postulates can be fulfilled (8).

MERS-CoV has been disseminated into 10 countries of the Middle East, North Africa and Europe. The virus may have crossed from bats to a domesticated or agricultural animal species and subsequently spread from there into humans. To date, the identities of both reservoir and intermediate hosts remain unknown, complicating virus control efforts. Human-to-human transmission has been documented in at least four independent hospital settings (9–12). As of 7 August 2013, a total of 94 cases have been confirmed, of which around 50% were fatal (<http://www.cdc.gov/coronavirus/mers/index.html>). This might be an overestimation of the case fatality risk, as many infected patients

likely have not required hospital assistance. In fact, recent data suggest that mild respiratory illness might also be part of the clinical spectrum of MERS-CoV infection (3). In addition to mild or acute respiratory illness, other reported clinical symptoms are abdominal pain and diarrhea, fever, and in some cases, renal failure (9). Many hospitalized cases occurred in persons with chronic underlying medical conditions or immunosuppression (3, 13). The virus loads are highest in lower respiratory tract samples, although low concentrations of viral RNA can also be found in stool, urine, and blood samples (12).

The genome of MERS-CoV includes more than 30,100 nucleotides and contains at least 10 predicted open reading frames (ORF1a, ORF1b, S, 3, 4a, 4b, 5, E, M, and N), 9 of which seem to be expressed from a nested set of eight mRNAs (14, 15). Interestingly, the partial genome sequences of three independent MERS-CoV isolates reveal that it evolved following a strict molecular clock model (6).

A functional receptor of MERS-CoV is dipeptidyl peptidase 4 (DPP-4) from both human and bat (16). This receptor binds to a 231-residue region in the spike (S) protein of MERS-CoV (17, 18), a domain different from the receptor-binding site of other *Beta-coronaviruses* (18). Infection of human airways by MERS-CoV prevents the induction of interferon-regulating factor 3 (IRF-3)-mediated antiviral alpha/beta interferon (IFN- α/β) responses. However, MERS-CoV was markedly more sensitive to the antiviral state established by ectopic IFN than severe acute respiratory syndrome CoV (SARS-CoV) (14, 19, 20).

Soon after MERS-CoV emergence, a diagnostic assay was designed (21). Similarly, antivirals inhibiting virus replication, such as cyclosporine A, IFN- α , or ribavirin, have been described (14, 22, 23). In contrast, reliable vaccines have not yet been developed, although the S protein and the receptor-binding site within this protein induce neutralizing antibodies and, in principle, could serve as a subunit vaccine (17). CoVs infect respiratory and enteric mucosal areas, and thus, induction of mucosal immunity is necessary to protect these tissues from infection. Live attenuated viruses are expected to elicit mucosal immunity more efficiently than nonreplicating antigens, which elicit reduced secretory immune responses. Live attenuated viruses can be generated by the deletion of genes conferring virulence, a procedure that requires the availability of a reverse genetics system for MERS-CoV. In this article, we describe the construction of an infectious cDNA clone of MERS-CoV using a bacterial artificial chromosome (BAC). Using this clone, recombinant MERS-CoV (rMERS-CoV) deletion mutants were constructed lacking genes nonessential for virus replication. In addition, we deleted the structural envelope (E) protein gene, because in previous work from our laboratory, deletion of the E gene in two other CoVs led to mutants that were either replication-competent, propagation-defective viruses or attenuated viruses (24–26). All deletion mutants efficiently replicated and spread in cell cultures except the one in which the E gene was deleted, which was replication competent but propagation defective. This virus was propagated in cells by providing E protein in *trans*. Therefore, this deletion mutant missing the E gene can serve as the basis for a safe vaccine candidate.

RESULTS

Construction of a MERS-CoV infectious cDNA clone as a BAC.

An infectious cDNA clone was assembled as a BAC under the control of the cytomegalovirus (CMV) immediate-early pro-

motor, based on the genome sequence of the MERS-CoV-EMC12 strain (GenBank accession number JX869059) (15). To this end, the same approach described for the generation of other CoV infectious cDNA clones (27–30) was used. This system allows the efficient intracellular production of viral RNA from the cDNA clone without the need for *in vitro* ligation and transcription steps.

The BAC clone carrying the MERS-CoV infectious cDNA was generated in several steps (Fig. 1). After selection of appropriate restriction sites in the viral genome (Fig. 1A), the intermediate plasmid pBAC-MERS-5'3' (Fig. 1B) was generated as the backbone to assemble the full-length cDNA clone. This plasmid contained the first 811 nucleotides of the viral genome fused to the CMV promoter, a multicloning site containing the restriction sites selected in the first step (BamHI, StuI, SmaI, and PacI), and the last 4,272 nucleotides of the genome, followed by a 25-nucleotide (nt) poly(A) stretch, the hepatitis delta virus (HDV) ribozyme, and the bovine growth hormone (BGH) termination and polyadenylation sequences. Finally, the full-length MERS-CoV infectious cDNA clone (pBAC-MERS^{FL}) was assembled by sequential cloning of four chemically synthesized overlapping DNA fragments (MERS-1 to MERS-4) into the plasmid pBAC-MERS-5'3' (Fig. 1C). The full-length clone sequence was identical to that reported for the MERS-CoV-EMC12 strain (15), with the exception of a silent point mutation (T to C) introduced in the cDNA at position 20,761 (Fig. 1C). This mutation, which eliminates an additional SmaI restriction site at position 20,760, was introduced to facilitate the cloning process and was used as a genetic marker to identify the virus recovered from the cDNA clone.

The assembled infectious cDNA clone was stable during its propagation in *Escherichia coli* DH10B cells for more than 200 generations, as determined by restriction endonuclease analysis (data not shown).

Rescue of infectious rMERS-CoV from the cDNA clone in Vero A66 and Huh-7 cells. Infectious viruses were recovered from the full-length cDNA clone, using susceptible Vero A66 and Huh-7 cells, with titers of around 10^6 PFU/ml at 72 h posttransfection (h.p.t.). The recovered viruses were cloned by three rounds of plaque purification, and their phenotypic and genotypic properties were determined. Viruses rescued from both cell lines (rMERS-CoV) induced a clear cytopathic effect (CPE), characterized by the induction of cell fusion, which was more apparent in Huh-7 cells (Fig. 2). In addition, the replication of the rMERS-CoV was confirmed by indirect immunofluorescence microscopy using a nucleocapsid (N) protein-specific antibody, showing a cytoplasmic staining pattern in both cell lines (Fig. 2).

