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A Novel Life Cycle Modeling System for Ebola Virus Shows a Genome Length-Dependent Role of VP24 in Virus Infectivity

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ABSTRACT

Work with infectious Ebola viruses is restricted to biosafety level 4 (BSL4) laboratories, presenting a significant barrier for studying these viruses. Life cycle modeling systems, including minigenome systems and transcription- and replication-competent virus-like particle (trVLP) systems, allow modeling of the virus life cycle under BSL2 conditions; however, all current systems model only certain aspects of the virus life cycle, rely on plasmid-based viral protein expression, and have been used to model only single infectious cycles. We have developed a novel life cycle modeling system allowing continuous passaging of infectious trVLPs containing a tetracistronic minigenome that encodes a reporter and the viral proteins VP40, VP24, and $GP_{1,2}$. This system is ideally suited for studying morphogenesis, budding, and entry, in addition to genome replication and transcription. Importantly, the specific infectivity of trVLPs in this system was ~500-fold higher than that in previous systems. Using this system for functional studies of VP24, we showed that, contrary to previous reports, VP24 only very modestly inhibits genome replication and transcription when expressed in a regulated fashion, which we confirmed using infectious Ebola viruses. Interestingly, we also discovered a genome length-dependent effect of VP24 on particle infectivity, which was previously undetected due to the short length of monocistronic minigenomes and which is due at least partially to a previously unknown function of VP24 in RNA packaging. Based on our findings, we propose a model for the function of VP24 that reconciles all currently available data regarding the role of VP24 in nucleocapsid assembly as well as genome replication and transcription.

IMPORTANCE

Ebola viruses cause severe hemorrhagic fevers in humans, with no countermeasures currently being available, and must be studied in maximum-containment laboratories. Only a few of these laboratories exist worldwide, limiting our ability to study Ebola viruses and develop countermeasures. Here we report the development of a novel reverse genetics-based system that allows the study of Ebola viruses without maximum-containment laboratories. We used this system to investigate the Ebola virus protein VP24, showing that, contrary to previous reports, it only modestly inhibits virus genome replication and transcription but is important for packaging of genomes into virus particles, which constitutes a previously unknown function of VP24 and a potential antiviral target. We further propose a comprehensive model for the function of VP24 in nucleocapsid assembly. Importantly, on the basis of this approach, it should easily be possible to develop similar experimental systems for other viruses that are currently restricted to maximum-containment laboratories.

Ebola virus is a negative-sense RNA virus (NSV) and the causative agent of a severe hemorrhagic fever in humans and nonhuman primates, with case fatality rates being up to 90% (1). The VP24 protein is unique to filoviruses and has no known homologues among other NSVs. In Ebola virus it has been identified as an interferon antagonist capable of blocking both the JAK-STAT and p38 mitogen-activated protein kinase pathways (2, 3), and extensive work has been undertaken to further characterize this role and connect it to the structure of VP24 (4–7). In addition to this, there is an increasing body of evidence suggesting that VP24 fulfills an additional function that is related to the formation of nucleocapsids; however, a comprehensive model of the function of VP24 with respect to such a role is still lacking.

Ebola virus is classified as a biosafety level 4 (BSL4) agent, and work with infectious Ebola virus is therefore restricted to a few maximum-containment laboratories worldwide, which is a significant hindrance to the study of Ebola virus biology and the development of countermeasures. The same limitations exist for a number of other viruses that are classified as BSL4 organisms, including other filoviruses, paramyxoviruses, New and Old World arenaviruses, and some bunyaviruses. While a recombinant Ebola virus in which an essential viral gene had been deleted from the genome was recently developed, this virus remains classified as a BSL3 agent (8). In order to study the Ebola virus life cycle under BSL2 conditions, a number of life cycle modeling systems have been developed over the years (reviewed in reference 9). These systems are generally based on minigenomes, i.e., miniature ver-

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sions of the virus genome from which viral genes have been removed and replaced by a reporter gene (10). Minigenomes are expressed in mammalian cells (most often by means of initial RNA transcription from a cDNA plasmid by a coexpressed T7 RNA polymerase) together with the nucleoprotein NP, the viral polymerase L, the polymerase cofactor VP35, and the transcriptional activator VP30 (together referred to as ribonucleoprotein complex [RNP] proteins), resulting in minigenome replication and transcription and, ultimately, reporter activity reflecting these processes (10).

As an extension of minigenome systems, transcription- and replication-competent virus-like particle (trVLP) systems have been developed (11, 12). These systems are based on a minigenome system, but in addition to expressing a minigenome and the RNP proteins from expression plasmids, the other viral structural proteins—VP40, $GP_{1,2}$, and, in some cases, VP24—are also expressed in mammalian cells, which are called producer cells. Since expression of VP40 is sufficient for the formation of virus-like particles (13), this leads to the formation of trVLPs, which are coated with $GP_{1,2}$ and harbor minigenome-containing nucleocapsids. These trVLPs can infect target cells and deliver the minigenome into these cells. In a trVLP system with untransfected or naive target cells, the minigenome undergoes primary transcription mediated by the RNP proteins brought into the target cells within the trVLPs (in the form of nucleocapsids). In contrast, in trVLP systems with pretransfected target cells, the minigenome is replicated and undergoes secondary transcription mediated by RNP proteins provided in *trans* from expression plasmids. In both cases, this results in reporter activity that reflects genome replication in producer cells, the production of infectious trVLPs, their entry into target cells, and either primary transcription in naive target cells or genome replication and secondary transcription in pretransfected target cells (11, 12).

While minigenome and trVLP systems are powerful tools to study the life cycle of Ebola viruses under BSL2 conditions, they are restricted in that they model only individual aspects of the virus life cycle and have thus far been used to model only a single infectious cycle. Moreover, both minigenome and trVLP systems rely on the plasmid-based overexpression of viral proteins, which does not accurately reflect the viral life cycle, where expression of these proteins is tightly regulated with respect to both quantity and timing. Consequently, trVLP systems produce an abundance of noninfectious particles, making biochemical analyses of trVLP preparations challenging (14).

Here we report a novel life cycle modeling system for Ebola virus and its application to the study of VP24. This system is based on the use of multicistronic minigenomes that, in addition to a reporter, express some of the viral proteins from the minigenome rather than from expression plasmids. Using this system, it was possible to model almost all aspects of the Ebola virus life cycle over the course of several infectious cycles and to do so safely under BSL2 conditions. Importantly, this concept should be easily applicable to other BSL4 NSVs, allowing comprehensive studies of their biology under BSL2 conditions as well. Using this system, we have analyzed the function of VP24 in the viral life cycle, and we show that, in contrast to what has been reported in previous studies, VP24 has only a very modest influence on genome replication and transcription when expressed in a regulated fashion, findings that were confirmed using a recombinant Ebola virus. In addition, we discovered a previously unknown, genome length-dependent role of VP24 for the infectivity of virus particles and show that this role can be at least partially explained by a previously undescribed function of VP24 in the packaging of genomic RNA into particles. Based on our findings, we propose a model that explains the function of VP24 in nucleocapsid assembly.

MATERIALS AND METHODS

Cells and viruses. Vero E6 (African green monkey kidney; ATCC CRL-1586) and 293 (human embryonic kidney; ATCC CRL-1573) cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS; Life Technologies), 2 mM L-glutamine (Q; Life Technologies), and 1× penicillin-streptomycin (PS; Life Technologies) and were grown at 37°C with 5% CO_2.

The recombinant Ebola viruses expressing firefly luciferase (Ebola virus H.sapiens-rec/COD/1976/Mayinga-rgEBOV-luc2, GenBank accession number KF990214; here called rgEBOV-luc2) and green fluorescent protein (GFP; Ebola virus H.sapiens-rec/COD/1976/Mayinga-rgEBOV-GFP, GenBank accession number KF990213; here called rgEBOV-GFP) have been previously described (15). Construction of full-length cDNA plasmids and rescue of recombinant viruses were performed as previously described (16). All work with infectious Ebola viruses was performed in the BSL4 laboratory at the Rocky Mountain Laboratories (RML), Division of Intramural Research (DIR), National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), following approved standard operation protocols.

Plasmids. Expression plasmids for Ebola virus proteins and the monocistronic minigenome encoding *Renilla* luciferase, as well as T7 polymerase, have been previously described (11). The multicistronic minigenomes were constructed by consecutive deletion of viral genes from a plasmid containing a full-length cDNA copy of the Ebola virus genome (17) with an 8A editing site in the GP gene by PCR and replacement of the first coding sequence (CDS) with a *Renilla* luciferase CDS, followed by subcloning into the monocistronic minigenome expression vector, resulting in the multicistronic minigenomes p4cis-vRNA-RLuc, p3cis-vRNA-RLuc, and p2cis-vRNA-RLuc. For the first gene junction in the minigenomes, the equivalent first gene junction of the full-length genome (i.e., NP-VP35) was used, whereas all other gene junctions were chosen so that the viral genes had authentic upstream 3′ noncoding regions (i.e., the VP40-$GP_{1,2}$ gene junction before $GP_{1,2}$ and the VP30-VP24 gene junction before VP24). The monocistronic minigenome encoding firefly luciferase that was used for the mixed mono- and tetracistronic minigenome assay was generated by replacing the *Renilla* CDS of the monocistronic minigenome with a firefly luciferase CDS using standard PCR-based standard cloning techniques and pGL4.10 (Promega) as the template. *Cercopithecus aethiops* T-cell immunoglobulin and mucin domain 1 (Tim1; GenBank accession number D88585.1) was cloned from Vero E6 cells into pCAGGS via EcoRI and XhoI using the primers 5′-GAT CCT CGA GCC GAT ATG TTC AGT CTT CTG CAG-3′ and 5′-GCC CGA ATT CTT GGA TCT GAA CGC GGA TCC-3′. Three point mutations (t392c:I131T, t50c:F17S, t126c:silent; positions are relative to the start of the coding sequence) compared to the reference sequence were observed in the cloned sequence and were confirmed to also be present in the Vero E6 cell line by direct sequencing. MicroRNAs (miRNAs) were designed on the basis of miRNA124 (18) as well as known Ebola virus small interfering RNA (siRNA) targets (19), and their variable portion was generated by hybridization of two oligonucleotides (Table 1) and cloned into an assembly vector, which was synthesized by Blue Heron. For expression, miRNAs were subcloned into pCAGGS via XhoI and NheI. The correct sequence of all plasmids was confirmed by Sanger sequencing. All recombinant work was approved by the Institutional Biosafety Committee following NIH guidelines for research involving recombinant or synthetic nucleic acid molecules.

