Ebola Virus (EBOV) VP24 Inhibits Transcription and Replication of the EBOV Genome

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The roles of Ebola virus (EBOV) VP24 in nucleocapsid (NC) formation and the effect of VP24 on transcription and replication of the viral genome during NC formation remain unknown. We therefore examined the effect of VP24 on the expression of a reporter gene (luciferase), viral RNA, and messenger RNA from the EBOV minigenome. VP24 inhibited the expression of luciferase and both RNAs in a dose-dependent manner, suggesting that VP24 inhibits transcription and replication of the EBOV genome. By contrast, FLAG-tagged VP24, which cannot support NC-like structure formation, did not appreciably decrease luciferase expression, indicating that association of VP24 with the ribonucleoprotein complex is required for inhibition. Glycoprotein and VP40 did not affect VP24-mediated inhibition of transcription and replication. Together, these results suggest that VP24 reduces transcription and replication of the EBOV genome by direct association with the ribonucleoprotein complex in virus-infected cells.

Ebola virus (EBOV) is a filamentous, enveloped, non-segmented, negative-strand RNA virus of the family *Filoviridae* in the order *Mononegavirales.* The RNA genome of EBOV encodes at least 7 structural proteins. The ribonucleoprotein (RNP) complex that mediates transcription and replication of the viral genome comprises 4 of these proteins: nucleoprotein (NP), VP35, VP30, and the RNA-dependent RNA polymerase (L)

[1]. The 3 other proteins—glycoprotein (GP), VP40, and VP24—are membrane-associated proteins [2, 3].

Recently, some of the functions of EBOV VP24, known as a minor matrix protein, have been reported. First, Reid et al. [4] demonstrated that EBOV VP24 inhibits interferon (IFN) signaling. The binding of IFN-α/β and IFN-γ to their receptors triggers the formation of STAT1:STAT2 heterodimers and STAT1:STAT1 homodimers, which are transported to the nucleus by karyopherin $\alpha 1$, an essential step in the activation of numerous genes involved in antiviral activities. VP24 competes with tyrosine-phosphorylated STAT1 to bind to karyopherin $\alpha 1$, blocking the nuclear accumulation of tyrosine-phosphorylated STAT1 and leading to the inhibition of IFN signaling. This finding suggests that VP24 is important for EBOV to evade the antiviral effects of IFNs. Second, Huang et al. [5] showed that VP24, together with NP and VP35, is involved in the formation of nucleocapsid (NC). Third, Hoenen et al. [6] demonstrated that (1) VP24-deficient infectious virus-like particles (VLPs) contain less VP30 than do VLPs containing VP24, and (2) VP24-deficient infectious VLPs could not support the initial transcription of the EBOV minigenome in VLP-infected cells, indi-

cating that VP24 is necessary for the correct assembly of a fully functional NC.

Although VP24 is important for NC assembly, its roles in the formation of NC structures and in transcription and replication of the viral genome remain unknown. To better understand the function of VP24, we examined the effect of VP24 on the transcription and replication of the viral genome by use of the EBOV minigenome system.

MATERIALS AND METHODS

Cells. Human embryonic kidney 293T cells were cultured in high-glucose Dulbecco's modified Eagle's medium containing 10% fetal calf serum, L-glutamine, and penicillin-streptomycin. The cells were grown in an incubator at 37°C under 5% CO_2.

Plasmids. To generate the EBOV minigenome containing the firefly luciferase gene, the firefly luciferase gene was amplified by polymerase chain reaction (PCR) using the plasmid pGAS-luc (Stratagene) as a template, and the green fluorescent gene in p3E5EGFP [7] was replaced with the PCR-amplified firefly luciferase gene. The resulting plasmid was designated "p3E5EF.luc" (figure 1*A*). The PCR product was also cloned into the pT7BlueBlunt vector (Novagen) to generate an RNA probe for Northern blot analysis; this plasmid was designated "pT7F.luc." The open reading frames encoding *Zaire ebolavirus* (ZEBOV) NP, VP35, VP40, glycoprotein (GP), VP30, VP24, and L were cloned into the expression vector pCAGGS/MCS [8, 9] as described elsewhere [7, 10–12]. Plasmids for the expression of the T7 polymerase and M1 of influenza A virus (A/WSN/33) were generated as described elsewhere [12, 13]. A plasmid encoding FLAG-tagged VP24 at the C terminus was constructed using PCR and standard cloning techniques.

