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# A reconstituted replication and transcription system for Ebola virus Reston and comparison with Ebola virus Zaire

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## Abstract

The only known filovirus, which presumably is not pathogenic for humans, is Ebola virus (EBOV) Reston. When EBOV Reston and the highly pathogenic EBOV Zaire were grown in cell culture, comparison of the replication kinetics showed a clear growth impairment of EBOV Reston, indicating that the replication cycle of EBOV Reston might be delayed. In addition, the cytopathic effect caused by the virus was much milder with EBOV Reston than with EBOV Zaire. To compare replication and transcription of EBOV Reston and Zaire, a reconstituted minigenomic replication and transcription system based on reverse genetics has been established for EBOV Reston. This system was used to exchange the EBOV Zaire and EBOV Reston nucleocapsid (NC) proteins NP, VP35, VP30, and L, which catalyze replication and transcription. Furthermore, chimeric minigenomes were constructed containing the *cis*-acting replication signals of EBOV Zaire combined with those of EBOV Reston. Surprisingly, the *cis*-acting signals as well as almost all NC proteins could be exchanged between EBOV Reston and Zaire, suggesting a high degree of functional homology of the replication/transcription complexes of EBOV Zaire and EBOV Reston. Only the combination of EBOV Zaire VP35 and EBOV Reston L did not result in replication and transcription activity. Although these two proteins did not constitute an active polymerase complex, it was shown by immunofluorescence analysis that they were still able to interact.

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## Introduction

Ebola viruses (EBOV) are subdivided into four species. Three of these species, EBOV Zaire (EBOV-Z), Sudan, and Ivory Coast, are originated from Africa whereas the fourth subtype, EBOV Reston (EBOV-R), is the only filovirus originated from Asia. EBOV-R was first isolated in 1989/1990 from *Cynomolgus* monkeys imported from the Philippines to the United States (Geisbert et al., 1992; Jahrling et al., 1990). In 1992 and 1996, respectively,

EBOV-R-infected monkeys emerged in Italy and the United States. These cases of EBOV-R-infected monkeys could be traced back to Philippine export facilities (Rollin et al., 1999). All African EBOV subtypes cause a severe hemorrhagic disease in humans and nonhuman primates with extraordinarily high fatality rates. Although EBOV-R also induces hemorrhagic fever in monkeys, it seems to be less pathogenic than the African filoviruses. Comparative studies with monkeys experimentally infected with the different EBOV subtypes revealed that the time course of the disease was delayed and the number of survivors was higher with EBOV-R infection (Fisher-Hoch and McCormick, 1999). In order to investigate the role of the type I interferon response in filovirus-infected mice, Bray (2001) infected immunocompetent adult mice and knockout mice lacking the interferon- $\alpha/\beta$  receptor with different Marburg

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virus (MBGV) and EBOV isolates. While the immunocompetent mice survived the infection, the knockout mice were killed by most filoviruses but not by EBOV-R, a finding which again underlines the reduced virulence of this viral subtype. As of today, no human disease is associated with EBOV-R. Moreover, accidental human infections during the outbreak in 1989/1990 were all asymptomatic (Miranda et al., 1991). This observation suggests that EBOV-R is not only less virulent than the African filoviruses but possibly nonpathogenic for humans.

Due to their genome organization, filoviruses are grouped in the order Mononegavirales. The EBOV-R genome consists of a single-stranded nonsegmented negative-sense RNA molecule, which is about 19 kb in length with a coding capacity for seven structural proteins (Groseth et al., 2002; Ikegami et al., 2001). For EBOV-Z and the closely related MBGV, it has been shown that four viral proteins, namely NP, VP35, VP30, and L, are associated with the viral genome and form the nucleocapsid complex (Becker et al., 1998; Elliott et al., 1985). The nucleocapsid proteins play a dual role in the viral replication cycle: they are structural components of the nucleocapsid complex and therefore involved in virus morphogenesis. Functionally, they catalyze replication and transcription of the RNA genome. In a reconstituted replication/transcription system, MBGV replication and transcription was mediated by NP, VP35, and L (Mühlberger et al., 1998). In the case of EBOV-Z, replication was supported by the same three proteins; however, transcription was strongly dependent on the fourth nucleocapsid protein VP30 (Mühlberger et al., 1999; Volchkov et al., 2001). Recently, it has been shown that EBOV-Z VP30 acts as a transcription antitermination factor immediately after transcription initiation while transcription elongation is not affected by the protein (Modrof et al., 2002, 2003; Weik et al., 2002). The function of MBGV VP30 has not been determined yet. The nucleoprotein NP encapsidates the viral RNA and is the driving force for nucleocapsid formation (Kolesnikova et al., 2000; Mavraklis et al., 2002). VP35 is homologous to the phosphoprotein P of other Mononegavirales and together with the L protein constitutes the polymerase complex (Mühlberger et al., 1998, 1999). EBOV-Z VP35 has been shown to act as a type I interferon antagonist (Basler et al., 2000). Interestingly, the protein VP35, from the less pathogenic EBOV-R, is also able to inhibit the type I interferon immune response (Basler et al., 2003). Amino acid sequence comparison of EBOV-R and EBOV-Z proteins revealed a degree of homology ranging from 58% identity for the glycoprotein (GP) to up to 80% for VP24 (Groseth et al., 2002; Ikegami et al., 2001). The degree of homology of NP, VP35, and VP30, respectively, ranges from 67.6% to 68.8% identity. The L protein is one of the best conserved EBOV proteins with 74.8% identity. The genomic ends contain important *cis*-acting signals for encapsidation, replication, and transcription initiation and are also highly conserved between EBOV-Z and EBOV-R. Thus, the first and the last 55

nucleotides of both genomes show a sequence identity of 87% and 75%, respectively.

In order to compare the replication and transcription strategy of EBOV-R and EBOV-Z, a reconstituted replication/transcription system was established for EBOV-R. This system will be a useful tool to investigate whether the lower virulence of EBOV-R is due to a delay of the replication and transcription processes.

## Results

### *Replication kinetics of EBOV-Z and EBOV-R in cell culture*

First, TCID<sub>50</sub> assays were performed to determine the titer of EBOV-Z and EBOV-R virus stocks, respectively. Therefore, 10-fold dilutions of the virus stocks were used to infect vero cells, and infection of the cells was analyzed visually every second day up to day 14 postinfection (pi). The determined peak TCID<sub>50</sub> titer of the EBOV-Z stock (10<sup>8</sup> TCID<sub>50</sub>/ml) was about 100-fold higher than that of the EBOV-R stock. In addition, the cytopathic effect caused by the virus infection was much milder with EBOV-R than with EBOV-Z, confirming the reduced cytopathogenicity of EBOV-R (Fig. 1A). To compare the replication kinetics of EBOV-Z and EBOV-R in cell culture, vero cells were infected with either EBOV-Z or EBOV-R at a multiplicity of infection (MOI) of 0.01. Virus growth was examined from days 1 to 7 pi by indirect immunofluorescence staining using virus-specific antibodies. The relative percentage of infected cells was determined by counting fluorescent cells. In Fig. 1B, a clear growth impairment of EBOV-R is shown in comparison to EBOV-Z, indicating that indeed the replication cycle of EBOV-R might be delayed.