To further confirm the identity of the rMERS-CoV, the full-length genome sequences of two independent clones rescued in Vero A66 and Huh-7 cells were analyzed. The recombinant viruses rescued in Huh-7 cells presented the same sequence as the cDNA clone, including the genetic marker at position 20,761. However, in the case of the viruses rescued in Vero A66 cells, several changes in the region of the accessory genes were detected in both clones. One of them presented a deletion of 179 nucleotides (from nucleotide 26,721 to 26,900) that disrupted gene 4b and eliminated the transcription-regulating sequence (TRS) and the first 20 amino acids of gene 5. The second clone presented a 1-base insertion at position 27,143 that changed the reading frame and promoted the expression of a truncated gene 5. Taking these data into consideration, two new virus clones rescued in Vero A66 cells were sequenced, and only one of them presented the wild-

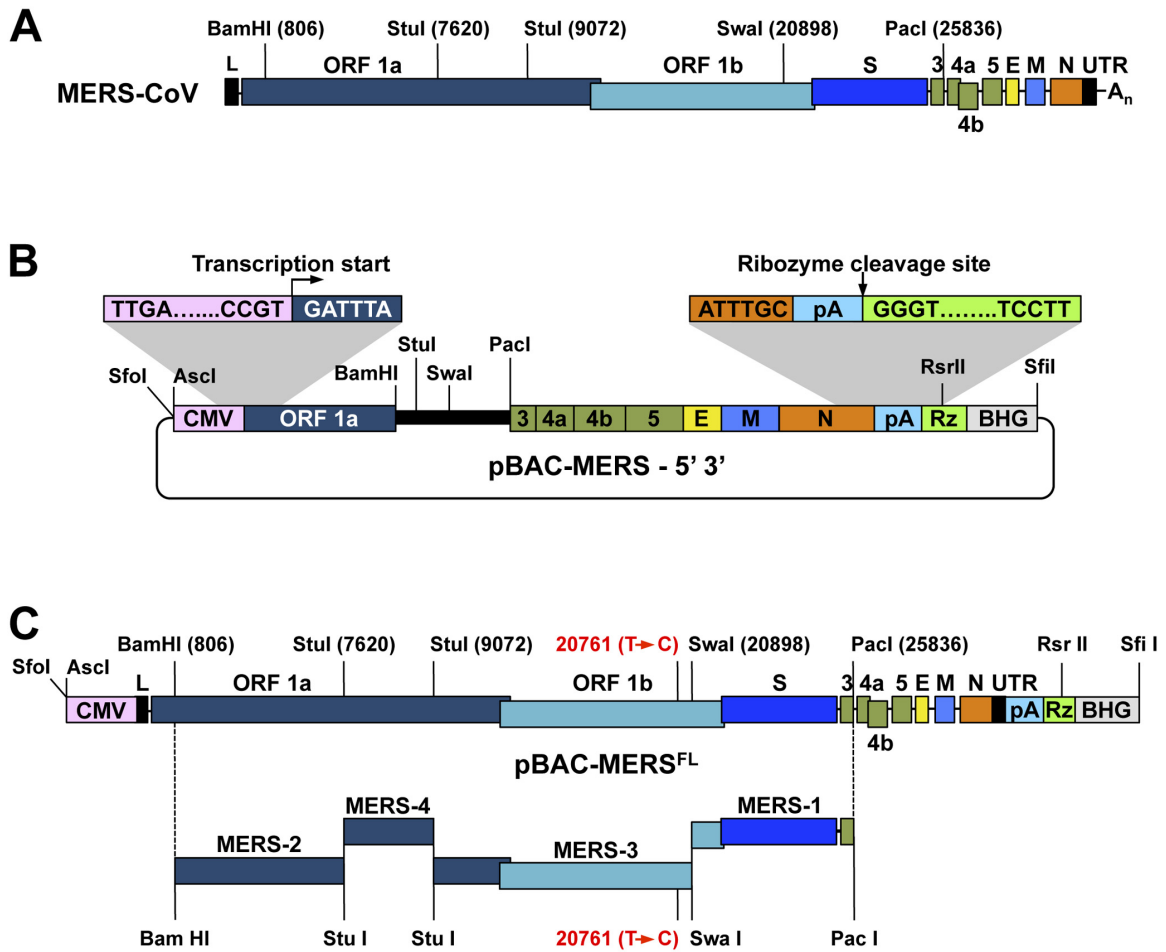


FIG 1 Assembly of a MERS-CoV full-length cDNA clone as a BAC. (A) Genome organization of the MERS-CoV-EMC12 strain. Viral genes (ORF 1a, ORF 1b, S, 3, 4a, 4b, 5, E, M, and N) are illustrated by boxes in this genome scheme. The relevant restriction sites used for the assembly of the infectious cDNA clone and their genomic positions (first nucleotide of the recognition sequence) are indicated. L, leader sequence; UTR, untranslated region; A_n, poly(A) tail. (B) Schematic representation of pBAC-MERS-5'3'. Relevant restriction sites, the CMV transcription start, the HDV ribozyme (Rz), and the BGH termination and polyadenylation sequences (BGH) are shown. (C) Strategy to assemble the MERS-CoV infectious cDNA clone. Four overlapping DNA fragments (MERS-1 to MERS-4), generated by chemical synthesis, were sequentially cloned into the plasmid pBAC-MERS-5'3' to generate the MERS-CoV infectious cDNA clone (pBAC-MERS^{FL}). Relevant restriction sites and the genetic marker (T to C) introduced at position 20,761 to abrogate the SwaI restriction site at position 20,760 are indicated. Acronyms for viral genes and regulatory elements are as described for panels A and B.

type sequence, suggesting that the MERS-CoV was more stable in Huh-7 cells. Therefore, Huh-7 cells were selected for further work.

Rescue of infectious rMERS-CoVs lacking accessory genes 3, 4a and 4b, and 5. The availability of the pBAC-MERS^{FL} infectious clone opened the door to investigate the importance of accessory genes 3, 4a, 4b, and 5 for MERS-CoV replication. To this end, cDNA clones with genes 3 (pBAC-MERS-Δ3), 4a and 4b (pBAC-MERS-Δ4ab), or 5 (pBAC-MERS-Δ5) deleted were constructed from pBAC-MERS^{FL} (Fig. 3A). The expression of gene 3 was abrogated by deletion of its TRS and coding sequence, with the exception of the 3' last 41 nucleotides, containing the 4a TRS, in order to preserve the expression of gene 4a. In the case of genes 4a and 4b, the majority of both coding sequences was deleted, except for the last 81 nucleotides of gene 4b, which overlap the gene 5 TRS. Finally, the expression of gene 5 was avoided by deletion of its TRS and complete coding sequence.