Minigenome assay. Firefly luciferase expression from the EBOV minigenome was tested as detailed below (figure 1*B*). First, 4×10^5 293T cells were transfected with different amounts of plasmids (0.125, 0.25, and 0.5 μg, respectively) encoding wild-type *(wt)* VP24, FLAG-tagged VP24, or influenza virus M1, along with plasmids for the production of ZEBOV NP (0.25 μg), VP35 (0.25 μg), VP30 (0.15 μg), L (2 μg), the EBOV minigenome (0.5 μg), and the T7 polymerase (0.5 μg). At 48 h after transfection, the cells were lysed, and the luciferase activity was measured using the Steady-Glo luciferase assay system (Promega) according to the manufacturer's instructions. These cell lysates were also subjected to SDS-PAGE, followed by Western blot analysis, to examine the expression of the EBOV proteins.

Preparation of strand-specific RNA probes. To generate strand-specific RNA probes, p3E5EF.luc or pT7F.luc was digested with *Sal*I or *Bam*HI, which is used to detect positive-sense (mRNA) or negative-sense RNA (viral RNA [vRNA]), respectively. The linearized DNA was then transcribed in vitro

to make RNA probes by use of a DIG Northern starter kit (Roche).

RNA isolation. At 48 h after transfection, cell lysates were treated with micrococcal nuclease (MCN) according to the protocol of Mühlberger et al. [1]. MCN-resistant RNA was isolated using the TRIsol reagent (Invitrogen) to analyze the synthesis of vRNA. For analysis of polyadenylated mRNA, total RNA was extracted using an RNeasy kit (Qiagen), and the polyadenylated mRNA was purified from total RNA by use of an Oligotex mRNA kit (Qiagen).

Northern blot analysis. RNA samples were denatured and separated on 1% denaturing agarose gels containing formaldehyde, transferred onto a Hybond-N+ membrane (GE Healthcare Life Sciences), and fixed by UV cross-linking. Northern blot analysis was performed with strand-specific RNA probes for the sense or antisense firefly luciferase open reading frame, by use of a DIG Northern starter kit (Roche) according to the manufacturer's protocol.

Electron microscopy. Ultrathin-section electron microscopy was performed as described elsewhere [14]. In brief, the plasmid-transfected 293T cells were fixed with 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer and postfixed with 2% osmium tetroxide in the same buffer. The cells were then dehydrated by use of a series of ethanol gradients, followed by propylene oxide treatment; embedded in Epon 812 resin mixture (TAAB Laboratories Equipment); and polymerized at 70°C. Thin sections were stained with uranyl acetate and lead citrate and examined with a HITACHI H-7500 electron microscope at 80 Kv.

Immunoprecipitation. 293T cells, which were cotransfected with plasmids expressing NP, VP35, and VP24 in various combinations, were collected 48 h after transfection and lysed in lysis buffer (50 mmol/L Tris-HCl [pH 7.5], 0.5% NP40, and 0.15 mol/L NaCl) containing protease inhibitors (complete protease inhibitor cocktails [Roche] and 1 mmol/L phenylmethylsulphonylfluoride). After being clarified by centrifugation, the supernatants were incubated with a rabbit anti-NP or an anti-VP35 antibody at 4°C overnight. Protein A agarose beads were added to the reactions, which were then incubated at 4°C for 1 h. The beads were washed 4 times with lysis buffer and suspended in SDS-PAGE sample buffer. The samples were then subjected to SDS-PAGE, followed by Western blot analysis.