### *Development of a reconstituted replication and transcription system for EBOV Reston*

In order to compare the replication/transcription strategies of EBOV-R and EBOV-Z, a reconstituted replication and transcription system for EBOV-R was established. First, the sequence of the used virus isolate EBOV-R Pennsylvania, 1989, was determined (GenBank accession number: AY769362). Subsequently, the genes coding for the nucleocapsid proteins NP, VP35, VP30, and L were amplified by RT-PCR and inserted into the expression vector pTM1 under the control of a T7 RNA polymerase promoter. Mutations within the cloned genes were removed by *in vitro* mutagenesis. As template for the nucleocapsid proteins, an EBOV-R-specific minigenome was designed containing the 3' and 5' ends of the viral genome flanking the CAT gene as a reporter gene (Fig. 2). These genetic elements were cloned into the vector 2,0 (kindly provided by A. Ball, Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL, USA) between the T7 RNA polymerase promoter and the hepatitis delta

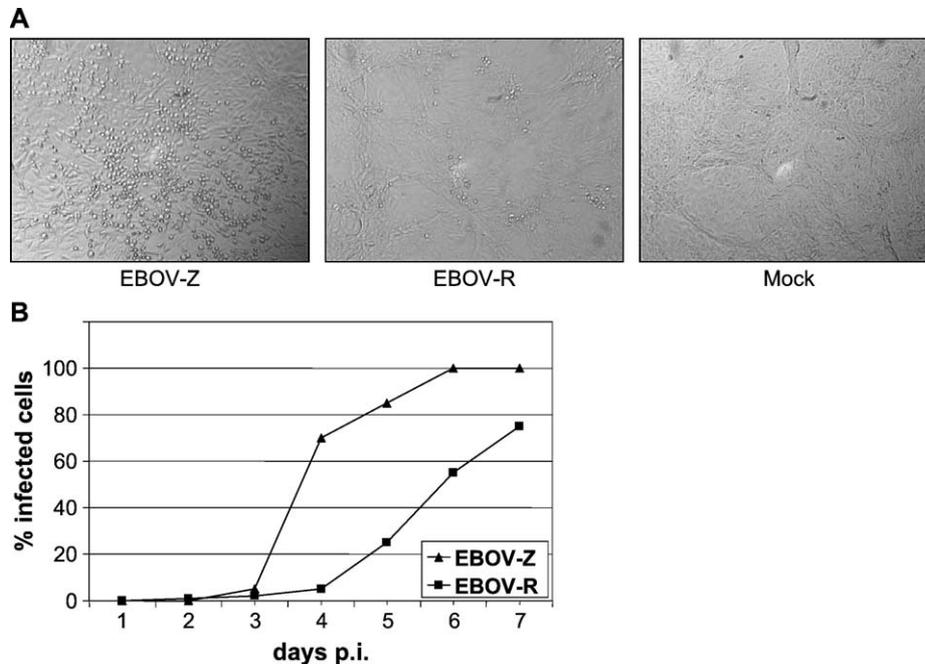


Fig. 1. Replication kinetics of EBOV-Z and EBOV-R. (A) Cytopathic effects caused by EBOV-Z and EBOV-R. Vero cells were infected with either EBOV-Z or EBOV-R at an MOI of 0.1. At 4 days pi, cells were examined by light microscopy. (B) Comparison of infectivity of EBOV-Z and EBOV-R. Vero cells were infected with either EBOV-Z or EBOV-R at an MOI of 0.01. Up to day 7 pi, cells were harvested daily and subjected to indirect immunofluorescence analysis by using antibodies directed against EBOV-Z NP or EBOV-R NP, respectively. In addition, cell nuclei were stained with DAPI. Infectivity of the viruses was determined by counting fluorescent cells.

ribozyme (Pattnaik et al., 1992). Transcription of the plasmid resulted in the negative-sense minigenomic RNA 3R–5R with exact 5' and 3' ends. To test whether the nucleocapsid proteins and the minigenome were functional, BSR T7/5 cells (Buchholz et al., 1999) constitutively expressing the T7 RNA polymerase were transfected with the plasmids coding for NP, VP35, VP30, L, and 3R–5R. At 2 days posttransfection, the cells were lysed and CAT activity was determined. In Fig. 3, it is shown that four nucleocapsid proteins were essential to support replication

and transcription. In the absence of the fourth nucleocapsid protein, VP30, no CAT activity could be detected, indicating that EBOV-R VP30 serves as a transcription activator similarly to EBOV-Z VP30.

Previous observations with the MBGV and EBOV-Z minigenome systems revealed that the relative and absolute amounts of the different plasmids used for transfection were critical for replication and transcription efficiency (Mühlberger et al., 1998, 1999). Thus, the EBOV-R-specific plasmids were titrated (Fig. 4A). While the optimal amount

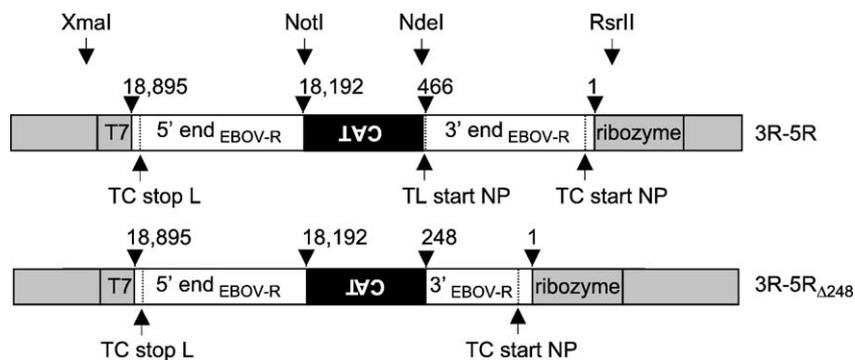


Fig. 2. Sketch of the EBOV-R-specific minigenomes 3R–5R and 3R–5R $_{\Delta 248}$ . The 3' and 5' ends of the EBOV-R genome (white bars) were cloned into the vector 2,0 (gray bar) in negative orientation under the control of the T7 RNA polymerase promoter (T7). The 3' end of minigenome 3R–5R comprises nucleotides 1–466, the 5' end nucleotides 18192–18895. Minigenome 3R–5R $_{\Delta 248}$  contains a truncated 3' region comprising nucleotides 1–248. The hepatitis delta ribozyme sequence is located downstream of the leader. As a reporter gene, the CAT gene (black bar) was inserted between the EBOV-R-specific sequences. Transcription with T7 RNA polymerase resulted in the synthesis of negative strand minigenomes. Exact 3' ends were then generated by autocleavage of the ribozyme. Used restriction sites are indicated by arrows. TC start NP: transcription start site of the EBOV-R NP gene; TL start NP: translation start site of the EBOV-R NP gene; TC stop L: transcription stop site of the EBOV-R L gene. Numbers refer to the genomic sequence of EBOV-R, isolate Pennsylvania, 1989 (GenBank accession number: AY769362).

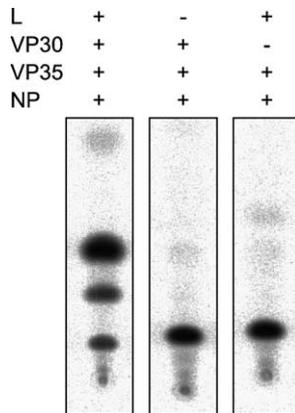


Fig. 3. Reporter gene expression mediated by EBOV-R nucleocapsid proteins. BSR T7/5 cells were transfected with plasmids coding for the EBOV-R minigenome 3R–5R and the nucleocapsid proteins NP, VP35, VP30, and L as indicated in the figure. At 2 days posttransfection, cells were harvested and CAT activity was determined.

for the plasmids encoding NP and L ranged from 1 to 2  $\mu\text{g}$ , the system was much less tolerant towards the plasmids encoding VP30 and VP35, with DNA amounts peaking at 0.5 and 0.1  $\mu\text{g}$  input DNA, respectively. The results of the first titration experiments were used to carry out a second round of titration so as to optimize the amount of plasmid DNA used (Fig. 4B). For the subsequent experiments, the following amounts of plasmid DNA were used: 1  $\mu\text{g}$  pT/ NP<sub>RES</sub>, 0.5  $\mu\text{g}$  pT/VP35<sub>RES</sub>, 0.5  $\mu\text{g}$  pT/VP30<sub>RES</sub>, 2  $\mu\text{g}$  pT/ L<sub>RES</sub>, and 2  $\mu\text{g}$  3R–5R.

