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Rescue of Recombinant Marburg Virus from cDNA Is Dependent on Nucleocapsid Protein VP30

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Here we report recovery of infectious Marburg virus (MARV) from a full-length cDNA clone. Compared to the wild-type virus, recombinant MARV showed no difference in terms of morphology of virus particles, intracellular distribution in infected cells, and growth kinetics. The nucleocapsid protein VP30 of MARV and Ebola virus (EBOV) contains a Zn-binding motif which is important for the function of VP30 as a transcriptional activator in EBOV, whereas its role for MARV is unclear. It has been reported previously that MARV VP30 is able to support transcription in an EBOV-specific minigenome system. When the Zn-binding motif was destroyed, MARV VP30 was shown to be inactive in the EBOV system. While it was not possible to rescue recombinant MARV when the VP30 plasmid was omitted from transfection, MARV VP30 with a destroyed Zn-binding motif and EBOV VP30 were able to mediate virus recovery. In contrast, rescue of recombinant EBOV was not supported by EBOV VP30 containing a mutated Zn-binding domain.

The filoviruses Marburg virus (MARV) and Ebola virus (EBOV) cause a severe hemorrhagic fever in humans and nonhuman primates with extraordinarily high fatality rates. MARV was first isolated in 1967, when 31 laboratory workers in Germany and Yugoslavia handling MARV-infected African green monkeys imported from Uganda became ill. Despite aggressive supportive treatment, seven of the patients died (14, 23). The largest MARV outbreak to date took place from 2004 to 2005 in Angola, when 252 people became infected. The case fatality rate of this outbreak was 91%.

The nonsegmented negative-sense RNA genome of MARV is 19,111 bases in length and encodes seven proteins (9). Four of these proteins (NP, VP35, L, and VP30) constitute the nucleocapsid complex (1). NP, VP35, and L are sufficient to mediate viral transcription and replication in a MARV-specific minigenome system, while the fourth component of the nucleocapsid complex, VP30, acts as a transcription activator for EBOV (17, 18, 28). Hence, the role of VP30 in the life cycle of MARV VP30 has not yet been determined. It has been reported that MARV VP30 interacts with NP-derived inclusions, indicating that VP30 might be involved in nucleocapsid maturation (16). RNA interference-based down-regulation of VP30 in MARV-infected cells resulted in significant reduction of all viral proteins, suggesting an important role for VP30 in viral replication and/or transcription (10). EBOV VP30 contains a Cys3-His motif comprising amino acids 68 to 95 which was shown to bind zinc ions. The integrity of the Zn-binding motif was crucial for the function as a transcriptional activator but not for the interaction with NP-derived inclusion bodies. Sequence comparison revealed that this motif is also present in MARV VP30 (amino acids 74 to 99) (15). The only other nonsegmented negative-strand RNA viruses possessing a fourth nucleocapsid protein are the pneumoviruses. For human respiratory syncytial virus, it was shown that the M2-1 protein serves as an elongation and antitermination factor during transcription (6, 8, 13). Interestingly, M2-1 contains a Zn finger motif similar to the motif found in VP30 which was shown to be essential for the function of the protein (12).

To study aspects of filovirus replication and transcription without biosafety level 4 containment, minigenome systems were established for MARV and EBOV (2, 11, 17, 18). However, a full-length rescue system is desirable to investigate all aspects of the viral life cycle in an authentic context. Rescue of negative-strand RNA viruses from cDNA was facilitated by using the antigenomic instead of the genomic sequence (22). Since then, full-length rescue systems have been established for several Mononegavirales (for reviews, see references 7 and 20), including EBOV (19, 26). These systems allow the specific mutation of proteins of interest (19, 26) or introduction of foreign reporter genes like enhanced green fluorescent protein (25).

In this study, we present a system which allows the recovery of infectious MARV entirely from cDNA. Using this system, the role of VP30 for the rescue of recombinant MARV was investigated.

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Cloning of the full-length MARV clone. The complete genomic sequence of MARV strain Musoke was determined and submitted as a reference sequence to GenBank (accession number DQ217792). A set of five cassettes using a pBlueScript II KS(+) backbone (Stratagene) was designed which could be combined to generate a full-length cDNA of the complete MARV antigenome termed pMARV(+). Reverse transcrip-
tion (RT)-PCR with viral RNA as the template and PCR using already existing plasmids containing MARV-specific sequences were used to generate 2.1- to 7.8-kb fragments flanked by unique restriction sites (Fig. 1). All five MARV-specific plasmids were digested with the respective enzymes shown in Fig. 1 and ligated to yield pMARV(+). The correct sequence was verified by automated sequencing on a MegaBACE sequencer (Amersham). To discriminate recombinant from wild-type virus, a silent mutation (viral RNA: A6225→G) was introduced into the GP gene by QuikChange mutagenesis (Stratagene), generating an additional SspI restriction site at nt 6220. Furthermore, the first nucleotide of the MARV-specific sequence was mutated (A→G) to enhance the nucleotide (nt) 6220. The SspI site shown in pMARV Apal/Sacl serves as a genetic marker. Restriction sites flanking the cassettes were used for construction of full-length pMARV(+) (right).