RESULTS

VP24 inhibits luciferase expression from the EBOV minigenome. To elucidate whether VP24 affects EBOV genome replication, we used the EBOV minigenome system, which we established elsewhere [7]. In the present study, we constructed the EBOV minigenome possessing the firefly luciferase gene instead of the green fluorescent protein (GFP) gene. To examine
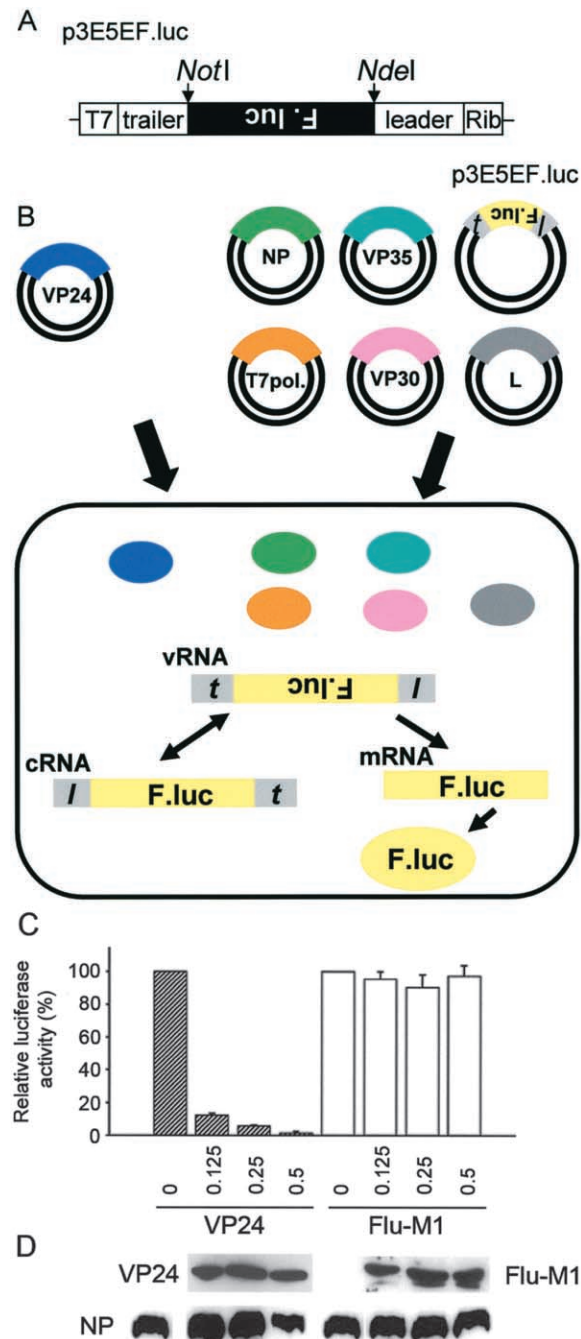
Figure 1. Inhibition of luciferase expression from the Ebola virus (EBOV) minigenome by VP24. *A,* Schematic diagram of the construct for the production of the EBOV minigenome p3E5EF.luc. This construct contains the firefly luciferase gene in the antisense orientation (as denoted by the inverted letters) between the leader and trailer sequences of the EBOV genome, flanked by the T7 RNA polymerase promoter (T7) and a ribozyme (Rib). *Not*I and *Nde*I are the restriction enzyme sites used to clone the luciferase gene. *B,* Schematic diagram of the system used for the minigenome assay. 293T cells were transfected with plasmids for the expression of nucleoprotein (NP), L, VP35, VP30, the EBOV minigenome, T7 polymerase (T7 pol.), and VP24. *C* and *D,* 293T cells were transfected with various amounts of plasmid for the expression of VP24 or influenza virus M1 (Flu-M1), along with plasmids for the expression of NP, VP35, VP30, L, the T7 polymerase, and an EBOV minigenome possessing the firefly luciferase gene. The total amount of DNA in all samples was kept constant (4.15 μg) by adding the appropriate amount of plasmid pCAGGS/MCS. At 48 h after transfection, luciferase activity *(C)* and NP, VP24, and Flu-M1 expression levels *(D)* in the transfected cell lysates were determined. For detection of proteins, 0.3 μg (for NP) and 10 μg (for VP24 and M1) of total protein were applied to gels. The *X*-axis in panel *C* denotes the amount of plasmid (expressed in micrograms). The mean $\pm$ SD values were calculated from data from 3 experiments in the minigenome assay.
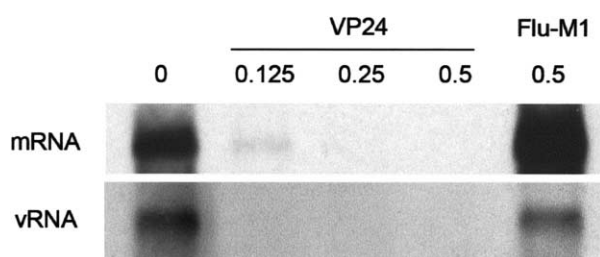
Figure 2. Inhibition of expression of mRNA and viral RNA (vRNA) from the Ebola virus (EBOV) minigenome by VP24. 293T cells were transfected with various amounts of plasmid for the expression of VP24 or a plasmid for the expression of influenza virus M1 (Flu-M1), along with other plasmids as described in the figure 1 legend. At 48 h after transfection, mRNA or micrococcal nuclease (MCN)–resistant RNA was extracted from the plasmid-transfected cells and then was analyzed by Northern blot analysis. A total of 50 ng and 1 μg of mRNA and MCN-resistant RNA, respectively, was applied to gels. The no. in each lane denotes the amount of plasmid (expressed in micrograms).