Infectious viruses were recovered in Huh-7 cells from plasmids pBAC-MERS-Δ3, pBAC-MERS-Δ4ab, and pBAC-MERS-Δ5 with

virus titers similar to that of the parental rMERS-CoV (around 10⁶ PFU/ml). After one passage on fresh cell monolayers, the recombinant viruses were cloned by three plaque isolation steps and their genetic structure was confirmed by sequencing. All the deletion mutant viruses (rMERS-CoV-Δ3, rMERS-CoV-Δ4ab, and rMERS-CoV-Δ5) were identical to the parental virus (rMERS-CoV) in terms of CPE and plaque morphology (data not shown). The growth kinetics of these viruses were also similar, reaching maximum virus titers at 72 h postinfection (h.p.i.) (Fig. 3B). In the case of rMERS-CoV-Δ4ab, the viral titer was around 10-fold lower than that obtained from the parental virus (Fig. 3B). These data indicated that the proteins encoded by genes 3, 4a, 4b, and 5 were not essential for MERS-CoV replication in cell cultures.

Generation of a rMERS-CoV mutant lacking the structural E protein gene. Based on published data showing that the deletion of CoV E protein resulted in either replication-competent, propagation-defective viruses (24) or attenuated viruses (25, 26, 31), a cDNA clone with the E gene deleted (pBAC-MERS-ΔE) was

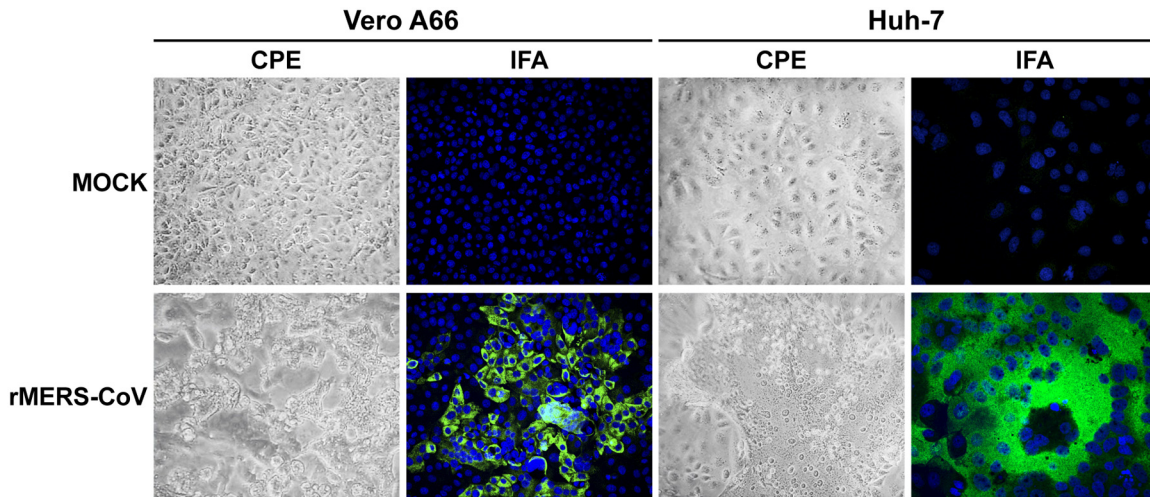


FIG 2 Identification of the virus recovered from the cDNA clone. Vero A66 and Huh-7 cells were mock infected or infected with the rMERS-CoV rescued in these cell lines at an MOI of 0.001 PFU/cell. The induction of syncytium formation (CPE) and N protein expression were analyzed 48 h.p.i. by light microscopy and indirect immunofluorescence assay (IFA), respectively. Pictures were taken with a 40 \times objective.

constructed from pBAC-MERS^{FL}. The expression of the E gene was abrogated by the deletion of its TRS and coding sequence, with the exception of the 3' last 49 nucleotides, in order to preserve the expression of gene M (Fig. 4A). To recover infectious virus, BHK cells were transfected with pBAC-MERS- Δ E or the full-length cDNA clone pBAC-MERS^{FL}. Six h.p.t. the transfected cells were overlaid on Vero A66 cell monolayers, and at 72 h.p.t., the supernatants were harvested and serially passaged three times on fresh Huh-7 cells. Infectious rMERS-CoV was recovered with titers of around 10^6 PFU/ml, whereas visible plaques were not detected for rMERS-CoV- Δ E virus throughout these passages (data not shown). Since CPE was observed at passages 0 and 1, the cell supernatants from the different passages were titrated in Huh-7 cells by limiting dilution. In contrast to the wild-type virus, which was recovered with high titers (around 1×10^6 50% tissue culture infection dose [TCID₅₀]/ml), the rMERS-CoV- Δ E was detected only at passage 0 with apparent low titers (around 2×10^3 TCID₅₀/ml) (Fig. 5A). This apparent low titer was most probably due to the transfer of detached cells transfected with the pBAC-MERS- Δ E. These cells were taken with the supernatant used to infect the next cell monolayer and formed syncytia with the non-transfected cells, giving the impression of virus production. In fact, rMERS-CoV- Δ E virus was lost at subsequent passages in several independent experiments performed in two different cell lines, Vero A66 and Huh-7 cells (data not shown). The presence of viral proteins was analyzed by immunofluorescence microscopy in Huh-7 cells infected with either rMERS-CoV or rMERS-CoV- Δ E from passage 0. As expected, E protein was detected in cells infected with rMERS-CoV but not in those infected with rMERS-CoV- Δ E (Fig. 4B). Viral N protein was detected in the cytoplasm of both rMERS-CoV- and rMERS-CoV- Δ E-infected cells (Fig. 4B). Interestingly, whereas the N protein was detected all over the cell monolayer in rMERS-CoV-infected cells, it was only detected in small syncytia in cells infected with rMERS-CoV- Δ E. Altogether, these data suggested that E protein was required for efficient virus propagation.

Complementation of rMERS-CoV- Δ E in cells expressing E protein in *trans*. Previous reports from our laboratory showed