FIG. 1. Cloning strategy for the full-length clone. (Left) Schematic drawing of the cassettes used for cloning. The numbers indicate nucleotide positions in the MARV genome (strain Musoke; GenBank accession number DQ217792). The MARV-specific sequence was obtained either by RT-PCR with viral RNA as the template or PCR assay of already existing plasmids derived from viral RNA. The SspI site shown in pMARV Apal/Sacl serves as a genetic marker. Restriction sites flanking the cassettes were used for construction of full-length pMARV(+) (right).
further characterized by immunofluorescence analysis. Vero cells were grown on glass coverslips and infected with recMARV or wt MARV or incubated with medium (mock) or the sample in which the L plasmid was omitted (pT/L). At 48 h p.i., the samples were inactivated in 4% paraformaldehyde overnight. Cells were incubated with a monoclonal antibody raised against MARV NP (1:100 dilution). As a secondary antibody, a rhodamine-conjugated goat anti-mouse antibody (1:200 dilution; Dianova) was used. Additionally, nuclei were stained with 0.1 g/ml 4', 6'-diamidino-2-phenylindole hydrochloride (DAPI). As shown in Fig. 2D, the typical inclusion bodies formed by the nucleocapsid proteins were observed in cells infected with either recMARV or wt MARV. No specific staining was observed in mock-infected cells or the sample transfected without the L gene. Lastly, virions were purified through a sucrose cushion as described above, inactivated in 4% paraformaldehyde overnight, and analyzed by electron microscopy as previously described (26). Pictures were taken on a Zeiss 109 electron microscope at a magnification of ×50,000.

Integrity of the zinc-binding motif Cys3-His within VP30 is not needed for interaction with NP but for transcriptional activity in an EBOV-specific minigenome system. Rescue of recMARV was only supported in the presence of all nucleocapsid proteins. When the fourth nucleocapsid protein, VP30, was omitted, it was not possible to generate recombinant MARV (see below and Fig. 4). Similarly, recovery of recombinant EBOV was also dependent on the presence of VP30.
Interestingly, the necessity of the fourth nucleocapsid protein M2-1 for virus rescue is different among the pneumo-
viruses. While human respiratory syncytial virus M2-1 was found to be necessary for production of recombinant virus, recombinant human metapneumovirus lacking the M2-1 open reading frame was successfully recovered in cell culture (3, 5). Concerning MARV, VP30 was not needed for replication and transcription of MARV minigenomes (17). Thus, the question arose of whether VP30 is involved in transcription and/or replication of the recombinant virus or whether it acts as a structural component during nucleocapsid formation.