the effect of VP24 on luciferase expression from the EBOV minigenome, we transfected 293T cells with various amounts of plasmid for the expression of VP24, together with plasmids for the expression of NP, VP35, VP30, L, and the EBOV minigenome (figure 1*B*). A plasmid for the expression of the T7 polymerase was also transfected into these cells to drive the expression of the EBOV minigenome under the control of the T7 promoter (figure 1*B*). We also used a plasmid for the expression of the influenza virus matrix protein M1 as a control. At 48 h after transfection, cells were harvested and lysed, and luciferase activity, reflecting luciferase expression from the minigenome, was determined (figure 1*C*). Expression of VP24 or M1 with NP in the same cell lysate was also examined by Western blot analysis (figure 1*D*). NP expression was used as a measure of the expression of the RNP complex, which mediates the replication of the EBOV minigenome. Although the influenza virus M1 protein had no effect on luciferase activity or NP expression, expression of VP24 caused a decrease in luciferase activity in a dose-dependent manner (figure 1*C*). The highest amount of plasmid for VP24 expression (0.5 μg) led to a decrease in NP expression (figure 1*D*); however, this decrease in NP expression was not responsible for the repression of the luciferase activity, because the luciferase activity was reduced even when NP expression was not affected—that is, when smaller amounts of VP24-expression plasmid were used (0.125 and 0.25 μg). These results indicate that VP24 inhibits luciferase expression from the EBOV minigenome.

VP24 inhibits transcription and replication of the EBOV minigenome. Repression of luciferase expression from the EBOV minigenome by VP24 implies that VP24 affects either transcription or replication of the minigenome, or both. We therefore examined mRNA and vRNA levels from the EBOV minigenome by Northern blot analysis (figure 2). We trans-

fected cells with various amounts of VP24 expression plasmid together with plasmids for the expression of NP, VP35, VP30, L, the T7 polymerase, and the EBOV minigenome, as described above. At 48 h after transfection, total RNA was isolated from the transfected cells. Polyadenylated mRNA was purified from total RNA and was analyzed to measure the level of transcription by use of the luciferase gene–specific negative-sense probe. MCN-resistant vRNA was used to measure the level of RNA replication by use of a gene-specific positive-sense probe. Influenza virus M1 did not appreciably affect mRNA or vRNA levels, whereas VP24 strongly inhibited the expression of both RNAs (figure 2), indicating that VP24 inhibits transcription and replication of the EBOV minigenome.