#### *EBOV-R VP30 serves as a transcription activator*

To confirm the finding that EBOV-R VP30 might function as a transcription activator, replicated and transcribed RNA species were detected using Northern blot analysis. Discrimination of the RNA species was performed by nuclease treatment and oligo(dT) binding. Transcribed viral mRNA is polyadenylated and binds to oligo(dT) cellulose. Furthermore, the transcribed RNA species are not encapsidated and can be degraded by nucleases. In contrast to the polyadenylated transcribed RNA, replicated RNA is not polyadenylated but is tightly encapsidated by the nucleocapsid proteins and therefore nuclease resistant. To isolate mRNA, BSR T7/5 cells were transfected with plasmids coding for the nucleocapsid proteins and the minigenome. At 2 days posttransfection, cellular RNA was isolated, purified by oligo(dT) cellulose binding, and subjected to Northern hybridization. When BSR T7/5 cells were used for isolation of replicated RNA, the background staining in the Northern hybridization was very high. To avoid this problem, Huh-T7 cells were transfected with the EBOV-R-specific plasmids. Constitutive expression of the T7 RNA polymerase in Huh-T7 cells is less efficient than in BSR T7/5 cells. In order to boost expression of the T7 RNA polymerase, Huh-T7 cells were cotransfected with the plasmid pC-T7Pol encoding the T7 RNA polymerase.

Because Huh-T7 cells are more sensitive to the addition of the reagent FUGENE6 than BSR T7/5 cells, the amount of FUGENE6 and consequently the amount of input DNA were slightly reduced as indicated in the legend of Fig. 5A. At 2 days posttransfection, cells were lysed and the lysates were treated with micrococcal nuclease prior to RNA purification. The purified RNA samples were analyzed by Northern hybridization using a negative-sense-orientated probe directed against the CAT gene to detect positive-sense minigenome RNA representing a replicative intermediate and mRNA. As a control, *in vitro* transcribed positive-sense 3E–5E minigenomic RNA was used (Fig. 5A, lane 1). The upper band shown in lane 1 represents the uncleaved transcript consisting of the minigenome and the ribozyme whereas the lower band corresponds to the processed minigenome after autocleavage of the ribozyme. Fig. 5A shows that three nucleocapsid proteins, NP, VP35, and L, were sufficient to support replication. In addition to minigenome 3R–5R, minigenome 3R–5R $\Delta$ 248, which contains only 248 nucleotides of the 3' end, was used as a template (Fig. 2). Interestingly, this minigenome was as efficiently replicated as minigenome 3R–5R, indicating that the first 248 nucleotides of the EBOV-R genome are sufficient to support replication (Fig. 5A). In contrast, transcription was strongly dependent on the presence of the fourth nucleocapsid protein, VP30 (Fig. 5B). Thus, the results of the Northern blot assays confirmed that EBOV-R VP30 acts as a transcription activation protein. The observed difference in size of the EBOV-Z and EBOV-R mRNAs is due to the different localization of the transcription stop signal of the L gene within the respective genome. The transcription stop signal of the EBOV-Z L gene is located only 66 nucleotides downstream of the ORF, and that of the EBOV-R L gene 691 nucleotides downstream of the ORF. Interestingly, the amount of detected mRNA was lower for the EBOV-R system compared to EBOV-Z, suggesting that transcription and/or replication occurred less efficiently. However, it cannot be ruled out that the smaller amount of transcribed EBOV-R mRNA is due to other possibilities, such as differences in T7 RNA polymerase efficiency, encapsidation, or mRNA stability.

#### *Exchange of the nucleocapsid proteins between EBOV-R and EBOV-Z*

Perhaps, due to the close relationship of EBOV-Z and EBOV-R, it might be possible to exchange the respective nucleocapsid proteins and the minigenomes. To this end, BSR T7/5 cells were transfected with all possible combinations of EBOV-Z and EBOV-R nucleocapsid protein genes and with either the EBOV-Z minigenome 3E–5E or the EBOV-R minigenome 3R–5R. Fig. 6A shows that it is indeed possible to exchange the EBOV-Z nucleocapsid proteins with those of EBOV-R when the 3E–5E minigenome was used with one exception: the combination of EBOV-Z VP35 and EBOV-R L did not work at all (Fig. 6A,