The Zn-binding motif Cys-Ser-His of EBOV VP30 was shown to be necessary for transcriptional activity but not for binding to NP (15). First, the importance of the Zn-binding motif of MARV VP30 was examined with regard to binding to NP and transcriptional activity in an EBOV-specific minigenome system (18). The expression plasmid pT/VP30M Zn-finger knockout contained two substitutions (genomic positions G\_9143\_C, A\_9155\_G, mRNA sense) introduced by QuikChange mutagenesis on pT/VP30M (17), which resulted in the disruption of the putative Zn-finger motif (Cys\_92-Ser, His\_96-Leu) (Fig. 3A). It has been shown previously for EBOV VP30 that mutation of the homologous amino acids led to a loss of function as transcription activator, while binding to NP-derived inclusion bodies was not impaired (15). Expression of VP30M and VP30M Zn-finger knockout was verified by Western blot analysis. Therefore, BSR T7/5 cells were transfected with 1.0 and 2.0 \( \mu \text{g} \), respectively, of either pT/VP30M or pT/VP30M Zn-finger knockout and lysed at 2 days posttransfection. VP30M was detected using a monoclonal mouse anti-VP30M antibody (1:1000) and a peroxidase-labeled goat anti-mouse antibody (1:40,000). As shown in Fig. 3C, both constructs were expressed at comparable levels. Immunofluorescence analysis was employed to study the interaction of VP30M Zn-finger knockout with NP. BSR T7/5 cells were grown on coverslips and transfected with either 0.5 \( \mu \text{g} \) pT/NPM, 1.5 \( \mu \text{g} \) pT/VP30M, 1.5 \( \mu \text{g} \) pT/VP30M Zn-finger knockout, or a combination of NP and VP30 plasmids. After 2 days, cells were fixed and permeabilized. VP30 was detected as described above, and VP30 was detected using a guinea pig anti-VP30 antibody (1:100) and fluorescein isothiocyanate-conjugated goat anti-guinea pig antibodies (1:200; Dianova). Upon single expression, NP formed inclusion bodies (Fig. 3B, panel 1), whereas VP30M and VP30M Zn-finger knockout were homogenously distributed in the cytoplasm (Fig. 3B, panels 2 and 3). When NP and VP30M or the mutant VP30M Zn-finger knockout were coexpressed, both VP30 variants were redistributed into the NP-derived inclusion bodies (Fig. 3B, panels 4 and 5). These data indicate that an intact Zn-finger motif is not important for binding to NP, as also described for EBOV VP30 (15). The next aim was to determine the functionality of VP30M Zn-finger knockout with respect to transcription activation. As mentioned above, MARV VP30 was not required for transcription activation in a MARV-specific minigenome system. However, it has been described previously that MARV VP30 was able to mediate transcription to some extent when used in an EBOV-specific minigenome system. When cells were transfected with plasmids encoding EBOV NP, VP35, L, the EBOV-specific minigenome, and MARV VP30, transcription activation was observed (18). Following the transfection procedure of BSR T7/5 cells described by Weik et al. (27), we replaced pT/VP30EBO with 2.0 \( \mu \text{g} \) of either pT/VP30M, pT/
VP30<sub>M Zn-finger knockout</sub> or pT/VP30<sub>M H90G</sub> + , an EBOV VP30 mutant with a disrupted Zn-finger motif (15). Transfected cells were lysed on day 2 posttransfection. The used minigenome 3E-5E consists of the 3' and 5' ends of the EBOV genome and a chloramphenicol acetyltransferase (CAT) gene as reporter gene (18). Transcription of the minigenome leads to CAT gene expression and was assayed by CAT activity (17). While MARV VP30 exhibited between 10 and 15% activity (Fig. 3D, lane 3), neither of the Zn-finger knockout mutants was able to activate transcription (Fig. 3D, lanes 4 and 5). Hence, the Zn-binding motif of MARV and EBOV VP30 seems to have a similar function, although the details are not yet understood (15).

**MARV can be rescued with EBOV VP30 and VP30<sub>M Zn-finger knockout**. Next, we examined the role of VP30 in the full-length rescue system. BSR T7/5 cells were transfected in triplicate as described above. Plasmid pT/VP30<sub>M</sub> was substituted with either 0.1 μg pT/VP30<sub>EBO</sub> coding for EBOV VP30 or 0.5 μg pT/VP30<sub>M Zn-finger knockout</sub>. Supernatants from mixed BSR T7/5 and Vero cells were used to infect fresh Vero cells, and at 10 days p.i., total cellular RNA was isolated and RT-PCR was performed as described earlier. As mentioned above, no signal was detected when VP30 was omitted from the transfection mixture (Fig. 4A, lanes 2 to 4). These data demonstrate that MARV VP30 is necessary for rescue of full-length virus, although it is not needed for transcription and replication in the minigenome system (17). Strong signals were obtained from all samples transfected with pT/VP30<sub>M Zn-finger knockout</sub> (Fig. 4A, lanes 5 to 7), indicating that the Zn-binding motif was not essential for recovery of recMARV, although it was necessary for transcription activation in the EBOV-specific minigenome system (Fig. 3D). Rescue was also observed in one of three samples when the EBOV VP30 plasmid was transfected (Fig. 4A, lanes 8 to 10). In contrast, it was not possible to generate recombinant EBOV when MARV VP30 was used in an EBOV rescue system (24).

To compare the effect of the VP30 Zn-binding domain on virus recovery between MARV and EBOV, similar experiments were performed by using the EBOV-specific rescue system. BSR T7/5 cells were transfected with plasmids coding for the EBOV nucleocapsid proteins NP, VP35, and L, the EBOV-specific full-length clone pFL-EBOVe as positive orientation, EBOV VP30, or the Zn-finger mutant VP30<sub>EBO H90L</sub> as described earlier (15, 26). Briefly, BSR T7/5 cells were grown overnight in 25-cm<sup>2</sup> flasks to about 60% confluence and transfected with a plasmid mixture containing 4 μg of the full-length plasmid pFL-EBOVe+, 1 μg of pT/VP35<sub>EBO</sub>, 1 μg of pT/NEBO, 2 μg of pT/L<sub>EBO</sub>, and 0.2 μg of either pT/VP30<sub>EBO</sub> or VP30<sub>EBO H90L</sub> by using Fugene 6 reagent (15, 26). After 6 days, culture medium was collected and used for inoculation of Vero E6 cells. At 6 days p.i., supernatants were used to infect Vero E6 cells. CPE caused by virus infection was determined after an incubation period of 6 days.

![FIG. 4. Influence of VP30 on recovery of recombinant MARV and EBOV.](http://jvi.asm.org/)
Nucleotide sequence accession number. The complete genomic sequence of MARV strain Musoke was submitted to GenBank under accession number DQ217792.

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