Tetracistronic trVLP assays. trVLP assays were performed as previously described (11), with some modifications (Fig. 1). 293 producer cells

TABLE 1 Oligonucleotides encoding the variable portions of miRNAs used to knock down viral mRNAs

miRNA	Oligonucleotide no.	Sequence[a]
Anti-L miRNA	1	5′-CTCCTACGAAGCGGTAGCTAAATATTTAATGTCATACAATATTTATATACAGCTTCGTAC-3′
	2	5′-TCTTGTACGAAGCTGTATATAAATATTGTATGACATTAAATATTTAGCTACCGCTTCGTA-3′
Anti-VP24 miRNA	1	5′-CTCCCCTCGACAAGAAGCCAAAGATTTAATGTCATACAATCTTTGCATTCGTGTCGAGGA-3′
	2	5′-TCTTTCCTCGACACGAATGCAAAGATTGTATGACATTAAATCTTTGGCTTCTTGTCGAGG-3′
Anti-GFP miRNA	1	5′-CTCCCCACAACGGCTACCTCATGATTTAATGTCATACAATCATGATATAGACGTTGTGGC-3′
	2	5′-TCTTGCCACAACGTCTATATCATGATTGTATGACATTAAATCATGAGGTAGCCGTTGTGG-3′
Anti-Luc2 miRNA	1	5′-CTCCCTATGGGCGGAAGCCAAACATTTAATGTCATACAATGTTTGTATTCAGCCCATAGC-3′
	2	5′-TCTTGCTATGGGCTGAATACAAACATTGTATGACATTAAATGTTTGGCTTCCGCCCATAG-3′

[a] RNA target sequences are underlined.

(also called passage 0, or p0, cells) were seeded in 2 ml medium into 6-well plates 24 h prior to transfection for a confluence of ~50%. p0 cells were transfected with previously optimized amounts of expression plasmids (11, 12), i.e., 125 ng pCAGGS-NP, 125 ng pCAGGS-VP35, 75 ng pCAGGS-VP30, 1000 ng pCAGGS-L, 250 ng tetracistronic minigenome expression plasmid, and 250 ng pCAGGS-T7 (for initial transcription of the minigenome), using 7.5 μl TransIT-LT1 transfection reagent (Mirus) per well. At 24 h after transfection, the medium was exchanged against 4 ml DMEM–PS–Q–5% FBS. At 72 h after transfection, the trVLP-containing supernatant of producer cells was clarified by centrifugation for 5 min at 800 × *g* and room temperature, and 3 ml of the clarified supernatant was transferred onto 293 cells (p1 to p5 cells) that had been transfected 24 h previously with 125 ng pCAGGS-NP, 125 ng pCAGGS-VP35, 75 ng pCAGGS-VP30, 1,000 ng pCAGGS-L, and 250 ng pCAGGS-Tim1. p0 cells were then lysed by applying 250 μl 1× GloLysis buffer (Promega) to the cells, incubating them for 10 min at room temperature, and resuspending the cells in the lysis buffer. Lysates were cleared by centrifugation for 3 min at 10,000 × *g*, and 40 μl of the lysate was added to 40 μl RenillaGlo substrate in opaque white 96-well plates (Costar). After incubation for 10 min, *Renilla* luciferase reporter activity was measured using a Modulus microplate luminometer (Turner Biosystems). At 24 h after infection, the supernatant of target cells (p1 to p5 cells) was exchanged against 4 ml DMEM–PS–Q–5% FBS. Reporter activity in the target cells was assessed 72 h after infection, and at the same time, trVLP-containing supernatant was passaged onto fresh target cells. For trVLP assays with different minigenome constructs, the copy number of minigenome plasmids was kept constant. In the case of the monocistronic minigenome, some experiments also included 250 ng pCAGGS-VP40, 250 ng pCAGGS-$GP_{1,2}$, and 60 ng pCAGGS-VP24, as indicated below.

Analysis of trVLPs. For Western blotting of trVLPs, 33 ml of supernatant was concentrated by ultracentrifugation through a 20% sucrose cushion in an SW28 rotor at 83,000 × *g* and 4°C. Pellets were resuspended in 120 μl phosphate-buffered saline, and 40 μl 4× SDS sample buffer was added before samples were incubated at 95°C for 5 min. Western blotting

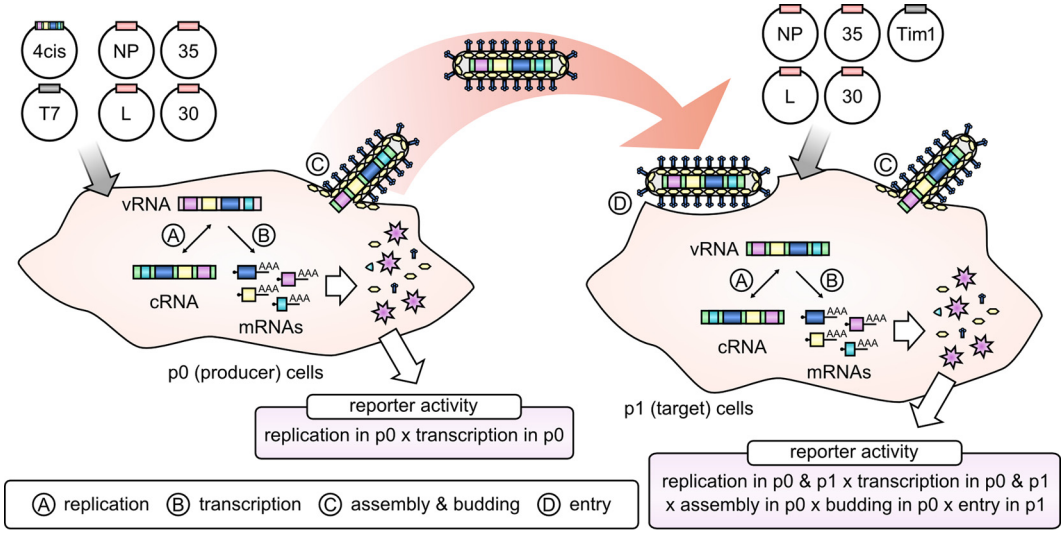

FIG 1 Schematic representation of a trVLP assay using a tetracistronic minigenome. 293 cells (called producer, or p0, cells) are transfected with expression plasmids for the RNP proteins NP, VP35, VP30, and L, as well as a tetracistronic minigenome (4cis) and the accessory T7 RNA polymerase (T7). After initial, T7-driven transcription, the vRNA minigenome is replicated through a cRNA intermediate by the viral proteins NP, VP35, and L and transcribed by these proteins and VP30 into mRNAs encoding a luciferase reporter as well as the viral proteins VP40, VP24, and $GP_{1,2}$. This leads to reporter activity (reflecting genome replication and transcription in p0 cells) as well as the formation of trVLPs that harbor a minigenome-containing nucleocapsid and are coated with $GP_{1,2}$. These trVLPs can infect and enter target cells, where their minigenome is replicated and transcribed by NP, VP35, VP30, and L provided in *trans* from expression plasmids. This leads to reporter activity in these target cells (reflecting genome replication and transcription in p0 and p1 cells, assembly and budding of trVLP from p0 cells, and their entry into p1 cells) as well as to the generation of new trVLPs that can infect new target cells, leading to ongoing infection over multiple infectious cycles, as long as NP, VP35, VP30, and L are supplied in target cells.

was performed as previously described (20) using mouse monoclonal antibodies against VP40 (2C4) and NP (B1C6), both at a dilution of 1:100. As secondary antibodies, Alexa Fluor 680-coupled goat anti-mouse (Molecular Probes) and IRDye800 goat anti-guinea pig (Rockland) antibodies diluted 1:5,000 were used, and fluorescent signals were detected and quantified using an Odyssey infrared imaging system (LI-COR Biosciences).

For RNA quantification, RNA was purified from 140 μl trVLP-containing supernatant using a QIAamp viral RNA (vRNA) minikit (Qiagen) according to the manufacturer's instructions. Sixteen microliters of RNA was subjected to a 30-min DNase digest using 2 μl DNase I (Thermo Scientific) in a total volume of 20 μl according to the manufacturer's instructions. Digested RNA (7.5 μl) was reverse transcribed using SuperScript III reverse transcriptase (Life Technologies) according to the manufacturer's instructions, but using 4.5 μl $MgCl_2$ per reaction mixture, with the primer 5′-CGG ACA CAC AAA AAG AAA GAA G-3′. Five microliters of the resulting cDNA was amplified in a touchdown PCR (10 cycles of annealing at 59 to 54°C for 30 s, followed by 10 cycles of annealing at 54°C for 30 s) using iProof polymerase (Bio-Rad) according to the manufacturer's instructions and the primers 5′-CTT GAC ATC TCT GAG GCA AC-3′ and 5′-ATG CAG GGG CAA AGT CAT TAG-3′. One microliter of PCR product was then subjected to quantitative PCR using a Rotor-Gene Sybr green PCR kit (Qiagen) according to the manufacturer's instructions and the primers 5′-CGA ACC ACA TGA TTG GAC CAA G-3′ and 5′-CTT ATC AGA CCT CCG CAT TAA TC-3′. Standards were included for each PCR at the stage of the first PCR amplification.