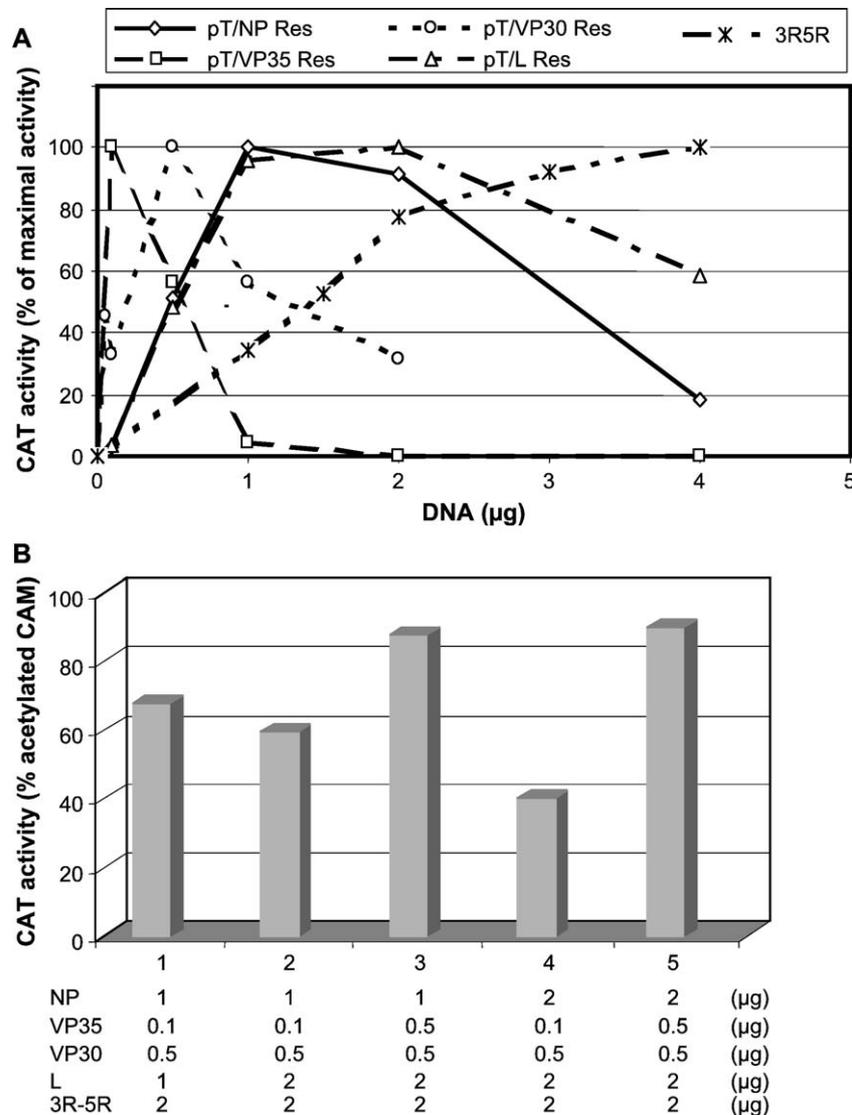


Fig. 4. Optimization of the reconstituted EBOV-R replication system by plasmid titration. BSR T7/5 cells were transfected with varying amounts of plasmids coding for the EBOV-R minigenome 3R–5R and the nucleocapsid proteins NP, VP35, VP35, and L. At 2 days posttransfection, cells were probed for CAT activity and acetylated chloramphenicol was quantified. (A) The first titration experiments were performed with set amounts of four of the plasmids (0.5 µg of pT/NP<sub>RES</sub>, 0.5 µg of pT/VP35<sub>RES</sub>, 0.5 µg pT/VP30<sub>RES</sub>, 1 µg of pT/L<sub>RES</sub>, and 2 µg of 3R–5R) and various amounts of the titrated plasmid as indicated in the figure. (B) Second round of plasmid titration based on the results shown in A. The amount of the different plasmids is indicated underneath the graph. The CAT assay is not in a linear range when the diacetylated form is synthesized. This was the case when values of more than 90% acetylation were achieved. Thus, the data shown here represent a qualitative rather than an exact quantitative comparison demonstrating clear differences in the replication/transcription efficiency of the different samples.

lanes 6, 9, 12, and 15). All of the other plasmid combinations resulted in CAT activity, however, the more EBOV-Z-specific plasmids were exchanged with those of EBOV-R, the less CAT activity was measured. Similar results were obtained when the EBOV-R-specific nucleocapsid protein genes were exchanged with those of EBOV-Z when minigenome 3R–5R was used (Fig. 6B). Again, the combination of EBOV-Z VP35 and EBOV-R L did not result in CAT activity (Fig. 6B, lanes 4, 7, 10, and 13), indicating that these two proteins are not able to constitute an active polymerase complex. In addition, the combination of EBOV-R NP and EBOV-Z L seemed to be largely nonfunctional in the EBOV-R minireplicon system (Fig. 6B,

lanes 6, 11, 12, and 16). Taken together, these results suggest a distinct functional and structural similarity of EBOV-R and EBOV-Z nucleocapsid proteins. Exchange of the EBOV-R nucleocapsid proteins with those of MBGV, however, did not result in any transcription activity (data not shown).

#### *EBOV-Z VP35 and EBOV-R L do not constitute an active polymerase complex but interact*

In order to analyze whether the lack of function of EBOV-Z VP35 and EBOV-R L as polymerase complex might be due to the incapability of both proteins to interact,

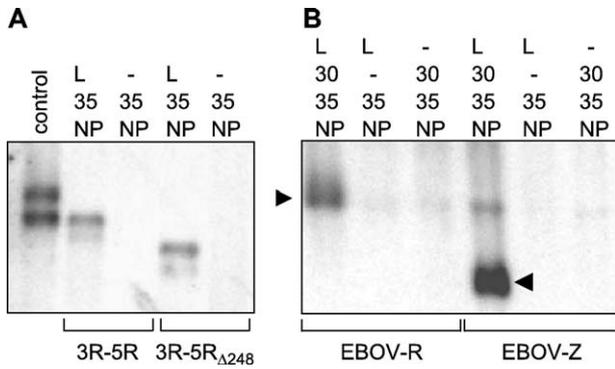


Fig. 5. Detection of replicated and transcribed RNA species. (A) Huh-T7 cells were transfected with plasmids encoding the EBOV-R nucleocapsid proteins (0.7  $\mu$ g of pT/NP<sub>RES</sub>, 0.35  $\mu$ g of pT/VP35<sub>RES</sub>, and 1.5  $\mu$ g of pT/L<sub>RES</sub>) and either minigenome 3R–5R or minigenome 3R–5R $\Delta$ <sub>248</sub> (1  $\mu$ g each) as indicated in the figure. In addition, cells were transfected with 0.5  $\mu$ g pC-T7Pol. At 2 days posttransfection, cells were lysed and treated with micrococcal nuclease. Subsequently, protected RNA was purified, subjected to Northern blot analysis, and detected by using a negative-sense RNA probe binding to the CAT gene. (B) BSR T7/5 cells were either transfected with plasmids coding for the EBOV-Z minigenome system (0.5  $\mu$ g of pT/NP<sub>EBO</sub>, 0.5  $\mu$ g of pT/VP35<sub>EBO</sub>, 0.1  $\mu$ g pT/VP30<sub>EBO</sub>, 1  $\mu$ g of pT/L<sub>EBO</sub>, and 1  $\mu$ g of 3E–5E) or with plasmids coding for the EBOV-R minigenome system. At 2 days posttransfection, cells were lysed, total cellular RNA was isolated, and mRNA species were extracted by oligo(dT) cellulose. Eluted mRNAs were subjected to Northern blot analysis as described above. Specific mRNA bands are marked by arrowheads. As a control, in vitro transcribed positive-sense 3E–5E minigenomic RNA was used.

protein binding studies were performed. For the closely related MBGV, it has been shown previously that VP35 interacts with L. Also, trimeric complexes consisting of NP, VP35, and L have been identified indicating that VP35 directs L to the NP-encapsidated RNA genomes by acting as a linker protein between NP and L (Becker et al., 1998). When filoviral NP is expressed in the absence of other viral proteins, it forms characteristic inclusion bodies that contain highly ordered tubular structures resembling the nucleocapsids in virus-infected cells (Kolesnikova et al., 2000). In contrast, the other nucleocapsid proteins are more or less homogeneously distributed in the cytoplasm when solely expressed in cells. In cells transfected with MBGV NP and VP35 genes, coexpression of NP and VP35 led to relocation of VP35 in NP-derived inclusion bodies. When MBGV NP, VP35, and L are coexpressed, L is also directed into NP-derived inclusions (Becker et al., 1998). Because EBOV-Z NP and VP35 are also found to be localized in inclusion bodies in EBOV-Z-infected cells (Bjornedal et al., 2003), it is possible that EBOV NP, VP35, and L would interact similarly to MBGV proteins. To investigate if EBOV-Z VP35 and EBOV-R L do interact, immunofluorescence analyses were performed. To this end, HeLa cells were infected with MVA-T7 and subsequently transfected with plasmids coding for the NP, VP35, and L proteins of EBOV-Z and EBOV-R in different combinations. Because no antibodies directed against the L proteins are available, all L constructs were tagged by a FLAG epitope. First, it was confirmed that the typical inclusion

bodies were formed when NP and VP35 were coexpressed in the absence of L (Fig. 7F). Subsequently, it is shown that the L proteins of EBOV-Z and EBOV-R are homogeneously distributed in the cytoplasm of infected and transfected cells when expressed in the absence of other filoviral proteins (Fig. 7A). Coexpression of autologous NP and VP35 with L