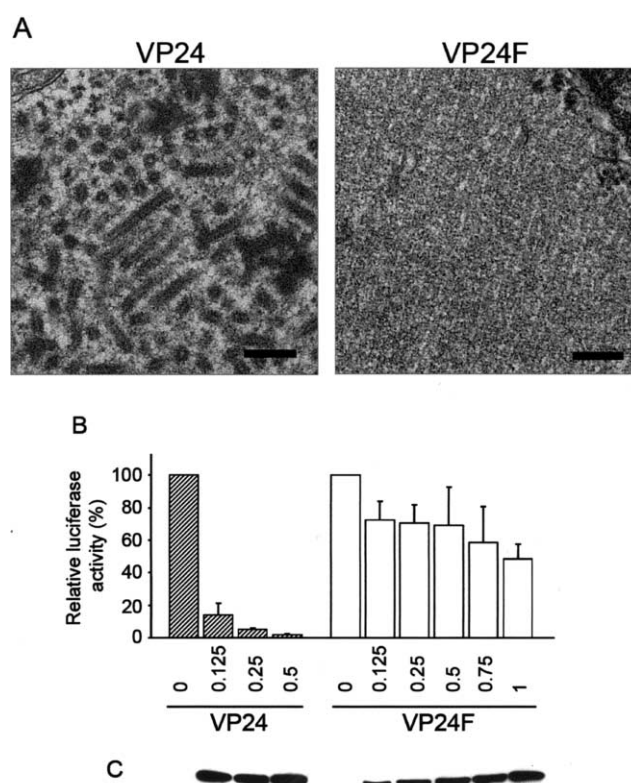***Association between the ability of VP24 to support NC for-***

Figure 3. No support of formation of nucleocapsid (NC) structures and no inhibition of luciferase expression from the Ebola virus (EBOV) minigenome by FLAG-tagged VP24. *A,* Electron microscopy of NC formation. 293T cells were transfected with 2 μg of a plasmid for the expression of VP24 or FLAG-tagged VP24 (VP24F), along with plasmids for the expression of nucleoprotein (NP) and VP35. Bars denote 200 nm. *B* and *C,* 293T cells were transfected with various amounts of a plasmid for the expression of VP24 or VP24F, along with other plasmids as described in the figure 1 legend. At 48 h after transfection, luciferase activity *(B)* and VP24 and VP24F expression levels *(C)* in the transfected cell lysates were determined. For detection of proteins, 10 μg of total protein was applied to gels. The *X*-axis in panel *B* denotes the amount of plasmid (expressed in micrograms). The mean ± SD values were calculated from data from 3 experiments in the minigenome assay.

NP	+	+		+		+		
VP35	+	+	+	+				+
VP24	+	+			+	+	+	+
IP with: anti-NP		+	+	+	+	+		
anti-VP35							+	+
Protein A	+	+	+	+	+	+	+	+
	1	2	3	4	5	6	7	8

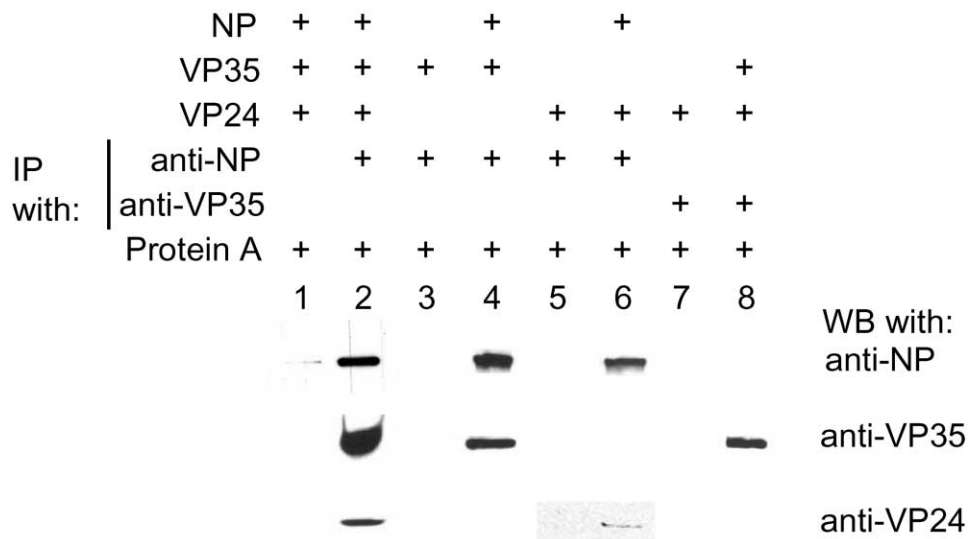WB with:
anti-NP

anti-VP35

anti-VP24

Figure 4. Interaction of VP24 with nucleoprotein (NP) but not VP35. 293T cells expressing NP, VP35, and VP24 in various combinations were lysed and subjected to immunoprecipitation with an anti-NP or an anti-VP35 antibody. The immunoprecipitated proteins were analyzed by Western blot (WB) analysis, by use of an anti-NP, anti-VP35, or anti-VP24 antibody, subsequent to SDS-PAGE. IP, immunoprecipitation.