RNAi experiments. For RNA interference (RNAi)-mediated knockdown, 293 cells in 96-well plates were transfected with 250 ng miRNA expression plasmid and 55 ng Tim1 expression plasmid per well, using 3 μl TransitLT1 transfection reagent per μg of DNA. At 24 h after transfection, cells were infected with recombinant Ebola viruses expressing luciferase or GFP (15) at the multiplicity of infection (MOI) indicated below.

In the case of infection with the luciferase-expressing virus, plates were coated with 50 μl poly-D-lysine (100 μg/ml) for 6 h at 37°C prior to the seeding of cells and transfection. Infection was performed for 1 h on ice, after which the inoculum was removed and 125 μl DMEM–PS–Q–5% FBS was added to the wells. At the indicated time points, the supernatant was removed from the cells and 100 μl GloLysis buffer was applied to the cells. After a 10-min incubation at room temperature, 75 μl of lysate was removed and frozen at −80°C. To measure reporter activity, samples were thawed and 40 μl of sample was added to 40 μl BrightGlo reagent (Promega) in opaque white 96-well plates. Luciferase activity was measured after 10 min using a GloMax-Multi microplate multimode reader (Promega).

In the case of infection with the GFP-expressing viruses, 5 random pictures per well were taken using an Axiovert 40-C microscope (Zeiss) and a Ds-F1 camera (Nikon). Pictures were analyzed with ImageJ software using the analyze particles function, after removing background noise with a border-limited mean filter, and generating a mask based on a brightness threshold of 50.

Statistical analysis. Student's *t* tests were performed using Prism (version 6) software (GraphPad Software).

RESULTS

Establishment of multicistronic minigenome systems. Current trVLP systems rely on the plasmid-driven overexpression of all viral proteins, including $GP_{1,2}$, VP40, and VP24, of which the last two have been shown to be strong inhibitors of genome replication and transcription (21, 22). In order to circumvent this requirement, we designed and cloned several multicistronic minigenomes to allow minigenome-based expression of these proteins. Specifically, we created expression plasmids for a bicistronic minigenome encoding a *Renilla* luciferase reporter and VP40; a tricistronic minigenome encoding a luciferase reporter, VP40, and $GP_{1,2}$; and a tetracistronic minigenome encoding a luciferase reporter, VP40, $GP_{1,2}$, and VP24 (Fig. 2A). As a first step in their characterization, each minigenome was expressed in 293 cells in the presence of the viral ribonucleoprotein complex (RNP) proteins required for genome replication and transcription, i.e., NP, VP35, VP30, and L. All minigenomes produced reporter activity; however, there was a strong inverse correlation between the observed reporter activity and minigenome length, with the monocistronic minigenome producing about 18-fold (1.25 $\log_{10}$ units) more reporter activity than the tetracistronic minigenome (Fig. 2B). It is currently not clear whether this decline is due to the action of the additionally expressed gene products (although, at least for $GP_{1,2}$, an influence on genome replication or transcription has never been reported) or due to the increased length of the minigenome, which might affect steps such as initial transcription by the T7 polymerase or illegitimate encapsidation of naked vRNAs prior to replication and transcription (9).

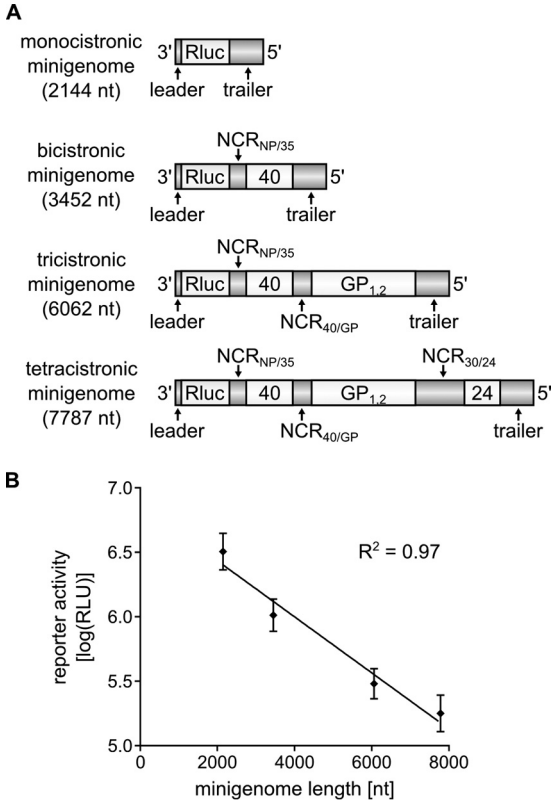

FIG 2 Design and evaluation of multicistronic minigenomes. (A) Schematic overview of the different mono- and multicistronic minigenomes used in this study. Open reading frames encoding *Renilla* luciferase (Rluc) and the viral proteins VP40, $GP_{1,2}$, and VP24 are shown as light boxes, and noncoding regions (NCR), including the terminal leader and trailer regions, are shown as dark boxes, with the subscript indicating which NCR was used to join the open reading frames. All minigenomes used in this study are negative-sense (vRNA) minigenomes. (B) Influence of minigenome length on reporter activity. Minigenome assays were performed in 293 cells using the different mono- and multicistronic minigenomes depicted in panel A, using the same molar amounts of minigenome plasmids for transfection. Reporter activity (in relative light units) was measured 3 days after transfection. The means and standard deviations from 6 independent experiments are shown, as are a linear regression curve and its coefficient of correlation (R^2).

trVLPs containing tetracistronic minigenomes can be used for highly efficient infection of target cells and can be passaged to model multiple infectious cycles. As the next step in charac-