that deletion of the transmissible gastroenteritis coronavirus (TGEV) E gene leads to a propagation-defective virus that can only spread from cell to cell by expression of the E protein in *trans* (24, 32). To analyze whether rMERS-CoV- Δ E could also be complemented in cells transiently expressing E protein, the rescue of rMERS-CoV- Δ E and of rMERS-CoV as a control was analyzed in Huh-7 cells that did not express E protein (E⁻) and in cells transiently expressing the E protein (E⁺). The transfection efficiencies in E⁺ cells varied between 40 and 50% in each independent experiment. Infectious rMERS-CoV was rescued from both E⁺ and E⁻ cells with virus titers of around 4×10^5 TCID₅₀/ml and 1×10^6 TCID₅₀/ml, respectively (Fig. 5A). In contrast, rMERS-CoV- Δ E was rescued in E⁺ cells with titers of around 1×10^3 TCID₅₀/ml but not in control E⁻ cells, in which the virus was not detectable from passage 1 (limit of detection, 50 TCID₅₀/ml) (Fig. 5A). These data indicated that the E protein was necessary for either viral RNA synthesis or virus propagation. To evaluate the role of the E protein in viral RNA synthesis, the level of genomic RNA (gRNA) was evaluated by quantitative reverse transcription-PCR (RT-qPCR) at each passage. Viral gRNA was detected for rMERS-CoV in both E⁻ and E⁺-expressing cells, as expected. However, MERS-CoV- Δ E viral RNA was detected at high levels in E⁺ cells at passages 0, 1, 2, and 3, whereas it was only detected at similar levels in E⁻ cells at passage 0, suggesting that MERS-CoV- Δ E was a replication-competent virus (Fig. 5B). To further confirm these data, viral RNA synthesis was analyzed in a single-cycle infection. E⁻ cells were infected with either rMERS-CoV or rMERS-CoV- Δ E grown in E⁺ cells. At 5 h.p.i., the levels of gRNA and subgenomic mRNA 8 (sgmRNA N) were evaluated by RT-qPCR (Fig. 6). Similar levels of gRNA and sgmRNA N were detected in cells infected with both viruses, indicating that E protein was not required for efficient viral replication and transcription. Overall, these data indicated that rMERS-CoV- Δ E was a replication-competent, propagation-defective virus.

DISCUSSION

The emergence of MERS-CoV represents a public health threat that requires further research to understand the virus biology and

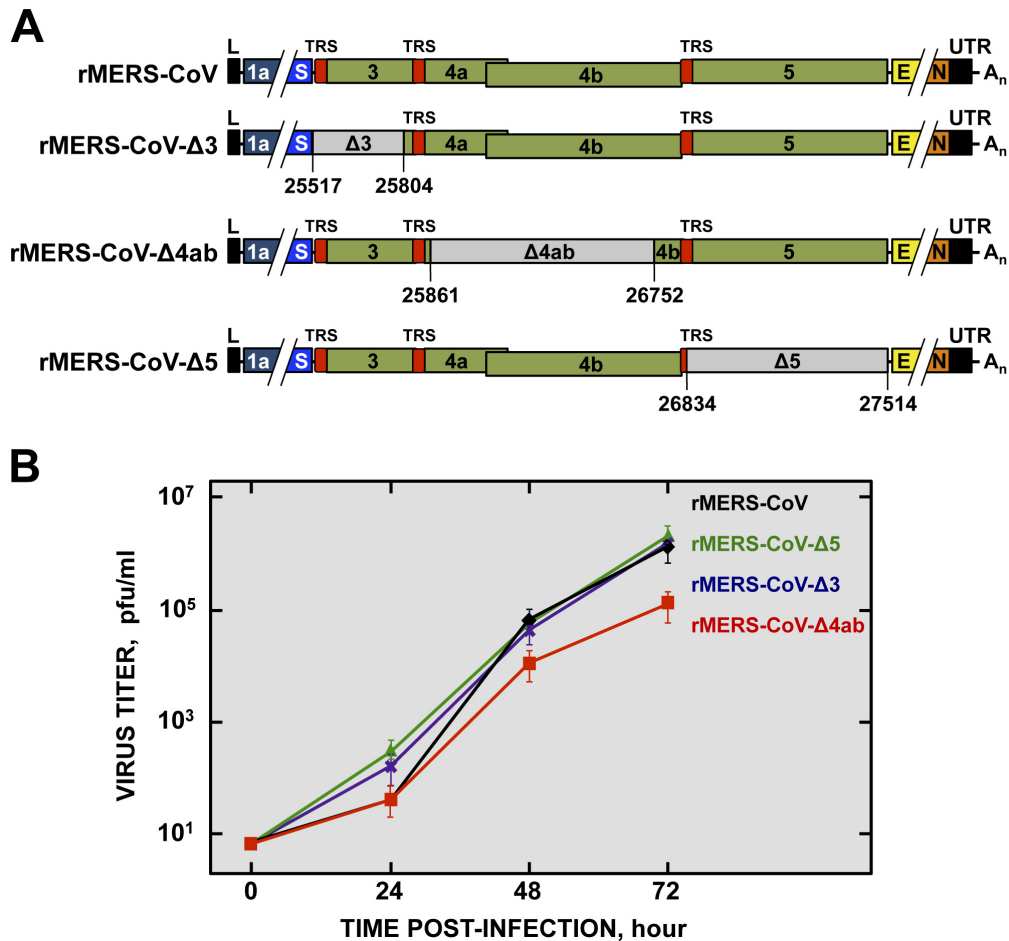


FIG 3 Rescue and growth kinetics of rMERS-CoV deletion mutants. (A) Genetic structure of rMERS-CoV-Δ3, rMERS-CoV-Δ4ab, and rMERS-CoV-Δ5 deletion mutants. TRSs and viral genes are depicted as boxes. The genomic positions of the deletions introduced (gray boxes) are indicated; the numbers correspond to the last and first nondeleted nucleotide in each case. Acronyms for viral genes are as defined in the legend to Fig. 1. (B) Growth kinetics of the deletion mutants. Huh-7 cells were infected at an MOI of 0.001 PFU/cell with rMERS-CoV-Δ3, rMERS-CoV-Δ4ab, rMERS-CoV-Δ5, or the wild-type virus (rMERS-CoV), and at the indicated times postinfection, virus titers were determined by plaque assay on Huh-7 cells. Error bars represent standard deviations of the mean from three experiments.

provide the basis for the development of control strategies. This paper describes for the first time the construction of a reverse genetics system for MERS-CoV, using BACs as vectors. The recombinant viruses described in this work were rescued using a combination of synthetic biology and reverse genetics techniques, as previously described for other CoVs (33). This system constitutes a valuable molecular tool for the rational design and launching of attenuated viruses that may serve as efficient and safe vaccine candidates. In addition, the infectious cDNA clone will be useful to study the role of specific viral genes in virus-host interactions in the context of the complete viral cycle.

A full-length cDNA copy of MERS-CoV-EMC12 was generated from synthetic fragments cloned downstream from the CMV promoter in a BAC. The BAC-based strategy allows the efficient and reproducible intracellular production of viral RNA, since it is first synthesized in the nucleus by the cellular RNA polymerase II (Pol II) and then amplified in the cytoplasm by the viral replicase encoded in the RNA itself (27, 28). The MERS-CoV infectious cDNA was stably maintained in bacteria for more than 200 generations, allowing the easy and direct manipulation of the viral

cDNA for molecular studies. In addition, this BAC-based system allows for the generation of viral replicons that may be used for the screening of drugs affecting viral RNA synthesis (27, 34).