mation with NP and VP35 and its inhibitory effect on the replication of the EBOV minigenome. Huang et al. [5] previously demonstrated that VP24, NP, and VP35 are required for NC formation. We confirmed this finding [15, 16]; however, we recently found that VP24 with a FLAG tag at its C terminus could not support NC formation (figure 3A) but that it retained the ability to inhibit IFN signaling (data not shown). This finding prompted us to examine whether FLAG-tagged VP24 (VP24F) inhibits transcription and replication of the EBOV minigenome. Therefore, we transfected various amounts of plasmid for the expression of VP24 or VP24F, together with plasmids for the expression of NP, VP35, VP30, L, the T7 polymerase, and the EBOV minigenome, as described above, and assessed luciferase activity 48 h after transfection (figure 3B). Expression of both *wt* VP24 and VP24F was also confirmed by Western blot analysis (figure 3C). Compared with the dose-dependent decrease in luciferase activity induced by the expression of VP24, the inhibitory effect of VP24F was substantially attenuated (although levels of expression of VP24F were not the same as those of VP24, despite the fact that we transfected the same amount of each plasmid) (figure 3B and 3C).

Huang et al. [5] previously demonstrated that NP directly interacts with VP35 or VP24; however, they did not examine the interaction between VP35 and VP24. Therefore, to further understand the interactions between NP, VP35, and VP24, we performed an immunoprecipitation analysis (figure 4). We confirmed that NP directly interacts with both VP35 and VP24 (figure 4, *lanes 1–6*). However, when cells expressing VP35 and VP24 were subjected to immunoprecipitation by use of an anti-VP35 antibody, VP24 was not precipitated (figure 4, *lane 8*),

indicating that the interaction between VP35 and VP24 did not occur or was not detectable under the conditions used.

Together, these data indicate that the association of VP24 with the RNP complex via its interaction with NP is required
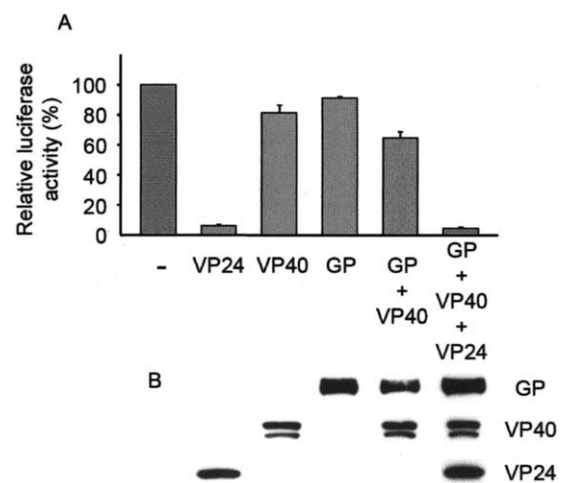

Figure 5. No effect of glycoprotein (GP) and VP40 on VP24-mediated inhibition of luciferase expression from the Ebola virus (EBOV) minigenome. 293T cells were transfected with the same amounts of a plasmid (0.25 μg) for the expression of VP24, VP40, or GP, along with other plasmids as described in the figure 1 legend. The total amount of DNA in all samples was adjusted to 4.4 μg. At 48 h after transfection, luciferase activity *(A)* and VP24, VP40, and GP expression levels *(B)* in the transfected cell lysates were determined. For detection of proteins, 10 μg of total protein was applied to gels. The mean $\pm$ SD values were calculated from data from 3 experiments in the minigenome assay.

for the inhibitory effect of VP24 on the transcription and replication of the EBOV minigenome.