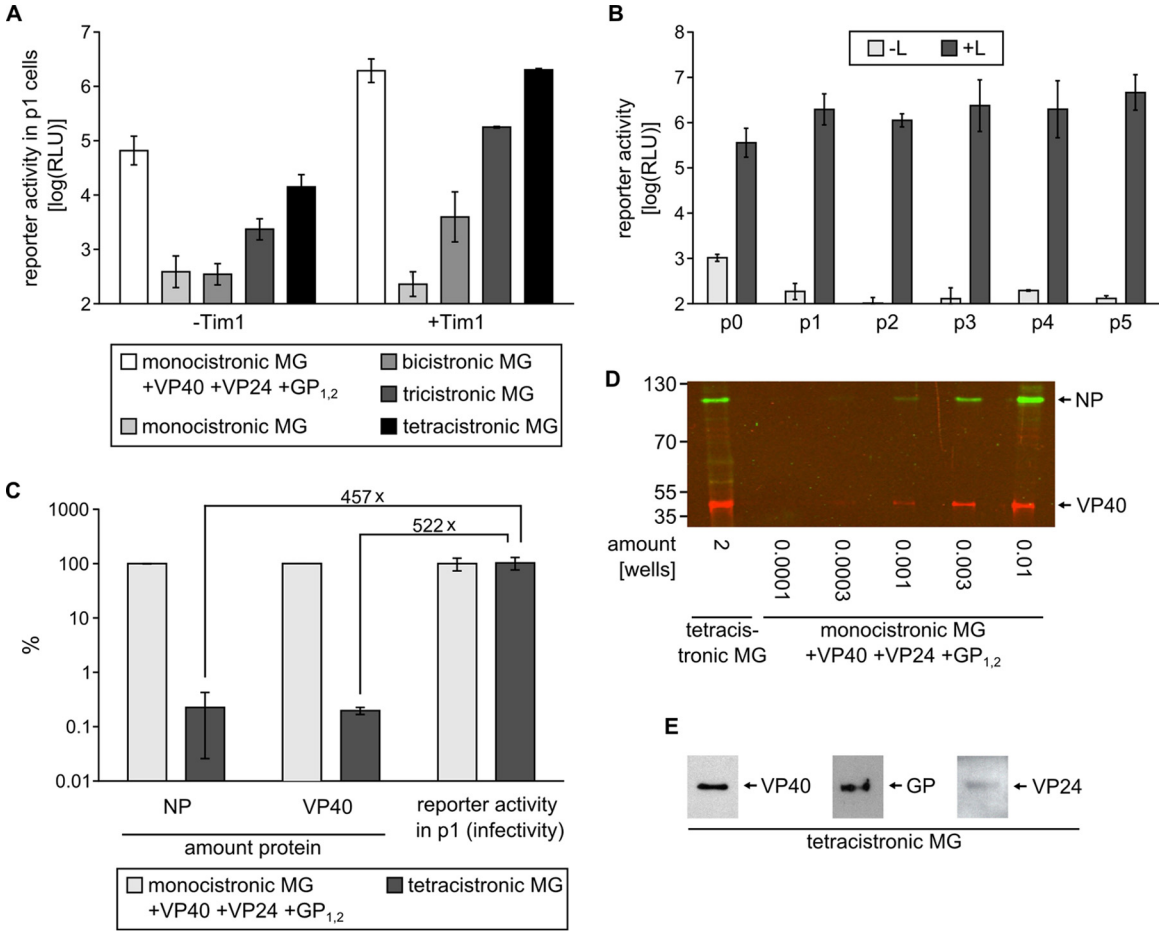

FIG 3 Characterization of trVLPs produced by multicistronic minigenomes. (A) Infection of target cells in the presence or absence of Tim1 with trVLPs produced using different multicistronic minigenomes. trVLPs containing different minigenomes (MGs) were produced and used to infect target cells that had been pretransfected to express the RNP proteins and either Tim1 or no Tim1. In the case of the monocistronic minigenomes, trVLP production was driven by expression of VP40, VP24, and $GP_{1,2}$ from plasmids. Reporter activity in target cells was determined 72 h after infection. The means and standard deviations from 3 independent experiments are shown. (B) Continuous passaging of tetracistronic trVLPs. trVLP assays were performed with a tetracistronic minigenome in the presence or absence of the viral polymerase L. Supernatant from p1 target cells was passaged 72 h after infection of the p1 cells onto p2 target cells pretransfected with expression plasmids encoding the Ebola virus RNP proteins NP, VP35, VP30, and L, as well as Tim1. This infection was repeated every 72 h for a total of 5 passages. The means and standard deviations from 3 independent experiments are shown. (C) Relative infectivity and trVLP amounts produced by a tetracistronic minigenome. trVLPs were produced with VP40, VP24, and $GP_{1,2}$ expressed either from expression plasmids (when using a monocistronic minigenome) or from a tetracistronic minigenome. trVLPs were then used to infect target cells in order to determine the total infectivity of the trVLP preparation, or they were purified through a sucrose cushion, followed by quantification of the VP40 and NP levels in the trVLP preparation (see also panel D). The levels of infectivity and the amounts of VP40 or NP in the trVLP preparation produced by a monocistronic minigenome and VP40, VP24, and $GP_{1,2}$ from expression plasmids were defined as 100%. The means and standard deviations from 3 independent experiments are shown, and fold changes in the ratios of the protein amount to trVLP infectivity are indicated. (D) Quantification of protein content in trVLPs. trVLPs which had been purified as described in the legend to panel C were subjected to Western blotting using antibodies directed against VP40 and NP. In order to compensate for different trVLP concentrations, different amounts of trVLP preparations were loaded. The numbers of wells (or the fraction of a well) of a six-well plate to which the loaded samples correspond are indicated. (E) Detection of VP40, $GP_{1,2}$, and VP24 in trVLPs. trVLPs which had been purified as described in the legend to panel C were subjected to Western blotting using antibodies directed against VP40, $GP_{1,2}$, and VP24. The equivalent of supernatant from 2 wells of a six-well plate was loaded.

terizing the multicistronic minigenomes, we assessed the production of trVLPs driven by minigenome-based expression of VP40, $GP_{1,2}$, and/or VP24. Supernatant from p0 producer cells expressing one of the minigenomes, as well as the RNP proteins, was passaged onto p1 target cells that had been pretransfected with expression plasmids encoding the RNP proteins. As a control, this experiment was also performed using a monocistronic minigenome with plasmid-based overexpression of VP40, $GP_{1,2}$, and VP24, leading to high levels of reporter activity in p1 cells (Fig. 3A, left). In contrast, the reporter activity matched the background noise of the luminometer ($\sim 10^{2.14}$ relative light units [RLU]) in p1 cells infected with the supernatant of p0 cells expressing only a monocistronic minigenome and the RNP proteins, but not VP40, $GP_{1,2}$, or VP24. Similarly, passaging of supernatant from p0 cells expressing a bicistronic minigenome and the RNP proteins did not lead to reporter activity in p1 cells, an expected result given the absence of $GP_{1,2}$, which mediates the entry of virus particles into target cells (23). When the experiment was performed using either a tricistronic or tetracistronic minigenome, reporter activity in p1 cells was readily detectable, showing that these minigenomes are able to produce infectious trVLPs.

293 cells, which were used in these experiments because of their

high transfectability, are known to be poorly susceptible to infection with Ebola viruses (24); however, expression of T-cell immunoglobulin and mucin domain 1 (Tim1) has been shown to increase their susceptibility (25). In order to assess whether Tim1 would also improve infection of target cells with trVLPs, p1 target cells were pretransfected with expression plasmids for the RNP proteins as well as Tim1 and then infected with the different minigenome-containing trVLPs. As expected, we observed a significant overall increase in reporter activity, indicating increased susceptibility of the 293 target cells to infection with trVLPs upon expression of Tim1 (Fig. 3A, right; $P = 0.0017$ for the monocistronic minigenome with VP40, VP24, and $GP_{1,2}$; $P < 0.0001$ for the tri- and tetracistronic minigenomes). Further, reporter activity in target cells infected with trVLPs containing a tetracistronic minigenome and minigenome-derived VP40, $GP_{1,2}$, and VP24 reached levels identical to those in cells infected with trVLPs containing a monocistronic minigenome and plasmid-derived VP40, $GP_{1,2}$, and VP24, demonstrating that this approach is highly efficient for the production of infectious trVLPs. Further, this suggests that the effect of minigenome length on reporter activity in a minigenome assay (Fig. 2B) might be due to effects on initial transcription by the T7 polymerase or illegitimate encapsidation, artificial processes that do not take place in p1 target cells in a trVLP assay. Surprisingly, some reporter activity was observed in cells pretransfected with Tim1 after infection with trVLPs produced from a bicistronic minigenome (i.e., in the absence of any $GP_{1,2}$). However, this reporter activity was 500-fold (2.7 $\log_{10}$ units) lower than the reporter activity observed after infection with trVLPs containing either tetracistronic or monocistronic minigenomes.

Since minigenome transcription in target cells results in expression of all the viral proteins required for the formation of infectious trVLPs, we next assessed whether trVLPs containing tetracistronic minigenomes could be passaged repeatedly, modeling multiple infectious cycles. To address this question, trVLP assays with tetracistronic minigenomes were performed as described above, and the supernatant of p1 cells was used to infect fresh p2 target cells that had been pretransfected with expression plasmids for the RNP proteins and Tim1. Reporter activity was readily detectable and remained stable over 5 passages (Fig. 3B), indicating that it is indeed possible to model multiple infectious cycles using tetracistronic minigenome-containing trVLPs and that this can be achieved without a loss of reporter activity between successive passages.

Given the fact that preparations of trVLPs containing monocistronic minigenomes or tetracistronic minigenomes showed similar infectivity in Tim1-expressing target cells, we next assessed whether there were differences in the total amount of VLPs produced by these two systems. To this end, VLPs were purified and concentrated from the p0 cell supernatant by ultracentrifugation through a sucrose cushion and subjected to Western blotting using antibodies against NP and VP40. We observed a dramatic reduction in the amount of NP and VP40 detected in trVLP preparations produced by tetracistronic minigenome-driven expression of VP40, VP24, and $GP_{1,2}$ compared to the amount detected in preparations produced by plasmid-based overexpression of these proteins (Fig. 3C and D; $P = 0.0025$). These results indicate that the tetracistronic minigenome-derived preparations contained a dramatically reduced amount of noninfectious VLPs, although they still contained comparable amounts of infectious trVLPs, resulting in an approximately 500-fold higher specific infectivity. Unfortunately, we were not able to directly compare the levels of VP40, $GP_{1,2}$, and VP24 in cell lysates since the minigenome-based expression of these proteins was below the detection limit of our Western blot assay, even though their presence in highly concentrated trVLP preparations provides clear evidence that these proteins are expressed (Fig. 3E). Particularly, we were not able to detect VP24 expressed either from minigenomes or from the small amount of expression plasmid used in monocistronic trVLP assays, despite numerous attempts, including acetone precipitation of proteins and isolation of highly concentrated cytosolic protein fractions (data not shown).

VP24 exerts only a very modest effect on genome replication and transcription during regulated expression. Previous studies by us and others showing a function of VP24 in regulating genome replication and transcription have relied on plasmid-based overexpression of this protein (11, 12). To investigate whether this function of VP24 can also be observed when VP24 is expressed in a fashion regulated by viral replication and transcription, rather than from plasmids, we performed minigenome assays using the tetracistronic minigenome to drive expression of VP24. Since there is a strong correlation between minigenome length and reporter activity (Fig. 2B), rather than deleting the VP24-coding sequence (CDS), we decided to inhibit expression of VP24 by introducing 3 stop codons immediately after the start codon of the VP24 CDS, thereby resulting in only minimal changes to the minigenome sequence and no change to its overall length. When performing minigenome assays using these two versions of the tetracistronic minigenome, we observed only a small increase in reporter activity in the absence of VP24 (2-fold, or 0.36 $\log_{10}$ unit) (Fig. 4A), although this difference was statistically significant ($P = 0.01$). In contrast, when we performed a minigenome assay with a tetracistronic minigenome unable to produce VP24 and provided VP24 in *trans* from an expression plasmid, we observed a dramatic reduction of reporter activity of 760-fold, or 2.88 $\log_{10}$ units (Fig. 4B), in line with previous reports (21, 22). However, it has to be kept in mind that in these assays both VP24 and the RNP proteins were expressed from plasmids, so that the lack of effect of VP24 expressed from a tetracistronic minigenome might be related to a relative excess of the RNP proteins.