The full-length sequence of rMERS-CoV, recovered from the infectious cDNA clone, was completely identical to that published for the original MERS-CoV-EMC12 isolate (15), except for the silent point mutation introduced as a genetic marker. Therefore, both viruses should have the same biological properties. In fact, the growth kinetics and CPE caused by both viruses were similar when the same multiplicity of infection (MOI) was used to infect Huh-7 cells (14).

Interestingly, the rMERS-CoV sequence seemed more stable in Huh-7 cells than in Vero A66 cells. However, the data presented in this article are statistically very limited to definitively conclude that virus genome stability depends on the cell type used. Based on the preliminary data presented here, it would be interesting to analyze the evolution of the MERS-CoV sequence in different cell types, including human respiratory epithelial cells.

The 3' third of the MERS-CoV genome contains a set of accessory genes encoding proteins with no similarity to other viral or

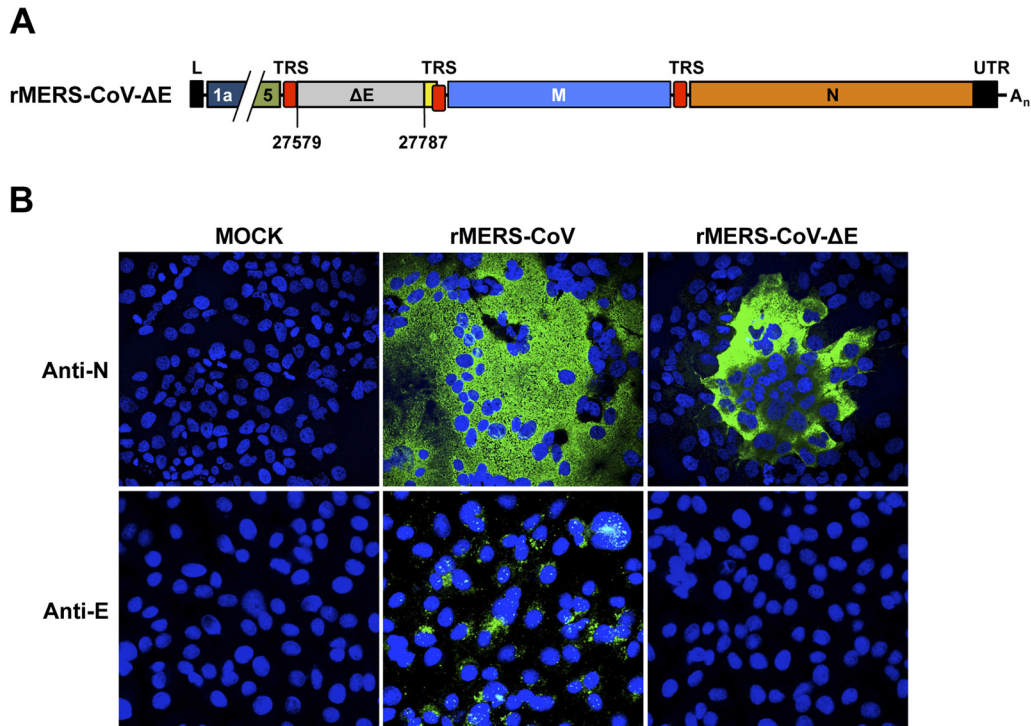


FIG 4 Rescue of rMERS-CoV-ΔE. (A) Genetic structure of rMERS-CoV-ΔE. TRSs and viral genes are illustrated as boxes. The genomic position of the introduced deletion (gray box) is indicated; the numbers correspond to the last and first nondeleted nucleotides. Acronyms for viral genes are as defined in the legend to Fig. 1. (B) Identification of the recovered viruses by immunofluorescence microscopy. Huh-7 cells were mock infected (MOCK) or infected with rMERS-CoV-ΔE and rMERS-CoV viruses from passage 0, and the expression of viral proteins E and N was analyzed 48 h.p.i. by immunofluorescence microscopy using specific antibodies.

mammalian known proteins (35). In general, CoV accessory genes are not essential for virus growth *in vitro* (36–39). The reverse genetics system described in this article was used to study the importance of these proteins in cell culture. MERS-CoV genes 3, 4a, 4b, and 5 were each found to be dispensable for virus replication in tissue cultures. Interestingly, some of the rMERS-CoV viruses recovered from Vero A66 cells contained mutations in the accessory gene genome region that would prevent the expression of any of these genes. Similar results were previously reported for the original MERS-CoV-EMC12 isolate after passage in Vero cells (15). These data suggested an apparent lack of selection pressure on MERS-CoV accessory genes during passages in cell culture and reinforced the dispensability of these genes for virus growth *in vitro*.

Although not essential in tissue culture, these MERS-CoV accessory genes could have an important role in virus-host interaction *in vivo*, leading to attenuated phenotypes. CoV accessory genes have been associated with the modulation of viral virulence (40). Among all CoVs, SARS-CoV contains the largest number of accessory genes, and it has been proposed that these genes may have important contributions to its high virulence (26, 39). To date, mouse hepatitis virus (MHV) ns2 and 5a, TGEV 7, and SARS-CoV 3b and 6 proteins have been implicated in the modulation of innate immune responses, using different mechanisms to influence virus virulence (36, 41–44).

rMERS-CoV-ΔE was a replication-competent, propagation-deficient virus and was only efficiently disseminated in cells expressing the E protein *in trans*. In the presence of transiently ex-

pressed E protein, rMERS-CoV-ΔE yielded maximum progeny viral titers of around 10^3 TCID₅₀/ml. This modest yield could be improved by the generation of cell lines stably expressing the E protein. In fact, a direct relation between viral titers and the amount of E protein expressed was previously observed for TGEV (45). However, high expression levels of E protein could induce apoptosis, as described for MHV E protein expression (46). To overcome this potential adverse effect in the case of MERS-CoV, an inducible system for E protein expression would have to be established.

rMERS-CoV-ΔE did not spread in cells in the absence of E protein, thus constituting a single-cycle replicative virus. However, infected cells produced syncytia, which suggests good expression of viral S protein. In addition, high levels of N protein were observed by immunofluorescence. These data suggested that the high expression levels of viral proteins might serve as potent immunogens to elicit a protective immune response. In the case of SARS-CoV, it has been shown that nonreplicating SARS-CoV-like particles bearing the E, S, and membrane (M) proteins induced immune responses that were protective against SARS in mice (47, 48). In addition, SARS-CoV inactivated viruses induced adaptive immunity that protected against challenge (49–52). The potential of rMERS-CoV-ΔE as a vaccine candidate is reinforced by previous observations indicating that a SARS-CoV lacking the E gene (SARS-CoV-ΔE) is attenuated and induces protection in hamsters, transgenic mice, and conventional aged mice (53–55).