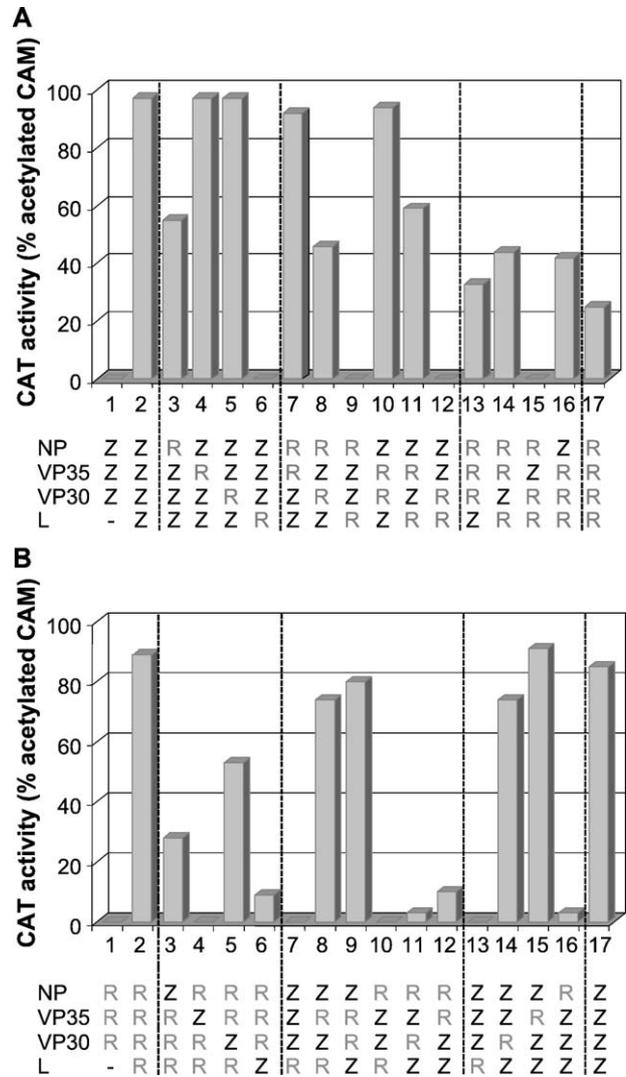


Fig. 6. Exchange of EBOV-Z and EBOV-R nucleocapsid proteins. BSR T7/5 cells were transfected with plasmids coding for EBOV-Z and EBOV-R nucleocapsid proteins, respectively, and with the plasmid encoding the respective minigenome. At 2 days posttransfection, cells were lysed and CAT activity was determined. (A) Exchange of EBOV-Z nucleocapsid proteins by EBOV-R nucleocapsid proteins. Cells were transfected with plasmids coding for the minigenome 3E–5E and EBOV-Z nucleocapsid proteins (lanes 1 and 2). The amount of used EBOV-Z plasmid DNA is given in the legend to Fig. 5. In the following lanes, one, two, three, or all nucleocapsid proteins were exchanged by the respective EBOV-R homologous proteins. The amount of the respective EBOV-R plasmid DNA used for transfection is given in the legend to Fig. 4. (B) Exchange of EBOV-R nucleocapsid proteins by EBOV-Z nucleocapsid proteins. Cells were transfected with plasmids coding for the minigenome 3R–5R and EBOV-R nucleocapsid proteins (lanes 1 and 2). In the following lanes, one, two, three, or all nucleocapsid proteins were exchanged by the respective EBOV-Z counterpart. Refer to the Fig. 4, legend, for a description of the quantification of the CAT assay.