To address this issue, we sought to verify the contribution of VP24 to regulating these processes during virus infection, ensuring physiological concentrations of all viral proteins. To this end, 293 cells were transfected with Tim1 and miRNA expression plasmids against VP24 or, as a control, against L and then infected at a high MOI with a recombinant Ebola virus expressing a luciferase reporter (15). The miRNAs used were based on published siRNA sequences and were shown to be highly efficient in the knockdown of VP24 or L expression, as shown by Western blotting and/or in infection-based assays (Fig. 4C and D). As previously reported (15), we were able to detect a small (1.6-fold, or 0.21-$\log_{10}$-unit) but highly significant ($P < 0.0001$) increase in reporter activity over that observed in mock-infected cells at 2 h after infection (Fig. 4E), corresponding to the onset of primary transcription. Reporter activity was monitored every 2 h and continued to increase until reaching a maximum of $10^{7.6}$ RLU at 32 h after infection, at which point the experiment was terminated. In cells expressing a miRNA against L, we observed a clear drop in the levels of reporter activity compared to that in cells expressing an unrelated control miRNA against GFP beginning at 14 h after infection (Fig. 4F). In contrast, cells expressing a miRNA against VP24

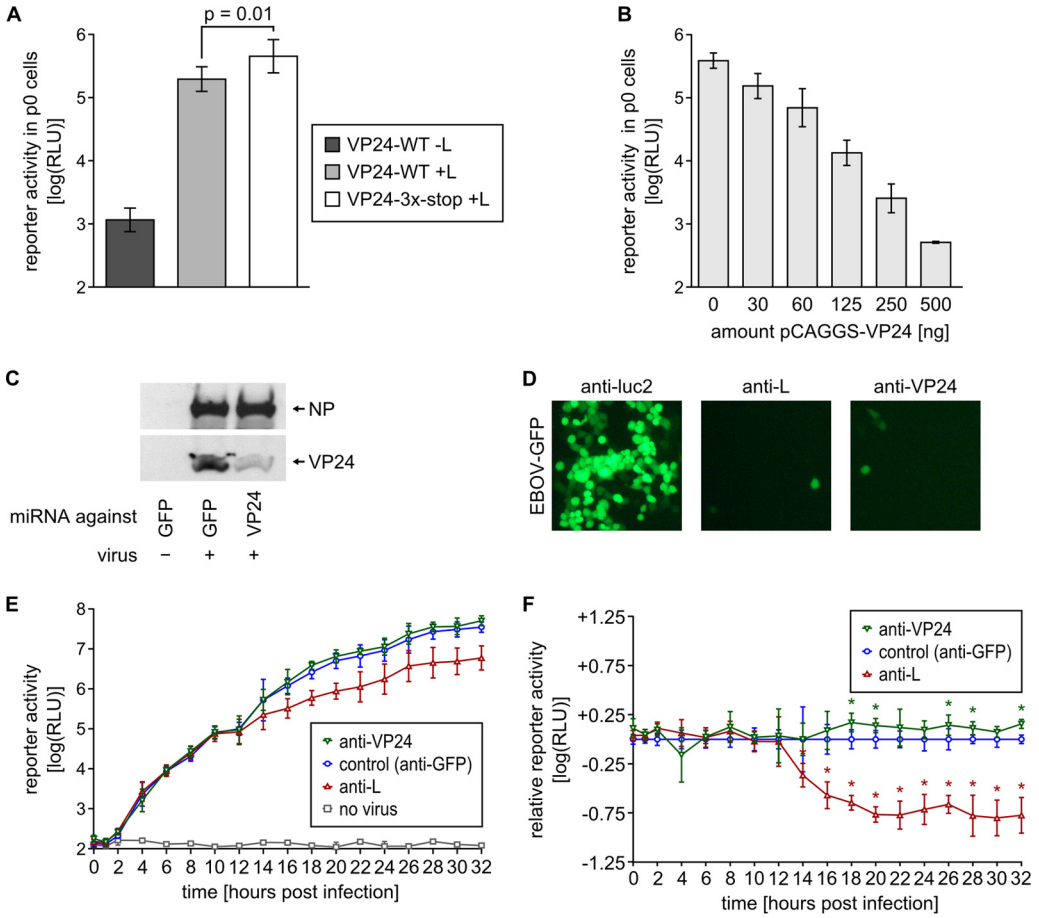

FIG 4 Role of VP24 in regulating genome replication and transcription. (A) Influence of minigenome-expressed VP24 on replication and transcription in a tetracistronic minigenome assay. 293 cells were transfected with expression plasmids encoding tetracistronic minigenomes containing a wild-type VP24 gene (VP24-WT) or a VP24 gene in which 3 stop codons were inserted immediately after the start codon (VP24–3×-stop), abolishing expression of VP24; expression plasmids encoding the Ebola virus RNP proteins NP, VP35, VP30, and L; and an accessory expression plasmid encoding T7 RNA polymerase. As a negative control, the expression plasmid for L was omitted (−L). After 72 h, reporter activity was determined. The means and standard deviations from 7 independent experiments are shown. (B) Influence of plasmid-expressed VP24 on replication and transcription in a tetracistronic minigenome assay. Minigenome assays using the tetracistronic minigenome with the VP24 gene in which 3 stop codons were inserted immediately after the start codon were performed as described in the legend to panel A. In addition, the indicated amounts of pCAGGS-VP24 were cotransfected. The means and standard deviations for 6 biological replicates from 2 independent experiments are shown. (C) Efficacy of miRNA-mediated knockdown of VP24. 293 cells expressing Tim1 and miRNAs against Ebola virus VP24 or GFP were infected with wild-type Ebola virus at an MOI of 1. At 24 h after infection, cells were lysed and lysates were analyzed by Western blotting using antibodies against NP or VP24. (D) Effect of miRNAs on virus replication. 293 cells expressing Tim1 and miRNAs against Ebola virus (EBOV) L (anti-L), Ebola virus VP24 (anti-VP24), or an unrelated protein (anti-luc2) were infected with a recombinant Ebola virus expressing GFP at an MOI of 0.1. At 4 days after infection, cells were visualized by fluorescence microscopy. Random fields are shown. (E) Influence of VP24 on genome replication and transcription in infection. 293 cells were transfected with expression plasmids encoding Tim1 and miRNAs directed against VP24 (anti-VP24), L (anti-L), or an unrelated protein (anti-GFP). After 24 h, cells were infected with a recombinant Ebola virus expressing luciferase at an MOI of 3. Reporter activity in cells was determined every 1 to 2 h for 32 h after infection. The means and standard deviations for 6 biological replicates from 2 independent experiments are shown as absolute values. (F) Relative influence of VP24 on genome replication and transcription in infection. The data from panel E are expressed relative to the reporter activity observed in cells transfected with an expression plasmid for an unrelated protein. *, significant differences ($P < 0.05$) in reporter activity compared to that for control cells.

showed only a very small increase in reporter activity starting at 18 h after infection. When combining data for all time points beginning at 18 h after infection (i.e., using 48 biological replicates), the mean of this increase was 1.34-fold (0.128 $\log_{10}$ unit), with a 95% confidence interval of 1.24- to 1.46-fold, and this difference was highly significant ($P < 0.0001$). However, when looking at data for individual time points (i.e., using only 6 biological replicates), a statistically significant difference ($P < 0.05$) was seen at only 5 of the 8 time points. In summary, during Ebola virus infection in the absence of VP24 synthesis, a detectable but very small increase in reporter activity is observed. This indicates only a minor role of VP24 in regulating genome replication and transcription and is consistent with our findings obtained using the tetracistronic minigenome system.

VP24 has a genome length-dependent role in infectivity of trVLPs. The results from the infection experiment over multiple infectious cycles clearly show that VP24 must play an essential role in the Ebola virus life cycle (Fig. 4D). Having shown that VP24 has only a very small effect on genome replication and transcription and, thus, reporter activity in p0 cells, we next assessed whether VP24 influences the infectivity of trVLPs and, thus, reporter activity in p1 target cells. We have previously shown that in a trVLP