GP and VP40 do not substantially hamper the inhibitory effect of VP24 on transcription and replication of the EBOV minigenome. To examine whether GP and VP40 influence the inhibitory effect of VP24, we cotransfected plasmids for the expression of GP, VP40, and VP24 with plasmids for the expression of NP, VP35, VP30, L, the T7 polymerase, and the EBOV minigenome, and we then determined levels of luciferase activity 48 h after transfection (figure 5A). Expression of GP, VP40, and VP24 was also confirmed by Western blot analysis (figure 5B). Unlike expression of VP24, expression of GP, VP40, or both did not dramatically decrease luciferase expression from the minigenome (figure 5A). Moreover, VP24 still efficiently reduced luciferase expression in the presence of both GP and VP40 (figure 5A). These results indicate that the inhibitory effect of VP24 is not affected by GP or VP40, suggesting that the inhibition by VP24 could occur in EBOV-infected cells.

DISCUSSION

We, as well as others, have demonstrated the involvement of VP24, together with NP and VP35, in the formation of the NC structures of EBOV [5, 16] and have shown that these structures form with or without VP30 and L, which are components of the RNP complex [15]. It is known, however, that VP24 is not required for transcription and replication of the viral genome. Therefore, in the present study, we examined the effect of EBOV VP24 on the transcription and replication of an EBOV minigenome and demonstrated VP24 inhibition of transcription and replication.

Although we did not prove the precise mechanism of this inhibition, association of VP24 with the RNP complex appeared to be important for this event (figure 3). We also demonstrated, by immunoprecipitation, that VP24 interacts with NP, but not with VP35 (figure 4). Whether VP24 interacts with VP30 (the viral-specific transcription activator), L (the viral RNA polymerase), or any host factors remains unknown; VP24 may hamper the functions of these proteins by binding to NP to inhibit transcription and replication of the viral minigenome.

We recently demonstrated, by use of electron microscopy, that NC-like structures are found at the edge of the NP tube bundle and that expression of VP40 along with NC-like structures results in the localization of NC-like structures at the plasma membrane, where EBOV budding occurs [15]. The results presented here, together with these recent data, suggest that the association of VP24 with the RNP complex may function as a signal to convert the viral genome from a transcription and replication-competent form to one that is ready for viral assembly. Although the inhibitory effect of VP24 on viral genome replication is clearly evident in the minigenome assay, it remains to be seen whether this effect occurs in viral-infected

cells. VP24 expression was detected later during infection than was NP, VP35, or VP40 expression [17]. In addition, VP24 repressed luciferase expression from the EBOV minigenome, even in the presence of GP and VP40 (figure 5), which mimics EBOV-infected cells. Thus, although we did not determine how much VP24 is needed for inhibition, this event likely occurs late in viral infection.

Recently, VP24 was shown to inhibit IFN signaling [4]. We demonstrated that VP24 with a FLAG-tag at its C terminus does not support the formation of NC-like structures, resulting in impaired inhibition of luciferase expression from the minigenome. However, this protein retained the ability to inhibit IFN signaling (data not shown). Thus, the region of VP24 that is important for NC formation may differ from the region involved in the inhibition of IFN signaling.

Among negative-sense RNA viruses, several proteins (such as proteins V and C of Sendai and measles viruses, nonstructural protein 1 [NS1] and M2-2 protein of respiratory syncytial virus, V protein of simian virus 5, Z protein of lymphocytic choriomeningitis virus, and nonstructural protein of Bunyamwera virus) have inhibitory effects on viral genome replication [18–27]. Sendai virus C protein binds to the viral polymerase L to inhibit viral RNA synthesis [28], and it has been suggested that the inhibitory effects of NS1 of respiratory syncytial virus, as well as those of the Z protein of lymphocytic choriomeningitis virus, could be responsible for the slower growth of these viruses, compared with that of other negative-sense RNA viruses [18, 22]. However, the biological significance and the mechanism of these effects are basically unclear. Further analyses are needed to define the biological significance and the mechanism by which VP24 exerts its inhibitory effect. Interestingly, some of these proteins, including VP24, also inhibit IFN signaling [29–32]. Thus, the inhibitory effect of these proteins on viral genome replication might also be a common feature among negative-strand RNA viruses to promote efficient virus amplification in the host.

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