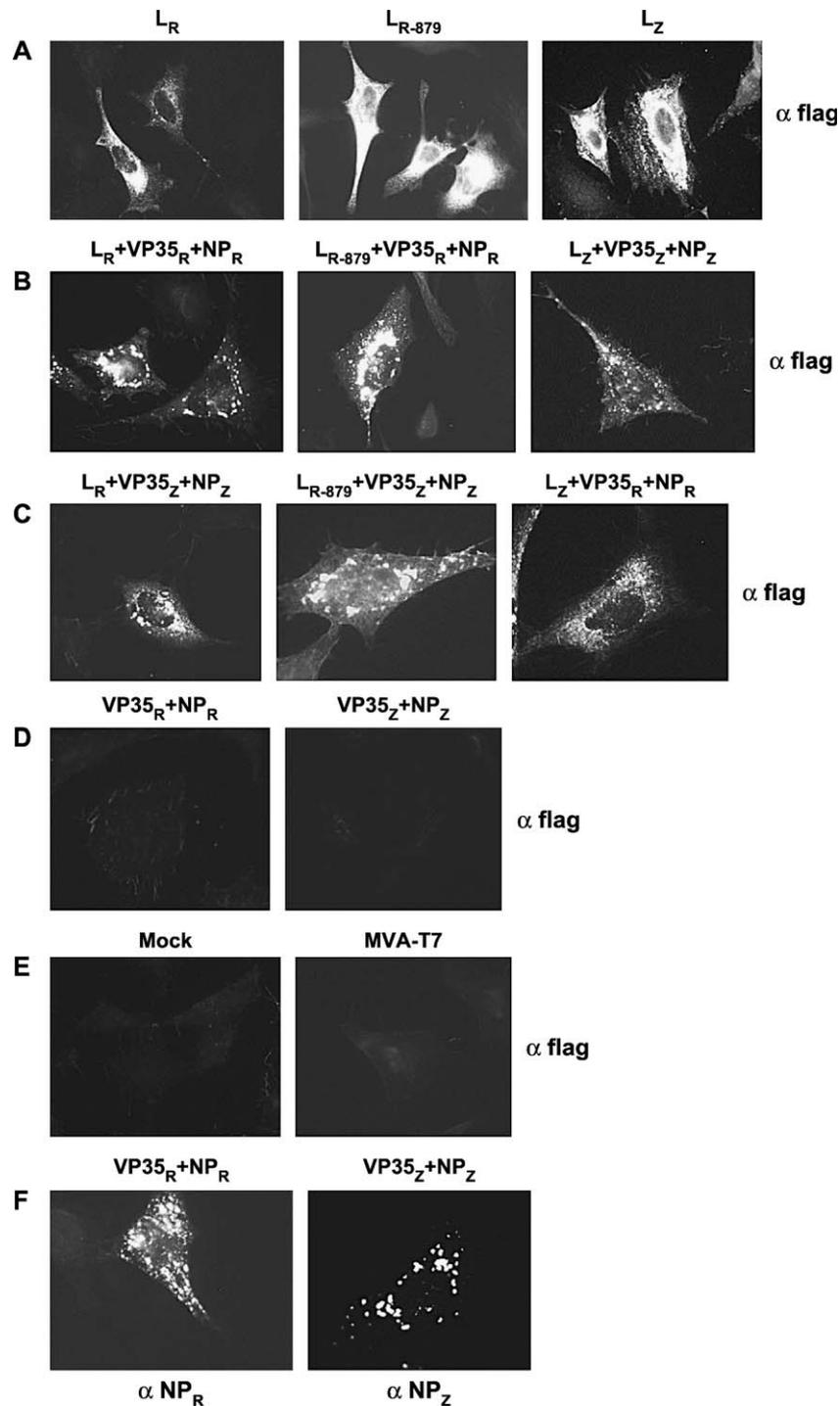


Fig. 7. Immunofluorescence analysis to investigate binding of L to VP35. HeLa cells grown on glass cover slips were infected with MVA-T7 and 1 h pi transfected with various combinations of plasmids coding for EBOV-Z and EBOV-R NP, VP35, and  $L_{Flag}$ . Because no antibodies directed against the L proteins are available, the EBOV-Z and EBOV-R L proteins were tagged by the FLAG epitope. In addition to the full-length EBOV-R L, the truncated protein EBOV-R  $L_{Flag-879}$  comprising the aminoterminal part of the protein up to amino acid 879 was used. At 8–12 h posttransfection, cells were fixed, permeabilized, and subjected to immunofluorescence analysis. The FLAG-tagged EBOV-Z and EBOV-R L proteins were visualized by using a monoclonal anti-FLAG antibody ( $\alpha$ -FLAG). EBOV-Z and EBOV-R NP were each stained with a specific monoclonal antibody ( $\alpha$ -NP<sub>Z</sub>,  $\alpha$ -NP<sub>R</sub>). Bound antibodies were detected with FITC- or rhodamine-conjugated goat anti-mouse IgG antibodies. (A) Solely expressed EBOV-R  $L_{Flag}$  ( $L_R$ ), EBOV-R  $L_{Flag-879}$  ( $L_{R-879}$ ), or EBOV-Z  $L_{Flag}$  ( $L_Z$ ), respectively; (B) coexpression of L with autologous NP and VP35; (C) coexpression of L with heterologous NP and VP35; (D) coexpression of EBOV-R NP ( $NP_R$ ) with VP35 ( $VP35_R$ ) and EBOV-Z NP ( $NP_Z$ ) with VP35 ( $VP35_Z$ ), respectively, stained with an anti-FLAG antibody; (E) mock- and MVA-T7-infected cells stained with an anti-FLAG antibody; (F) coexpression of  $NP_R$  and  $VP35_R$  stained with an anti- $NP_R$  antibody and coexpression of  $NP_Z$  and  $VP35_Z$  stained with an anti- $NP_Z$  antibody.

led to relocalization of L into NP-derived inclusion bodies (Fig. 7B). Interestingly, an EBOV-R-specific truncated L protein comprising the first 879 amino acids of L (L<sub>879</sub>) also relocalized into the NP-derived inclusions, indicating that the N-terminal region of EBOV-R L is sufficient for interaction with VP35. When EBOV-R L, EBOV-R L<sub>879</sub>, and EBOV-Z L, respectively, were coexpressed with heterologous NP and VP35, the L proteins were detected in NP-derived inclusions, suggesting that complex formation took place in each case (Fig. 7C). These data indicate that although EBOV-Z VP35 and EBOV-R L are able to interact, the formed complex is not functional.

### Chimeric minigenomes

In Figs. 6A (lanes 17) and 6B (lane 17), it was shown that the replication and transcription promoter sequences of EBOV-Z were accepted by EBOV-R and vice versa. It was now of interest to investigate whether chimeric minigenomes whose 3' or 5' ends were replaced by the respective 3' or 5' regions of the heterologous virus were accepted as templates for replication and transcription. Fig. 8 shows that the chimeric minigenomes were efficiently replicated and transcribed by both polymerase complexes, indicating that the 3' and 5' promoter regions of EBOV-R and EBOV-Z show a high degree of structural similarity. Again, the replication/transcription efficiency of the different minigenomes was always lower in the EBOV-R replication system.

### Discussion

In the present study, we have developed a reconstituted minireplicon system for EBOV-R. We showed that three out of the four nucleocapsid proteins were sufficient to support EBOV-R replication, whereas for transcription, the fourth nucleocapsid protein VP30 was also needed. These data indicate that the replication and transcription strategy of

EBOV-R is similar to that of EBOV-Z, thus the same proteins are required for replication and transcription (Mühlberger et al., 1999, 2002; Volchkov et al., 2001; Watanabe et al., 2004). In contrast, transcription of MBGV minigenomes occurs independently of VP30 (Mühlberger et al., 1998). It has been shown for EBOV-Z that VP30-dependent transcription is regulated by an RNA secondary structure formed by the transcription start signal of NP gene and downstream sequences (Weik et al., 2002). While the NP gene transcription start sites of EBOV-R and EBOV-Z are identical, there are two nucleotide exchanges within the downstream located sequences involved in stem-loop structure formation. Interestingly, these two nucleotides are located within the loop whereas the nucleotides involved in base pairing are conserved between EBOV-R and EBOV-Z, thus leading to formation of similar RNA structures. EBOV-Z VP30 is a zinc binding protein. Destruction of the zinc binding domain leads to a loss of function of the protein as transcription activator (Modrof et al., 2003). This VP30-specific zinc binding domain is well conserved between EBOV-Z and EBOV-R, suggesting that EBOV-Z and EBOV-R transcription processes might be regulated by the same mechanism.

Titration experiments performed with the plasmids encoding the EBOV-R nucleocapsid proteins revealed optimal amounts for NP, VP35, VP30, and L leading to maximal CAT activity. When the amount of input DNA was further increased, CAT activity declined, suggesting that overexpression of one nucleocapsid protein gene led to an imbalance between the different components of the replication complex. Similar findings were obtained concerning the NP, VP35, and VP30 plasmid DNA of EBOV-Z; however, the total amount of each EBOV-Z input DNA needed for maximal CAT activity was different compared to the EBOV-R system (Mühlberger et al., 1999). While large amounts of EBOV-Z L input DNA were tolerated in the EBOV-Z system without suppressing reporter gene activity (Mühlberger et al., 1999), increasing amounts of EBOV-R L input DNA above 2 µg led to a decrease of CAT activity. This difference might be due to different expression rates of these proteins.

The nucleocapsid proteins of EBOV-Z and EBOV-R show a varying degree of homology, ranging from 67.6% to 74.8% identity (Groseth et al., 2002; Ikegami et al., 2001). The leader and the trailer regions containing the signals for encapsidation, replication, and transcription initiation are also highly conserved between EBOV-Z and EBOV-R. This high degree of conservation is reflected by the fact that EBOV-Z nucleocapsid proteins were able to rescue the EBOV-R-specific minigenome and vice versa, thus accepting the replication and transcription promoters of the heterologous genome. Moreover, chimeric minigenomes containing the leader of EBOV-Z in combination with the trailer of EBOV-R and vice versa were replicated and transcribed by both sets of nucleocapsid proteins. Interestingly, the amount of CAT activity was lower with the

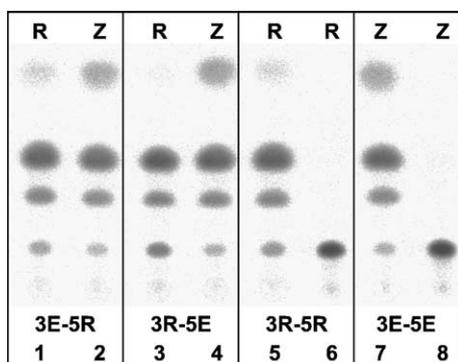


Fig. 8. Transcription and replication of chimeric minigenomes. BSR T7/5 cells were transfected with plasmids coding either for EBOV-Z (Z) or EBOV-R (R) nucleocapsid proteins NP, VP35, VP30, and L. In lanes 6 and 8, the plasmid coding for the respective L protein was omitted. Cells additionally were transfected with the respective minigenome as indicated in the figure. Two days later, cells were assayed for CAT activity.