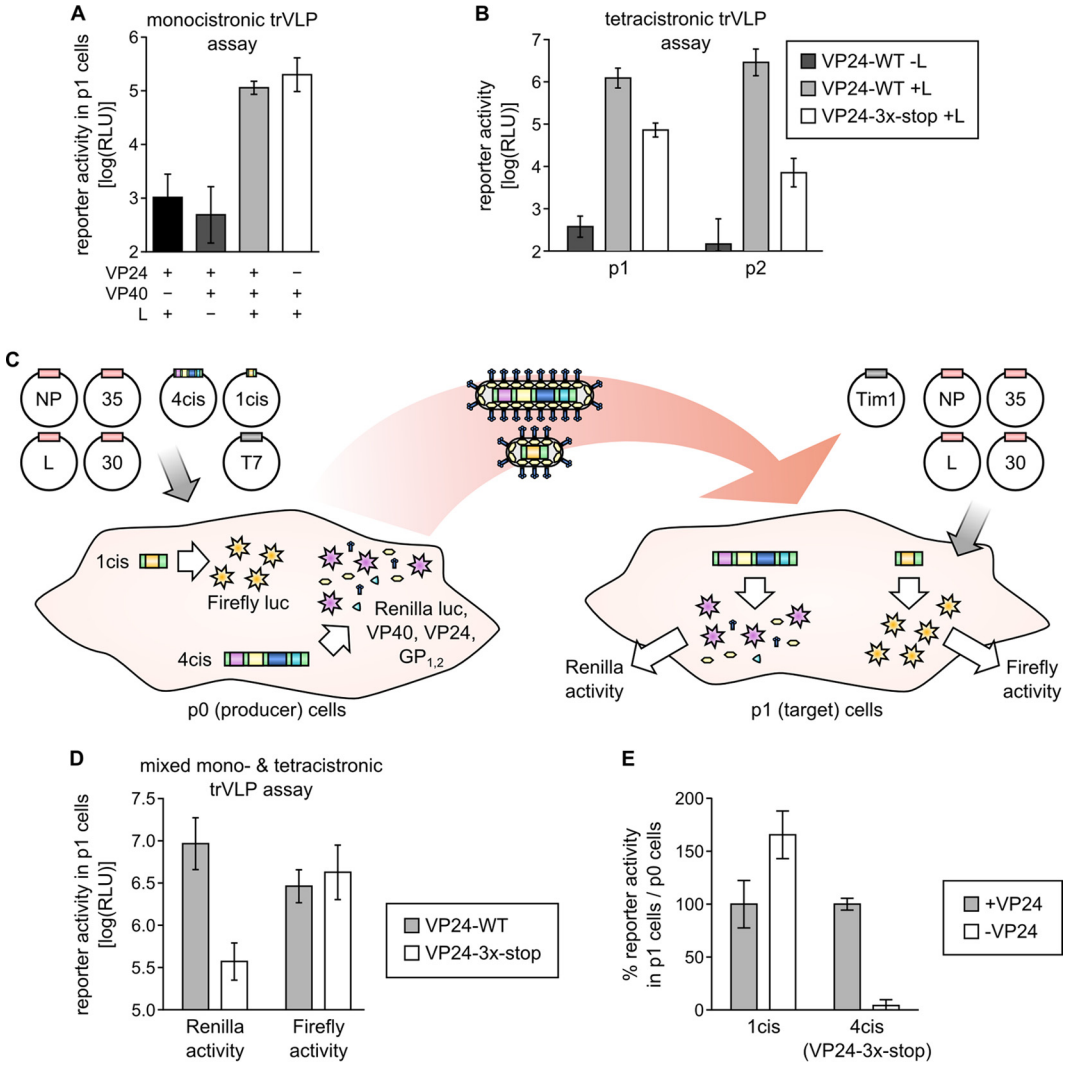

FIG 5 Role of VP24 in trVLP infectivity. (A) Effect of VP24 on trVLP infectivity in a monocistronic trVLP assay. 293 cells (p0 cells) were transfected with expression plasmids encoding a monocistronic minigenome and Ebola virus structural proteins (NP, VP35, VP30, L, VP40, VP24, $GP_{1,2}$), as indicated. As negative controls, either VP40 was omitted (−VP40) or L was omitted (−L) from the transfection. At 72 h after transfection, trVLPs were passaged onto pretransfected p1 target cells, and after 72 h, the reporter activity in p1 cells was measured. The means and standard deviations from 3 independent experiments are shown. (B) Effect of VP24 on trVLP infectivity in a tetracistronic trVLP assay. trVLP assays were performed as described in the legend to A, but omitting the expression plasmids for VP40, VP24, and $GP_{1,2}$ and using a tetracistronic minigenome instead of a monocistronic minigenome. These tetracistronic minigenomes contained either a wild-type VP24 gene (VP24-WT) or a VP24 gene in which 3 stop codons had been inserted immediately after the start codon (VP24–3×-stop), abolishing expression of VP24. As a negative control, the expression plasmid for L was omitted. After 72 h, trVLPs were passaged onto target cells (p1) that had been pretransfected with expression plasmids encoding NP, VP35, VP30, and L as well as Tim1. After 72 h, reporter activity in p1 cells was measured, and trVLPs produced by the p1 target cells were used to infect pretransfected target cells (p2). Reporter activity in p2 cells was measured 72 h after infection. The means and standard deviations from 5 independent experiments are shown. (C) Design of a mixed mono- and tetracistronic trVLP assay. A trVLP assay was performed as described in the legend to panel A; however, in addition to a tetracistronic minigenome (4cis) encoding *Renilla* luciferase, a monocistronic minigenome (1cis) encoding firefly luciferase was also coexpressed in the p0 producer cells. trVLPs containing minigenome-derived VP40 and $GP_{1,2}$ as well as either monocistronic or tetracistronic minigenomes were passaged onto pretransfected p1 target cells. (D) Dependence of VP24 function on minigenome length. Mixed mono- and tetracistronic trVLP assays were performed as described in the legend to panel C, using either a tetracistronic minigenome encoding wild-type VP24 or a VP24 gene in which 3 stop codons had been inserted immediately after the start codon, abolishing expression of VP24. *Renilla* luciferase activity derived from the tetracistronic minigenome and firefly luciferase activity derived from the monocistronic minigenome in p1 target cells 72 h after infection with trVLPs are shown. The means and standard deviations from 3 independent experiments are shown. (E) Effect of VP24 provided in *trans* on trVLP infectivity. trVLP assays were performed using either a monocistronic minigenome or a tetracistronic minigenome incapable of producing VP24. Assays were performed in either the presence of 60 ng pCAGGS-VP24 (+VP24) or the absence of pCAGGS-VP24 (−VP24). Reporter activity in p1 target cells was measured and normalized to the reporter activity in p0 producer cells to compensate for the strong inhibitory effect of VP24 on minigenome replication and transcription (cf. Fig. 4B) in those cells. For each of the minigenomes, reporter activity in the presence of VP24 was defined as 100%. The means and standard deviations from 3 independent experiments are shown.

assay using a monocistronic minigenome and pretransfected target cells VP24 is not required for trVLP infectivity (11), and we confirmed these results as part of this study (Fig. 5A). Surprisingly, when a trVLP assay was performed using tetracistronic minigenomes containing a wild-type VP24 CDS or a VP24 CDS in which expression was abolished by the introduction of stop codons, we observed a drastic drop in reporter activity in target cells of 17-fold (1.23 $\log_{10}$ units; $P = 0.0028$) in the first passage

and 405-fold (2.61 $\log_{10}$ units; $P = 0.0019$) in the second passage (Fig. 5B).

The differences between the trVLP assays using a monocistronic versus a tetracistronic minigenome are 2-fold: (i) the monocistronic minigenome is considerably shorter than the tetracistronic minigenome (2,144 nucleotides [nt] versus 7,787 nt), and (ii) VP40, VP24, and $GP_{1,2}$ are derived from constant, plasmid-based overexpression for the monocistronic minigenome and from temporally regulated, minigenome-based expression for the tetracistronic minigenome. To distinguish which of those two differences was responsible for the discrepancy between the monocistronic and tetracistronic minigenome assays, we designed the experiment depicted in Fig. 5C. In this experiment, the RNP proteins as well as both a monocistronic minigenome, containing a firefly luciferase reporter gene, and a tetracistronic minigenome, containing a *Renilla* luciferase reporter gene, were expressed in p0 producer cells. trVLP production in these cells was driven by the tetracistronic minigenome-derived VP40, VP24, and $GP_{1,2}$, and these trVLPs could package either monocistronic or tetracistronic minigenomes. Importantly, VP40, VP24, and $GP_{1,2}$ in trVLPs containing monocistronic minigenomes were not produced by plasmid-based overexpression but solely by the tetracistronic minigenome. In the producer cells, reporter activity reflecting replication and transcription of the monocistronic minigenome was completely unaffected by VP24 ($10^{7.3 \pm 0.1}$ RLU in the absence of VP24 versus $10^{7.3 \pm 0.3}$ RLU in the presence of VP24) and reporter activity reflecting replication and transcription of the tetracistronic minigenome was only minimally affected by VP24 ($10^{5.2 \pm 0.3}$ RLU in the absence of VP24 versus $10^{5.4 \pm 0.2}$ RLU in the presence of VP24), consistent with the previous results (Fig. 4A). Target cells were then infected with these trVLPs, and the levels of firefly and *Renilla* luciferase activity were determined, allowing us to distinguish reporter activity from monocistronic and tetracistronic minigenomes. The results of this mixed mono- and tetracistronic trVLP assay were indistinguishable from the results observed in the separate mono- and tetracistronic trVLP assays (Fig. 5D). *Renilla* luciferase reporter activity derived from the tetracistronic minigenome was dependent on VP24, with the reporter activity after infection with trVLPs produced in the absence of VP24 being reduced by 25-fold (1.39 $\log_{10}$ units; $P = 0.003$) compared to that after infection with trVLPs produced in the presence of VP24. In contrast, firefly luciferase reporter activity derived from monocistronic minigenomes was independent of VP24. Since in this experiment there were no differences in the levels or kinetics of VP40, VP24, and $GP_{1,2}$ expression leading to the production of trVLPs containing either monocistronic or tetracistronic minigenomes, this indicates that the difference in length between these two minigenomes is most likely responsible for the differing effects of VP24 on the infectivity of these two kinds of trVLPs. However, the possibility of sequence-specific effects, for example, effects due to the intergenic regions present in the tetracistronic minigenome but not in the monocistronic minigenome, also cannot completely be ruled out at this point.

To further confirm the genome length-dependent effect of VP24 on particle infectivity, we designed an experiment in which we produced trVLPs containing either a monocistronic minigenome or a tetracistronic minigenome incapable of producing VP24, and we then provided VP24 in *trans* from an expression plasmid. Since adding VP24 in *trans* strongly reduced reporter activity in producer cells (see also Fig. 4B), the reporter activity in target cells was normalized to that seen in producer cells (Fig. 5E). As expected, monocistronic minigenome reporter activity in target cells and, thus, trVLP infectivity were not decreased when VP24 was absent during trVLP production. In contrast, tetracistronic minigenome reporter activity in target cells was strongly dependent on the presence of VP24 during trVLP production (24-fold reduction in the absence of VP24; $P < 0.0001$), confirming the genome length-dependent effect of VP24 on trVLP infectivity.