MERS-CoV infects mucosal areas in the lungs and, probably, the enteric tract. Mucosal immunity in a specific tissue, such as in

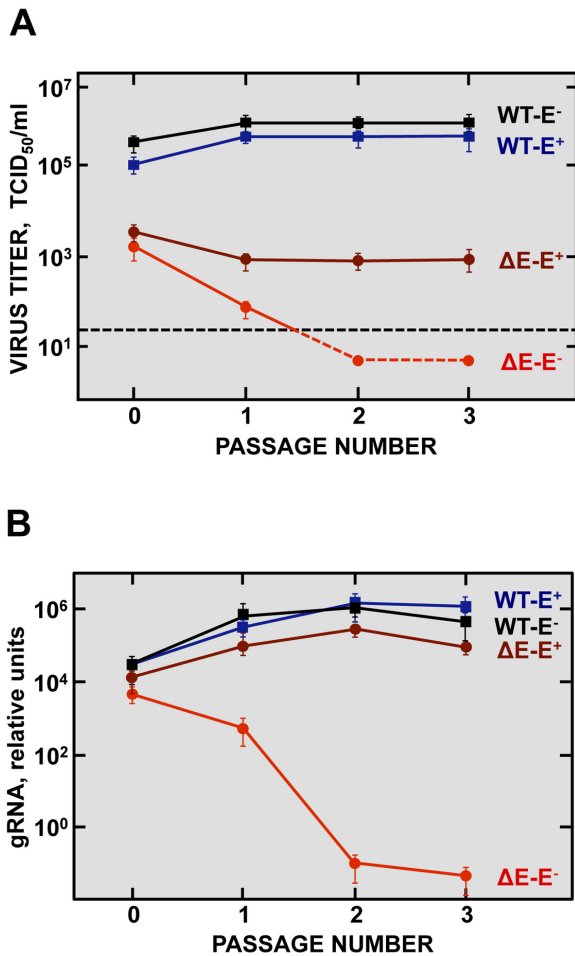


FIG 5 Rescue of rMERS-CoV-ΔE in cells expressing E protein in *trans*. (A) Virus rescue. After Huh-7 cells expressing (E⁺) or not expressing (E⁻) the E protein in *trans* were transfected with plasmids pBAC-MERS-ΔE and pBAC-MERS^{FL}, cell culture supernatants were serially passaged 3 times on fresh E⁺ and E⁻ cells every 72 h.p.i., and the virus titers of the rescued rMERS-CoV (WT) and rMERS-CoV-ΔE (ΔE) were determined by limiting dilution. The black dashed line represents the detection threshold of the virus titration assay (50 TCID₅₀/ml). Error bars represent standard deviations of the means from three experiments. (B) Viral gRNA analysis. The levels of viral gRNA in E⁺ and E⁻ cells infected with either rMERS-CoV (WT) or rMERS-CoV-ΔE (ΔE) were analyzed at each passage. Total RNA was extracted and analyzed by RT-qPCR. gRNA levels were normalized by 18S rRNA levels. Error bars represent standard deviations of the means from three experiments.

lung infections with MERS-CoV, is optimally induced by local stimulation. Therefore, immunization with live attenuated forms of rMERS-CoV-ΔE virus grown in a packaging cell line providing the E protein in *trans* may be a convenient option, particularly in comparison with purified MERS-CoV antigens, such as the S protein, that could serve as a subunit vaccine.

Vaccines based on live attenuated viruses may present biosafety problems associated with the possibility of reversion to virulent phenotypes or causing disease in immunocompromised individuals. In this sense, the use of rMERS-CoV-ΔE would be a safer option, as it does not propagate in the absence of E protein expression, preventing straightforward reversion to virulence. To increase the biosafety of a rMERS-CoV-ΔE-based vaccine, additional safety guards could be included, such as the previously de-

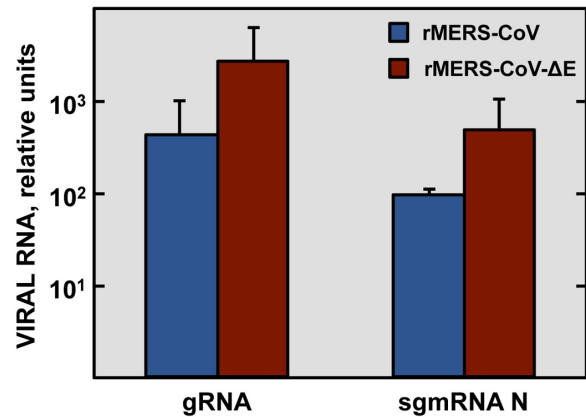


FIG 6 Analysis of replication and transcription levels in rMERS-CoV-ΔE-infected cells. Huh-7 cells were infected with rMERS-CoV-ΔE and rMERS-CoV at an MOI of 0.001 TCID₅₀/ml, and at 5 h.p.i., the levels of gRNA and sgRNA N were evaluated by RT-qPCR. Both gRNA and sgRNA N levels were normalized by 18S rRNA levels. In addition, sgRNA N levels were made relative to gRNA levels. Error bars represent standard deviations of the means from three experiments.

scribed attenuating mutations in distant genomic locations, like those encoding the nsp1 (56, 57) or nsp14 (58) replicase proteins, or by introducing genomic rearrangements (59). Overall, we consider rMERS-CoV-ΔE a promising vaccine candidate that should be further developed.

rMERS-CoV-ΔE could also be used as the starting point to generate an inactivated vaccine in case of an urgent need to control the disease. In order to guarantee the absence of virulent viruses after an incomplete chemical inactivation due to clump formation, potential noninactivated viruses would be propagation defective and, therefore, attenuated.

rMERS-CoV-ΔE, in which one of the nonessential accessory proteins was deleted, could be considered a marker vaccine, as it will allow the sera of field-infected patients to be distinguished from sera of vaccinated patients, based on the lack of antibodies specific for nonessential viral proteins (60).

The rMERS-CoV-ΔE could also be considered a viral replicon, as its genome self amplifies in infected cells but infection is not efficiently spread from cell to cell. The construction of a minimal replicon is also possible with reduced effort using the infectious clone, a project that is currently in progress in our laboratory. Therefore, the introduction of a reporter gene, such as green fluorescent protein, within this replicon could easily generate a useful tool for MERS-CoV antiviral drug screening.

In this paper, we describe for the first time a reverse genetics system of MERS-CoV engineered on BACs, which has allowed the generation of the first modified live vaccine candidate to protect against MERS-CoV. Furthermore, this reverse genetics system is a useful tool for the identification of viral genes involved in pathogenesis and the associated signaling pathways. Drugs inhibiting these pathways would be potential antivirals.