EBOV-R system, suggesting that the replication/transcription complex of EBOV-R might function less efficiently compared to EBOV-Z. Amino acid sequence comparison of the nucleocapsid proteins of MBGV and Ebola viruses revealed a degree of homology between 35% and 49% identity only. Consequently, it was not possible to exchange the nucleocapsid proteins of EBOV-R and MBGV. Similar results were obtained with MBGV and EBOV-Z NP, VP35, and L. In contrast, MBGV VP30 was able to support EBOV-Z-specific transcription, albeit with a low efficiency (Mühlberger et al., 1999). It has been reported for other members of the order Mononegavirales that they accept heterologous minigenomic RNA derived from closely related viruses as templates for replication and transcription. Thus, human parainfluenza virus (PIV) types 1 and 3 supported replication of a Sendai virus minigenome, whereas measles virus did not (Curran and Kolakofsky, 1991; Pelet et al., 1996). Yunus et al. (1999) demonstrated that a bovine respiratory syncytial virus (RSV) minireplicon was rescued in cells infected with human RSV and ovine RSV, respectively, but not in bovine PIV type-3-infected cells. It is noteworthy that the nucleocapsid proteins of human, bovine, and ovine RSV show a very high level of amino acid identity in the range of 80–93% (Yunus et al., 1999). On the other hand, Dimock and Collins (1993) observed that human PIV type 3 minigenomes were neither rescued by human RSV nor by the very closely related bovine PIV type 3. Taken together, these data indicate that the replication complexes are specific for the autologous RNA species but that occasionally very closely related viruses have the capacity to replicate and transcribe heterologous RNAs.

When the nucleocapsid proteins of EBOV-Z and EBOV-R were exchanged, almost all combinations were able to support replication and transcription; however, certain combinations significantly reduced replication efficiency. As for the EBOV-Z system, it seemed that the more heterologous the proteins the less efficient the replication. Especially, this was true for the exchange of NP: with one exception, swapping of the NP protein led to a remarkable decrease in CAT activity. For the EBOV-R system, it was shown that the combination of EBOV-R NP and EBOV-Z L dramatically diminished replication and transcription activity. These results have interesting connotations when applied towards construction of recombinant attenuated Ebola viruses. For a recombinant human PIV type 3 virus, in which the NP gene was replaced by the bovine PIV type 3 counterpart, it has been demonstrated that the virus was attenuated in primates (Bailly et al., 2000).

The only nucleocapsid combination not working at all was the combination of EBOV-Z VP35 and EBOV-R L. Filovirus VP35 is the equivalent of paramyxo- and rhabdovirus P proteins and constitutes together with L the polymerase complex (Mühlberger et al., 1999). For MBGV, it has been shown that VP35 binds to L and NP, thus directing L into NP-derived inclusion bodies (Becker et al.,

1998). Here, we have shown indirectly that EBOV-Z VP35 and EBOV-R L interaction still occurred; however, the enzymatic function of this complex was lost.

In summary our data revealed that the replication/transcription complexes of EBOV-Z and EBOV-R show a high degree of functional homology. The leader and trailer sequences are conserved and are accepted by the nucleocapsid proteins of the heterologous virus. In addition, it was possible to exchange almost all EBOV-R and EBOV-Z nucleocapsid proteins, again underlining the close relationship between these viruses. It seems, however, that at least in the minigenome systems, EBOV-R replication and transcription is less efficient compared to EBOV-Z. Future studies will examine if the delayed replication cycle and the reduced virulence of EBOV-R are due to decelerated replication and transcription processes.

## Materials and methods

### *Cell lines and viruses*

Vero and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Huh-T7 cells (derived from the human hepatoblastoma cell line Huh7) constitutively expressing the T7 RNA polymerase (kindly provided by V. Gaussmüller, Department of Medical Molecular Biology, University of Lübeck, Germany) were grown in DMEM supplemented with 10% FCS and 1 mg/ml geneticin (Schultz et al., 1996). BSR T7/5 cells (derived from a BHK-21 cell line), constitutively expressing the T7 RNA polymerase (kindly provided by K. K. Conzelmann, Max von Pettenkofer Institute and Gene Center, Munich, Germany), were cultured as described by Buchholz et al. (1999). The recombinant vaccinia virus MVA-T7 containing the T7 RNA polymerase gene was grown in chicken embryo fibroblasts (Sutter et al., 1995). EBOV-Z, strain Mayinga and the EBOV-R isolate Pennsylvania, 1989, were propagated in vero cells. For preparation of virus stocks, supernatants were harvested at 4 days (EBOV-Z) or at 14 days pi (EBOV-R). To determine the titer of the virus stocks, TCID<sub>50</sub> assays were performed. Briefly, vero cells seeded in 96-well plates were infected by 10-fold dilutions of EBOV-R or EBOV-Z virus stocks, respectively, and examined for cytopathic effects by light microscopy. The TCID<sub>50</sub> titer of the EBOV-Z stock was determined at 7–8 days pi and that of the EBOV-R stock at 14 days pi. Calculation of TCID<sub>50</sub> was performed by the Spearman–Kärber method.

For cloning of the nucleocapsid protein genes and the minigenome, the genomic RNA of EBOV-R was used. The virus was propagated in vero cells, supernatants were harvested at 10 days pi, clarified by a short centrifugation step, mixed with an equal volume of RLT buffer of an RNeasy kit (Qiagen), and supplied with 0.6 volume of 100% ethanol. Finally, the RNA was purified according to

the supplier's instructions. All work with infectious EBOV-R and EBOV-Z was performed in the BSL4 facility of the University of Marburg.

#### *Determination of the 3' and 5' ends of the EBOV Reston genome and sequencing of the nucleocapsid protein genes*

Determination of the 5' ends of the EBOV-R genome was performed by using a 5' RACE kit (Invitrogen). For cDNA synthesis, a primer was used which bound to nucleotides 18,483–18,506. Tailing of the cDNA was done with dGTP or dCTP, respectively. The 5' end was determined after nested PCR with the tailed cDNA as a template and subsequent sequencing of the resulting PCR fragments. To determine the 3' end, the genomic RNA was circularized by using T4 RNA ligase (Roche). The ligated RNA was then used as a template for RT-PCR using a primer pair binding to nucleotides 420–443 and 18,483–18,506, respectively. RT-PCR was followed by two nested PCRs using a primer pair binding to nucleotides 313–338 and 18,570–18,593, respectively, for the first nested PCR and a primer binding to nucleotides 116–140 and 18,772–18,794, respectively, for the second nested PCR. For sequencing of the nucleocapsid protein genes, RT-PCR was performed with the genomic EBOV-R RNA as template. Resulting fragments were subcloned into the vector TOPO TA (Invitrogen) and subjected to sequencing (GenBank accession number: AY769362).

#### *Cloning of the EBOV Reston nucleocapsid protein genes and the minigenomes*

To generate cDNA clones encoding the nucleocapsid protein genes NP, VP35, VP30, and L, the EBOV-R-specific genomic RNA was reverse transcribed with Omniscript Reverse Transcriptase (Qiagen) and amplified via PCR with Pwo polymerase (Roche) by using purified genomic EBOV-R RNA as a template. The amplified genes were inserted into the expression vector pTM1 under the control of the T7 RNA polymerase promoter. To insert additional restriction sites, a DNA linker containing the recognition sites for *Sfi*I, *Not*I, and *Sac*II was cloned into the *Bam*HI site of pTM1. The NP gene flanked by an *Eco*RI and a *Not*I site fragment comprised nucleotides 464–2683 of the viral RNA, the VP35 and VP30 gene fragments, respectively, were flanked by *Eco*RI and *Xho*I sites and comprised nucleotides 3155–4144 (VP35) and 8487–9353 (VP30), respectively. For cloning of the L gene, which is 6636 nucleotides in length, three fragments of about 2 kb were amplified by RT-PCR. These fragments were ligated by using internally located restriction sites (*Bst*BI and *Nru*I) and inserted in the pTM1 vector by using the flanking restriction sites *Xma*I and *Bam*HI. The generated plasmids were designated pT/NP<sub>RES</sub>, pT/VP35<sub>RES</sub>, pT/VP30<sub>RES</sub>, and pT/L<sub>RES</sub>. Mutations within the genes, which were introduced during cloning, were removed by *in vitro* mutagenesis using the Quickchange

mutagenesis PCR kit (Stratagene) according to the supplier's instructions.