The role of VP24 in trVLP infectivity is partially due to a function of VP24 in RNA incorporation into particles. A possible explanation for the length-dependent effect of VP24 on trVLP infectivity is that VP24 might be required for the incorporation of longer minigenomes (and full-length genomes) into particles. In order to test this hypothesis, an experiment was designed in which the minigenome RNA content of trVLPs produced in the presence and absence of VP24 was measured and correlated with the infectivity of these particles. To this end, we infected cells with trVLPs containing a tetracistronic minigenome encoding wild-type VP24 (Fig. 6A). These cells had been pretransfected with expression plasmids for the RNP proteins as well as expression plasmids for Tim1 and the previously validated miRNAs directed against L, VP24, or an unrelated protein (i.e., GFP). trVLPs in the supernatant of these p1 cells were then used to infect fresh p2 target cells and also analyzed for their minigenome RNA content. Importantly, the use of trVLPs produced in the presence of the anti-VP24 miRNA resulted in a 10-fold (0.95-$\log_{10}$-unit; $P < 0.0001$) reduction in reporter activity in p2 target cells compared to that for control trVLPs (Fig. 6B), indicating the efficacy of VP24 knockdown. While we did not observe a reduction in the amount of trVLPs produced, as indicated by the unchanged amounts of VP40 present in the supernatant, VP24 incorporation was reduced in the presence of the anti-VP24 miRNA (Fig. 6C). When analyzing the RNA content of trVLPs produced under these conditions, we also observed a significant reduction in the RNA content of trVLPs produced in the presence of the anti-VP24 miRNA (Fig. 6D; $P < 0.0001$). Interestingly, the extent of this difference in minigenome RNA content (2-fold) was not sufficient to fully explain the difference in trVLP infectivity observed (10-fold), suggesting that VP24 might have an additional length-dependent function in determining trVLP infectivity. Nevertheless, these results clearly indicate a new role for VP24 in the virus life cycle, namely, the length-dependent incorporation of RNA genomes into particles.

DISCUSSION

There is an increasing body of evidence suggesting that Ebola virus VP24 is involved in nucleocapsid formation. Upon recombinant expression in mammalian cells, it has been reported that VP24 is, along with NP and the viral protein VP35, necessary and sufficient for the formation of nucleocapsid-like structures that are clearly visible by electron microscopy (26, 27), and VP24 was recently found to be an integral part of nucleocapsids located inside virus particles, where it appears to bridge adjacent NP molecules (28, 29). In addition, using siRNA knockdown of VP24 during infection, it was further shown that VP24 is required for the assembly of nucleocapsids (30). However, functionally it has been shown that VP24 is not required for genome replication or secondary transcription (10) but, rather, exerts a strong negative impact on both these processes (21, 22), at least following overexpression of VP24 from expression plasmids. In contrast to this, VP24 was demon-

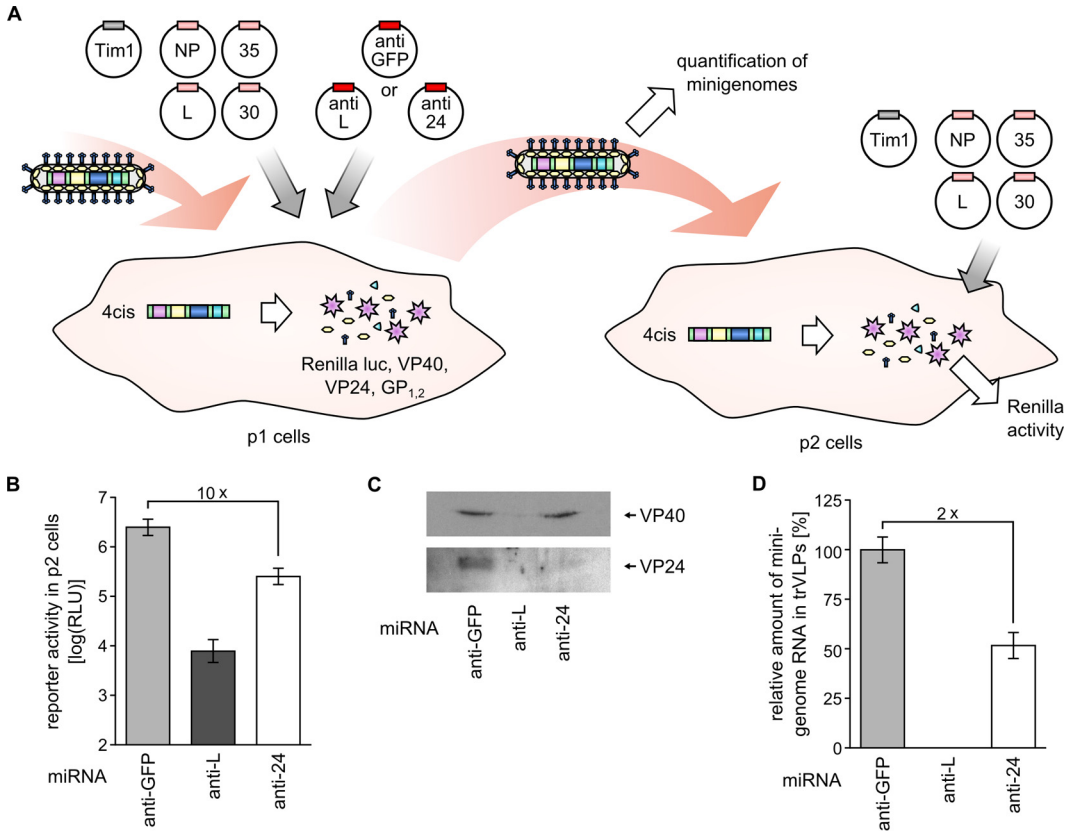

FIG 6 Role of VP24 in RNA incorporation. (A) Experimental design. 293 cells (p1 cells) pretransfected with expression plasmids for NP, VP35, VP30, L, and Tim1 as well as miRNAs directed against L (anti-L), VP24 (anti-VP24), or an unrelated protein (anti-GFP) were infected with trVLPs containing a tetracistronic minigenome. At 72 h after infection, trVLPs produced by the p1 cells were used to infect p2 target cells that had been pretransfected with expression plasmids for NP, VP35, VP30, L, and Tim1. (B) Efficacy of miRNA knockdown. Reporter activity in p2 cells was determined 72 h after infection to confirm the efficacy of miRNA knockdown in p1 cells. The means and standard deviations from 4 independent experiments are shown. (C) Influence of VP24 on trVLP budding. trVLPs produced by p1 cells were concentrated by centrifugation through a sucrose cushion and subjected to Western blotting using antibodies against VP40 and VP24. (D) Influence of VP24 on RNA incorporation into trVLPs. Minigenome RNA in trVLPs produced by p1 cells was extracted and quantified using quantitative reverse transcription-PCR. The means and standard deviations from 4 independent experiments are shown.

strated to be important for primary transcription, although the mechanism for this has remained unclear (11).

In this study, we have developed a novel life cycle modeling system based on tetracistronic minigenomes and applied it to further analyzing the function of VP24. Contrary to previous reports using monocistronic minigenomes and plasmid-based overexpression of VP24, we have shown that VP24 has only a very modest influence on genome replication and transcription in a tetracistronic minigenome, where it is expressed in a regulated fashion (i.e., as a product of viral genome replication and transcription), as well as during infection with live virus. In contrast to this very minor role for VP24 in downregulating replication and transcription, we observed a dramatic influence of VP24 on trVLP infectivity. However, this effect was observed only in the case of trVLPs containing tetracistronic minigenomes and not with trVLPs containing monocistronic minigenomes. Importantly, this difference in the effect of VP24 on infectivity between monocistronic and tetracistonic trVLPs was not due to differences in the levels of VP40, VP24, and $GP_{1,2}$ expression but, instead, was due to the minigenome length, highlighting the importance of the availability of a tetracistronic minigenome for these types of studies.

Based on the data from this study, as well as previously published findings, we propose a model (Fig. 7) in which VP24 is responsible for the structural rearrangement of the RNP from a relaxed to a condensed state, with the latter being represented by the structures observed as nucleocapsids using electron microscopy. According to this model, the viral polymerase complex can proceed along the RNP only in its relaxed state, making such relaxed RNPs active in replication and transcription. In contrast, RNPs condensed by the presence of VP24 prevent the polymerase complex from proceeding along the template, and polymerase complexes bound to the 3′ end of the genome are locked in that position. This model explains why VP24 is required for the formation of nucleocapsids and nucleocapsid-like structures (26, 27, 30) but not for genome replication and transcription (10) and why plasmid-based overexpression of VP24 dramatically impairs genome replication and transcription (21, 22).

In the case of short monocistronic minigenomes, our model predicts that the length difference of relaxed RNPs versus condensed RNPs would not be enough to impair packaging of the RNPs, such that there would be no differences in the amount of minigenome delivered to target cells, where the minigenome would then serve as the template for genome replication and transcription using the RNP proteins provided in *trans*. This explains