MATERIALS AND METHODS

Cells and viruses. Baby hamster kidney cells (BHK-21) were obtained from American Type Culture Collection (ATCC CCL-10). Human liver-derived Huh-7 cells were kindly provided by R. Bartenschlager (University of Heidelberg, Germany). African green monkey kidney-derived Vero A66 cells were kindly provided by A. Carvajal (University of Leon, Spain).

In all cases, cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 25 mM HEPES, 1% nonessential amino acids (Sigma), and 10% fetal bovine serum (FBS) (BioWhittaker). Virus titrations were performed on Vero A66 or Huh-7 cells following standard procedures and using closed flasks or plates sealed in plastic bags. For plaque assays, infected cells were overlaid with DMEM containing 0.6% low-melting agarose and 2% FBS, and at 72 h.p.i., cells were fixed with 10% formaldehyde and stained with crystal violet. For 50% tissue culture infectious dose (TCID₅₀) assays, CPE was recorded at 72 h.p.i. All work with infectious virus was performed in biosafety level 3 facilities by personnel wearing positive-pressure air-purifying respirators (high-efficiency particulate Air-Mate).

Plasmids and bacteria strains. Plasmid pBeloBAC11 (61), kindly provided by H. Shizuya (California Institute of Technology, Pasadena, CA), was used to assemble the MERS-CoV infectious cDNA clone. This plasmid is a low-copy-number plasmid (one to two copies per cell) based on the *E. coli* F factor (62) that allows the stable maintenance of large DNA fragments in bacteria. *E. coli* DH10B (Gibco/BRL) cells were transformed by electroporation using a MicroPulser unit (Bio-Rad) according to the manufacturer's instructions. BAC plasmid and recombinant BACs were isolated and purified using a large-construct kit (Qiagen), following the manufacturer's specifications.

Construction of a full-length cDNA clone of MERS-CoV. Based on the data of the full-length sequence of the MERS-CoV-EMC12 strain (GenBank accession number JX869059) (15), a MERS-CoV infectious cDNA clone was assembled in BAC using a three-step strategy. In the first step, the restriction sites BamHI (genomic position 806), StuI (genomic positions 7,620 and 9,072), SmaI (genomic position 20,898), and PacI (genomic position 25,836), present in the viral genome, were selected (Fig. 1A). Second, the intermediate plasmid pBAC-MERS-5'3' was constructed as the backbone for assembly of the full-length cDNA clone (Fig. 1B). To generate this plasmid, two DNA fragments were generated by chemical synthesis (Bio Basic, Inc.). The first fragment contained the CMV promoter fused to the first 811 nucleotides of the viral genome flanked by SfoI and BamHI sites, and the other one contained a multicloning site with the restriction sites selected in the first step (BamHI, StuI, SmaI, and PacI) followed by the last 4,272 nucleotides of the viral genome joined to a 25-nt poly(A), HDV ribozyme, and BGH termination and polyadenylation sequences. The first DNA fragment was cloned into pBeloBAC11^{-StuI} (a pBeloBAC without the StuI restriction site) and digested with SfoI and BamHI to generate the plasmid pBAC-MERS-5', and then the plasmid pBAC-MERS-5'3' was generated by cloning the second DNA fragment, digested with BamHI and SfiI, into pBAC-MERS-5' digested with the same restriction enzymes. Finally, the third step was the assembly of the full-length cDNA clone (pBAC-MERS^{FL}) by sequential cloning of four overlapping DNA fragments (MERS-1 to MERS-4) into the multicloning site of the intermediate plasmid pBAC-MERS-5'3' (Fig. 1C). The overlapping DNA fragments flanked by the appropriate restriction sites were generated by chemical synthesis (Bio Basic, Inc.). In the case of fragment MERS-3, a silent mutation (T to C) was introduced at position 20,761 in order to eliminate the SmaI restriction site at position 20,760 and to use it as a genetic marker. The genetic integrity of the cloned DNAs was verified throughout the assembly process by extensive restriction analysis and sequencing.

Construction of MERS-CoV cDNA clones lacking accessory genes 3, 4a, 4b, and 5. The deletion of gene 3 was generated by PCR-directed mutagenesis using the plasmid pUC-MERS-1 (a pUC plasmid containing the MERS-1 fragment spanning nucleotides 20,898 to 25,836 of the MERS-CoV genome) as the template and the oligonucleotides MERS-S-Tth1111-VS (5' TGCTATTTGACAAAGTCACTATAGCTGATC 3', where the restriction site Tth1111 is underlined) and MERS-S-PaCI-RS (5' CCCTTAATTAAGTCTGAGTAACCAACGTCAAAAGATTCACTATTAGTGAACATGAACCTTATGCGGCTCGAGGTCGATTCC 3', where the restriction site PaCI is underlined). The PCR product, including the deletion (from nucleotides 25,518 to 25,803), was digested with

Tth1111 and PaCI and cloned into the same sites of pUC-MERS-1, leading to pUC-MERS-1-Δ3. To generate pBAC-MERS-Δ3, the SmaI-PaCI digestion product from pUC-MERS-1-Δ3 was cloned into the same restriction sites of pBAC-MERS^{FL} (Fig. 3A).

The deletions of genes 4a, 4b, and 5 were introduced by PCR-directed mutagenesis, using as a template the plasmid pBAC-MERS-3' (a BAC plasmid containing the MERS-3' fragment spanning nucleotides 25,836 to 30,107 of the MERS-CoV genome). For deletion of genes 4a and 4b, overlapping PCR fragments were amplified using oligonucleotides del4ab-VS (5' GAACTCTATGGATTACGGTTGTCTCCATACGGTC 3') and del4ab-RS (5' GACCGTATGGAGACAACCGTAATCCATAGATT 3'). The final PCR product was amplified with outer oligonucleotides T7 and SA27201RS (5' CAAACAGTGGAAATGTAGG 3'), digested with PaCI and NheI, and cloned into the same restriction sites of pBAC-MERS-3', leading to pBAC-MERS-3'-Δ4ab that contains a deletion spanning nucleotides 25,862 to 26,751 of the MERS-CoV genome. For gene 5 deletion, a PCR fragment lacking gene 5 (nucleotides 26,835 to 27,513) was amplified using oligonucleotides SA25834VS (5' GTTAATTAACGA ACTCTATGGATTACG 3', where the restriction site PaCI is underlined) and del5-SanDI-RS (5' CACGGGACCCATAGTAGCGCAGAGCTGCT GTTAAATCCTGGATG 3', where the restriction site SanDI is underlined), digested with PaCI and SanDI, and cloned in the same sites of pBAC-MERS-3', leading to pBAC-MERS-3'-Δ5. To generate plasmids pBAC-MERS-Δ4ab and pBAC-MERS-Δ5, the PaCI-RsrII digestion products from plasmids pBAC-MERS-3'-Δ4ab and pBAC-MERS-3'-Δ5 were cloned in the same sites of pBAC-MERS^{FL} (Fig. 3A). All cloning steps were checked by sequencing of the PCR fragments and cloning junctions.