Because no antibodies directed against the L proteins of EBOV-Z and EBOV-R are available, both proteins were tagged with a FLAG epitope located at the aminoterminal end of the proteins. Construction of the FLAG-tagged L proteins was performed by using PCR technique with either pT/L<sub>RES</sub> or pT/L<sub>EBO</sub> (Mühlberger et al., 1999) as template. In addition, a truncated FLAG-tagged EBOV-R L protein was constructed containing the first 879 amino acids of the protein. The resulting plasmids were designated pT/L<sub>RES</sub>-Flag, pT/L<sub>RES</sub>-879-Flag, and pT/L<sub>EBO</sub>-Flag.

The EBOV-R-specific minigenome 3R–5R contains the 3' and 5' ends of the EBOV-R genome separated by the CAT gene as reporter gene. The 3' end comprises nucleotides 1–466, the 5' end nucleotides 18192–18895. As a backbone for generation of the EBOV-R-specific minigenome 3R–5R and the chimeric minigenomes 3R–5E and 3E–5R, the EBOV-Z-specific minigenome 3E–5E was used (Mühlberger et al., 1999). In this minigenome, the EBOV-Z 3' end was exchanged with the EBOV-R-specific 3' end by using the restriction sites *Rsr*II and *Nde*I, and the EBOV-Z 5' end was exchanged with the EBOV-R 5' end by using the restriction sites *Not*I and *Xma*I (Fig. 2). For generation of minigenome 3R–5R<sub>Δ248</sub> containing a truncated 3' region, the 3' end of minigenome 3R–5R was removed by digestion with *Nde*I and *Rsr*II and replaced by a PCR fragment containing nucleotides 1–248 of the EBOV-R sequence which was flanked by *Nde*I and *Rsr*II restriction sites.

#### *Transfection of BSR T7/5 and Huh-T7 cells*

Transfection of BSR T7/5 or Huh-T7 cells ( $5 \times 10^5$  in a 7-cm<sup>2</sup> well) was performed using FUGENE6 (Roche Molecular Biochemicals) as described previously (Modrof et al., 2002). The amount of plasmids used for the respective transfection procedure is indicated in the figure legends. Cells were harvested at 2 days posttransfection, and cell lysates were either used for CAT assays or for RNA analyses.

#### *Detection of transcribed and replicated RNA*

BSR T7/5 or Huh-T7 cells grown in 6-well plates were transfected with EBOV-R-specific plasmids as described above. When Huh-T7 cells were used, cells were transfected additionally with 0.5 μg of plasmid pC-T7Pol expressing the T7 RNA polymerase (kindly provided by T. Takimoto, St. Jude Children's Research Hospital, Memphis, TN, USA, and Y. Kawaoka, University of Wisconsin, Madison, WI, USA; Neumann et al., 2002). At 2 days posttransfection, cells were washed twice with PBS, scraped into the washing buffer, and pelleted. For mRNA isolation, total cellular RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer's instructions. Subsequently, polyadenylated RNA was purified by using oligo(dT) cellulose as

described previously (Mühlberger et al., 1999). Isolation of replicated RNA is described elsewhere (Grosfeld et al., 1995; Mühlberger et al., 1999). Briefly, cell pellets were resuspended in 200  $\mu$ l micrococcal nuclease buffer (10 mM NaCl, 10 mM Tris [pH 7.5], 10 mM MgCl<sub>2</sub>, 5% Triton X-100, 0.3% sodium deoxycholate, 10 mM CaCl<sub>2</sub>), sheared and sonicated for 1 min. After addition of 51 U micrococcal nuclease (Roche Molecular Biochemicals), cell lysates were incubated for 70 min at 33 °C. Thereafter, protected RNA species were purified by using an RNeasy kit (Qiagen). Finally, the replicated and transcribed RNA species were subjected to Northern blot analysis. To detect the specific RNA species, the negative-sense digoxigenin-labeled riboprobe DIG-BS/CAT, which binds to the CAT gene, was used (Mühlberger et al., 1998).

#### CAT assay

BSR T7/5 cells were transfected as described above. At 2 days posttransfection, cells were washed twice with PBS and scraped into 150  $\mu$ l of reporter lysis buffer (Promega) per well. CAT activity was determined using a standard protocol. The amount of acetylated chloramphenicol was quantified with a Bioimager Analyzer (Fuji BAS-1000) using the Raytest TINA software.

#### Immunofluorescence analysis

##### Infected and transfected HeLa cells

HeLa cells grown on glass coverslips to 50% confluence were infected with MVA-T7 at an MOI of 5. At 1 h pi, cells were transfected by using Lipofectin (GIBCO-BRL) with various combinations of the following plasmids: 1  $\mu$ g of pT/L<sub>RES</sub>-Flag, pT/L<sub>RES-879</sub>-Flag, or pT/L<sub>EBO</sub>-Flag, and 0.5  $\mu$ g of pT/NP<sub>RES</sub>, pT/VP35<sub>RES</sub>, pT/NP<sub>EBO</sub>, or pT/VP35<sub>EBO</sub>. At 8–12 h posttransfection, cells were washed three times with PBS and fixed for 15 min at room temperature in 3% paraformaldehyde. Thereafter, cells were treated with 0.1 M glycine and permeabilized for 15 min with 0.1% Triton X-100. Cells were incubated with the respective antibodies for 1 h at room temperature and then washed four times with PBS and stained with the appropriate secondary antibodies for 1 h at room temperature. To detect EBOV-Z NP, a monoclonal antibody directed against EBOV-Z NP was used (1:20 dilution). For staining of EBOV-R NP, a monoclonal antibody directed against EBOV-R NP was used (1:100 dilution; kindly provided by A. Sanchez, Centers for Disease Control, Atlanta, GA USA). Staining of the FLAG-tagged L proteins was performed with an anti-FLAG M2 monoclonal antibody (1:200 dilution, Sigma-Aldrich). Bound antibodies were detected with FITC- or rhodamine-conjugated goat anti-mouse IgG antibodies (1:200 dilution, Dianova). Finally, cells were washed four times with PBS, dipped into H<sub>2</sub>O, covered with mounting medium, and examined using a fluorescence microscope.

##### Infected vero cells

Vero cells seeded in chamber slides were infected with EBOV-Z and EBOV-R, respectively, at an MOI of 10<sup>-2</sup>. At 1–7 days pi, cells were inactivated by addition of 4% paraformaldehyde for at least 12 h. Cells were treated with 0.1 M glycine and permeabilized for 5 min at room temperature with 0.1% Triton X-100. Incubation with the respective antibody was performed as described above. To detect EBOV-Z infection, the abovementioned monoclonal antibody directed against EBOV-Z NP was used. For staining of EBOV-R-infected cells, the monoclonal antibody directed against EBOV-R NP was used. Bound antibodies were detected with a Texas red-conjugated goat anti-mouse IgG antibody (1:200 dilution, Dianova). To visualize the nuclei, cells were stained additionally with 0.1  $\mu$ g/ml 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) for 5 min at room temperature.

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