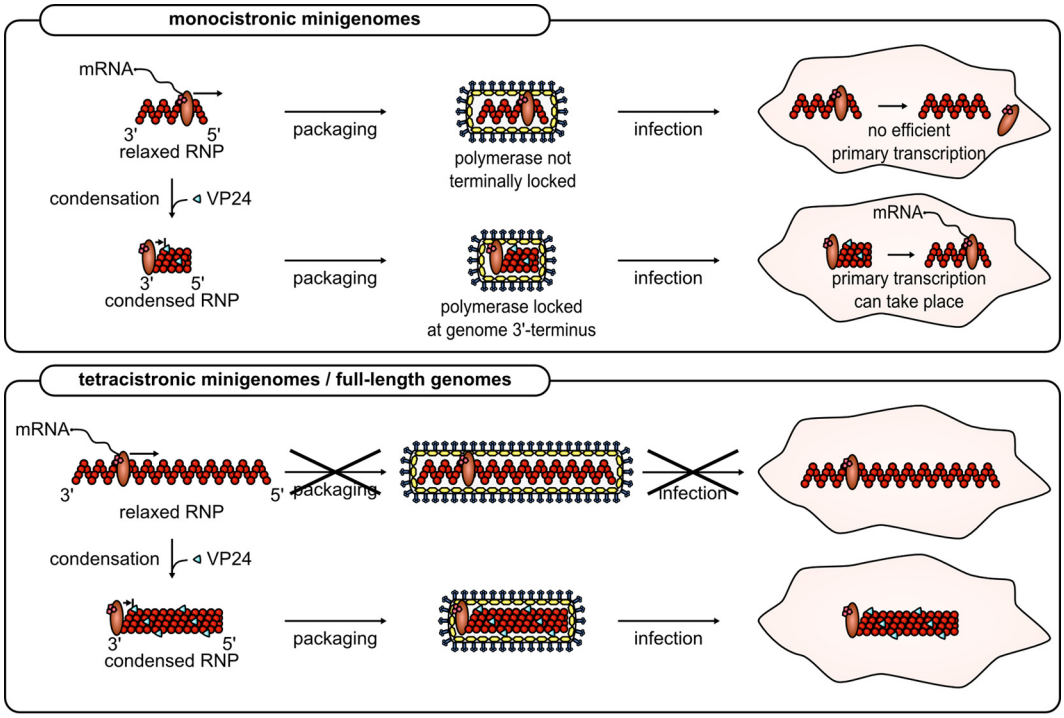

FIG 7 Proposed model for the function of VP24 in the regulation of genome replication and transcription and in morphogenesis. Genome replication and transcription of mRNAs take place using relaxed RNPs as the templates. VP24 leads to condensation of these RNPs, which locks the polymerase at the 3′ end of the genome and abolishes replication and transcription. For short minigenomes, both relaxed and condensed RNPs can be incorporated into trVLPs and infect target cells; however, only in condensed RNPs is the polymerase in the correct position to initiate primary transcription. For long minigenomes or full-length genomes, packaging of relaxed RNPs into particles is less efficient than for that of condensed RNPs due to their increased length, and infection of target cells with particles containing relaxed RNPs is less efficient than infection with trVLPs containing condensed particles.

why there are no differences after infection of pretransfected target cells with trVLPs containing a monocistronic minigenome and produced in the presence or absence of VP24 (11, 12). However, only when the particles contain condensed RNPs would the polymerase be locked in the right position to allow primary transcription to initiate correctly in target cells, presumably after dissociation of VP24 following the uncoating of nucleocapsids. Such a scenario is consistent with the observation that VP24 is required for primary transcription after infection of naive target cells with trVLPs containing monocistronic minigenomes (11), a finding that was previously difficult to rationalize in the context of known VP24 functions.

In contrast, for long tetracistronic minigenomes, our model predicts that packaging of relaxed RNPs into trVLPs would be impaired compared to that of condensed RNPs, which is why we observed fewer tetracistronic minigenomes in trVLP preparations produced in the absence of VP24. In addition, trVLPs that are produced in the absence of VP24 are expected to contain much longer, uncondensed RNPs and are, therefore, themselves longer, leading to reduced infectivity. This reduced infectivity is manifested as a reduction in the reporter activity in infected target cells which exceeds the reduction in RNA incorporation (a 10-fold reduction in reporter activity versus a 2-fold reduction in RNA content), as was observed in our study.

Until now, trVLP systems have been the most comprehensive systems for modeling the Ebola virus life cycle under BSL2 conditions; however, these systems rely on plasmid-based overexpression of all viral proteins. In particular, the proteins VP40 and VP24, which inhibit early stages of the viral life cycle (i.e., genome transcription and replication [21, 22]) but are required for late stages (i.e., morphogenesis and budding of infectious particles [31, 32]), are expressed at relatively high levels at all times in a classical trVLP system. As a consequence, there is a delicate balance that must be struck for the expression of VP40 and VP24 in producer cells in order to obtain a working trVLP system. Matrix proteins of other NSVs, including other viral hemorrhagic fever (VHF)-causing NSVs that are classified as BSL4 organisms, also show similar properties. For example, the arenavirus Z protein, which is the driving force for morphogenesis and budding (reviewed in reference 33), is at the same time a strong inhibitor of genome replication and transcription (34), and until now the only trVLP system established for arenaviruses relies on a chimeric approach in which the Junín virus Z protein is used to drive the formation of a Tacaribe virus minigenome containing trVLPs (35). For most other VHF-causing NSVs, minigenome systems are available (9, 36), but only rarely have trVLP assays been established. However, on the basis of the similarities in virus biology and the availability of monocistronic minigenome systems, the concept of using a multicistronic minigenome for the production of trVLPs should be readily transferable to all of these viruses.

A second problem associated with classical trVLP systems featuring monocistronic minigenomes is that plasmid-based overexpression of the proteins involved in morphogenesis and budding gives rise to large amounts of noninfectious VLPs, which can cause significant problems for downstream biochemical analysis of trVLP preparations (14, 37). We have shown that the use of a

tetracistronic minigenome to drive expression of VP40, $GP_{1,2}$, and VP24 can alleviate these problems. Using this strategy, VP40, $GP_{1,2}$, and VP24 are expressed only after viral transcription of their mRNAs and at much lower levels than after plasmid-based expression. In fact, while we were able to detect these proteins in highly concentrated trVLP preparations (Fig. 3D and E and 6C), providing evidence that these proteins are expressed from the tetracistronic minigenome, we were unable to do so in cell lysates. Consistent with this observation, trVLP preparations produced using tetracistronic minigenomes contained about 500-fold fewer VLPs (as determined by the amount of VP40 and NP in those preparations) than trVLP preparations produced using plasmid-based expression, but they had the same level of infectivity, indicating that the reduction in the overall amount of VLPs is due to a reduction in noninfectious VLP levels. Further, this demonstrates that the intracellular levels of VP40, $GP_{1,2}$, and VP24 produced from minigenomes are completely sufficient to drive the production of infectious trVLPs. Indeed, this makes sense, since the amount of VP40, $GP_{1,2}$, and VP24 produced is dependent on the amount of viral genome replication and transcription, such that the amount of these proteins correlates with the amount of nucleocapsids available for packaging, similar to what occurs during actual virus infection.

Interestingly, in the presence of Tim1 we observed a very small amount of infection by trVLPs produced from a bicistronic minigenome (i.e., in the absence of $GP_{1,2}$,); however, the extent of this infection was about 500-fold lower than that observed with trVLPs produced from a tetracistronic minigenome or a monocistronic minigenome with $GP_{1,2}$ provided in *trans*. This is consistent with a report that Tim1 can mediate the $GP_{1,2}$-independent but phosphatidylserine-dependent uptake of Ebola virus $GP_{1,2}$-containing vesicular stomatitis virus pseudovirions (38) and an entry model in which Tim1 facilitates phosphatidylserine-mediated macropinocytosis of virus particles in a $GP_{1,2}$-independent manner (38), but in which $GP_{1,2}$ and its interaction with a specific receptor, such as NPC-1 (39–41), are subsequently required for efficient fusion and release of nucleocapsids into the cytoplasm.

While trVLPs containing a tetracistronic minigenome can readily infect target cells, they require the presence of NP, VP35, VP30, and L in order for genome replication and transcription to take place, leading to the production of progeny trVLPs. These proteins must be provided in *trans* from expression plasmids. Importantly, these expression plasmids do not share any homologous sequences with the tetracistronic minigenome that would allow recombination events to occur, effectively excluding the possibility of the minigenome acquiring these genes and becoming self-replicating. Further, to the best of our knowledge, such recombination events have never been observed for filoviruses in any context. Therefore, trVLPs containing a tetracistronic minigenome are biologically restricted to infecting cells that express NP, VP35, VP30, and L, making them safe for use under BSL2 conditions.

To date, many studies on Ebola virus entry have been performed using surrogate systems to allow the study of these processes under BSL2 conditions. The most commonly used system involves the pseudotyping of retroviruses; however, the validity of this approach has been questioned (42–44). trVLPs produced from tetracistronic minigenomes represent a viable alternative to this system, with the clear advantage that, in contrast to pseudovirus particles, infectious trVLPs not only exhibit the unique threadlike morphology of Ebola viruses (14, 37), which has been suggested to strongly influence the uptake mechanism used by these viruses (44), but also do not contain any components from foreign viruses. As such, trVLPs containing tetracistronic minigenomes most closely resemble authentic Ebola virus particles and mimic their ability to continuously infect target cells over multiple infectious cycles. In addition, the viral proteins involved in morphogenesis, budding, and entry are produced through processes closely resembling infection, making them a very attractive and versatile tool to study these processes under BSL2 conditions. While we have developed this system using 293 cells, previous studies have shown that other target cells can be used for infection with trVLPs, as long as they are reasonably transfectable (11, 45). Alternatively, primary transcription can be used as a readout, which alleviates the need for transfection of target cells (11); however, in this case target cells are no longer able to produce progeny trVLPs. A possible alternative to our system is a recombinant Ebola virus in which the VP30 CDS is deleted from the genome, biologically containing this virus to cells that provide this protein in *trans* (8). While this virus has the advantage that only one instead of four viral proteins has to be provided in *trans*, it is presently classified as a BSL3 organism, thus restricting its use.

Overall, with the tetracistronic trVLP system, we have developed a novel life cycle modeling system that accurately reflects morphogenesis, budding, and entry over multiple infectious cycles and can also be used to study genome replication and transcription. There are no foreign viral components in this system, and plasmid-based overexpression is avoided for the proteins involved in morphogenesis, budding, and entry, resulting in a modeling of the virus life cycle more authentic than was previously possible. Using this system, we have described a previously unknown role for VP24 in the viral life cycle, and we propose a comprehensive model for the function of VP24 in nucleocapsid assembly. Importantly, the concept for this system should be easily transferable to other negative-strand RNA viruses, including other BSL4 viruses, and since this system can be safely used under BSL2 conditions, it will be of tremendous use in future studies of virus biology and for the development of novel antiviral approaches.

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