Construction of a MERS-CoV cDNA clone lacking the structural E gene. The pBAC-MERS-ΔE, encoding a MERS-CoV lacking the E gene, was constructed from the full-length plasmid pBAC-MERS^{FL}. To this end, the SanDI-RsrII DNA fragment (2,634 bp) from pBAC-MERS^{FL} was exchanged with a chemically synthesized (Bio Basic, Inc.) SanDI-RsrII DNA fragment with a deletion from nucleotides 27,580 to 27,786 that included the TRS core sequence and the first 197 nucleotides of the E gene (Fig. 4A). The genetic integrity of the cloned DNA was verified by restriction analysis and sequencing.

Recovery of recombinant viruses from the cDNA clones. To recover infectious virus, BHK cells were grown to 95% confluence in a 12.5-cm² flask and transfected with 6 μg of the infectious cDNA clone using 18 μg of Lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications. At 6 h.p.t., cells were trypsinized, plated over a confluent monolayer of either Vero A66 or Huh-7 cells grown in a 12.5-cm² flask, and incubated at 37°C for 72 h. The cell supernatants were harvested and passaged once on fresh cells, and the recovered viruses were cloned by three rounds of plaque purification, following standard procedures.

Virus genome sequencing. The complete genome sequence of each rescued recombinant MERS-CoV was determined by sequencing overlapping RT-PCR fragments of 2.5 kb covering the full-length viral genome. Reverse transcription and PCRs were performed with specific oligonucleotides using ThermoScript reverse transcriptase (Invitrogen) and the Expand high-fidelity PCR system (Roche), respectively, following the manufacturers' recommendations. The genomic 5'- and 3'-terminal sequences were determined using the 5'/3' RACE (rapid amplification of cDNA ends) kit (Roche) according to the manufacturer's specifications. Sequence assembly and comparison with the consensus sequence of the MERS-CoV-EMC12 strain were performed with the SeqMan and MegAlign programs (Lasergene, Madison, WI).

Generation of Huh-7 cells expressing MERS-CoV E protein. For the generation of Huh-7 cells transiently expressing E protein, cells were nucleofected with the plasmid pcDNA3-E (expressing the MERS-CoV E protein under the CMV promoter) by using a 4D Nucleofector device (Lonza) and the buffer and program recommended by the manufacturer. For the construction of plasmid pcDNA3-E, the E gene was amplified by PCR using pBAC-MERS^{FL} as the template and the specific oligonucleotides E1-EcoRI-VS (5' GTGCTGGAATTCGCCCATGTTACCTTT

GTCCAAGAACGAA 3', restriction site EcoRI is underlined) and E249-XhoI-RS (5' CGCCCAGCTCGAGTTAAACCCACTCGTCAGGTGG 3', restriction site XhoI is underlined) and cloned into the plasmid pcDNA3 (Invitrogen) digested with EcoRI and XhoI.

Analysis of viral RNA synthesis by RT-qPCR. Total intracellular RNA was extracted from transfected or infected cells with the RNeasy miniprep kit (Qiagen) according to the manufacturer's specifications. In the case of transfected cells, the residual DNA was removed from samples by treating 7 μ g of each RNA with 20 U of DNase I (Roche) in 100 μ l for 30 min at 37°C, and DNA-free RNAs were repurified using the RNeasy miniprep kit (Qiagen). Viral RNA synthesis was quantified by RT-qPCR. Total cDNA was synthesized with random hexamers from 100 ng of total RNA using a high-capacity cDNA reverse transcription kit (Invitrogen). Using this cDNA, the viral RNA synthesis was analyzed using two custom TaqMan assays specific for MERS-CoV gRNA (forward primer 5' GCACATCTGTGGTTCTCTCTCT 3', reverse primer 5' AAGCCCAGGCCCTACTAT TAGC 3', and MGB probe 5' TGCTCCAACAGTTACAC 3') and sgmRNA N (forward primer 5' CTCCCCTCGTTCTCTTGCA 3', reverse primer 5' TCATTGTTATCGGCAAAGGAAA 3', and MGB probe 5' CTTTGATTTTAACGAATCTC 3'). Data were acquired with an Applied Biosystems 7500 real-time PCR system and analyzed with ABI PRISM 7500 software, version 2.0.5. The relative quantifications were performed using the cycle threshold ($2^{-\Delta\Delta CT}$) method (63). To normalize for differences in RNA sampling, the expression of eukaryotic 18S rRNA was analyzed using a specific TaqMan gene expression assay (Hs99999901_s1; Applied Biosystems).

Generation of polyclonal antisera specific for MERS-CoV N and E proteins. Rabbit polyclonal antisera (pAb) specific for MERS-CoV N and E proteins were purchased from BioGenes. In brief, peptides NTGRS-VYVKFQDSKPPL (corresponding to E protein amino acids 60 to 76) and AAANKMHRHKRTST (N protein amino acids 244 to 257) were synthesized and used to immunize two rabbits with each peptide according to the company's standard protocol. The polyclonal antisera obtained were evaluated by enzyme-linked immunosorbent assay (ELISA) using the synthetic peptides, leading to titers ranging from 1:150,000 to 1:200,000 in all cases.

Indirect immunofluorescence assay. Vero A66 and Huh-7 cells were grown to 80% confluence on glass coverslips and infected with the recombinant MERS-CoVs. At 48 h.p.i., cells were fixed either with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 20 min or with methanol at -20°C for 15 min. For N protein immunodetection, paraformaldehyde-fixed cells were permeabilized with 0.2% saponin in PBS containing 10% FBS for 20 min and incubated with MERS-CoV N protein pAb (dilution 1:200) in PBS containing 10% FBS at room temperature for 90 min. For E protein immunodetection, methanol-fixed cells were incubated with MERS-CoV E protein pAb (dilution 1:500) in PBS containing 10% FBS overnight at 4°C. Coverslips were washed 4 times with PBS and incubated at room temperature for 45 min with goat anti-rabbit antibody conjugated to Alexa Fluor 488 (Invitrogen) diluted 1:500 in PBS containing 10% FBS. Nuclei were stained using DAPI (4',6'-diamidino-2-phenylindole) (1:200, Sigma). To fully inactivate the samples' infectivity, methanol-fixed cells were treated with 4% paraformaldehyde in PBS as described above. Finally, coverslips were mounted in ProLong Gold antifade reagent (Invitrogen) and analyzed on a Leica SP5 confocal microscope. Images were acquired with the same instrument settings and analyzed with Leica software.

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