JOURNAL OF VIROLOGY, Dec. 2008, p. 12569–12573 Vol. 82, No. 24
0022-538X/08/$08.00+0 doi:10.1128/JVI.01395-08

Role of Ebola Virus VP30 in Transcription Reinitiation▽

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Received 3 July 2008/Accepted 19 September 2008

VP30 is a phosphoprotein essential for the initiation of Ebola virus transcription. In this work, we have studied the effect of mutations in VP30 phosphorylation sites on the ebolavirus replication cycle by using a reverse genetics system. We demonstrate that VP30 is involved in reinitiation of gene transcription and that this activity is affected by mutations at the phosphorylation sites.

Ebola virus (EBOV) causes a severe hemorrhagic fever syndrome, characterized by high mortality rates, in humans and nonhuman primates (7, 13, 14). The EBOV replication cycle takes place in the cytoplasm of infected cells, where inclusion bodies are formed (4). The inclusions contain viral ribonucleoprotein (RNP) complexes consisting of viral RNA and four nucleocapsid proteins: the nucleoprotein (NP), VP35, the polymerase (L), and VP30 (15, 16). Using an EBOV minigenome system, it was demonstrated that NP, VP35, and L are sufficient for RNA replication, whereas the addition of VP30 is required for transcription initiation (12). Recently, it has been shown that VP30 provided in *trans* supports transcription of the minigenome delivered by infectious virus-like particles (VLPs) and replication of a recombinant EBOV lacking the VP30 gene (5). VP30 is phosphorylated at two serine clusters (amino acids 29 to 31 and 42 to 46), each containing three serine residues (Fig. 1A). Previously, it has been shown that mutants of VP30 with both serine clusters replaced by alanine residues supported transcription of an EBOV minigenome but did not accumulate in the NP-induced inclusions. In contrast, when all serine residues at the phosphorylation sites were replaced by aspartate residues, VP30 was unable to support transcription (10). While a Ser→Ala substitution mimics nonphosphorylated serine, a Ser→Asp substitution mimics constantly phosphorylated serine.

In this study, we investigated the effect of mutations simulating a constantly phosphorylated state of VP30 during a Zaire EBOV infection (Fig. 1A). VP30 mutants with nonphosphorylated and highly phosphorylated states are represented by VP30-AA and VP30-DD, respectively. Two other constructs, VP30-AD and VP30-DA, have one of the two serine clusters replaced by an alanine cluster and the other by an aspartate cluster.

In an attempt to generate recombinant viruses containing the designated mutations, we used a reverse genetics system for EBOV (17). Only the wild-type EBOV genome was rescued into infectious virus (Fig. 1B). Failure to recover a virus containing VP30-DD is likely explained by the lack of transcription initiation activity of VP30-DD (10). To assess whether the ability of VP30 to support viral transcription was altered with the other three mutants, we quantified their activities in an EBOV-specific minigenome system (8) (Fig. 2A and B). VP30-AA was 30% more active than the VP30 wild type (VP30-WT) in supporting EBOV transcription, and both VP30-AD and VP30-DA supported transcription of the reporter gene in the same range as VP30-WT. Thus, the experiments with minigenomes did not provide a plausible explanation for the failure to recover recombinant viruses containing the AA, AD, or DA mutations.

In addition to transcription initiation activity, VP30 is a structural protein (3, 6, 14). Since it has been shown that mutations at the phosphorylation site alter VP30's association with NP-induced inclusions (10), we presumed that mutations at the phosphorylation site would impact the incorporation of VP30 into viral particles and thus affect the efficiency of virus recovery. To assess the ability of VP30 mutants to support viral morphogenesis, we used an EBOV-specific VLP system (8). We found that all mutants of VP30 were incorporated into VLPs and were protease protected (Fig. 2C). This indicated that VP30 mutants were localized inside the particles. To confirm these results, we tested whether the mutants of VP30 were also incorporated into authentic EBOV. Subconfluent 75-cm^2 flasks of Vero cells were transfected with 8 μg of plasmids encoding myc-tagged mutants of VP30 and were then infected with EBOV at a multiplicity of infection (MOI) of 1 at 6 hours posttransfection. Virions released into the culture medium during 24 h of infection were collected by ultracentrifugation through a 20% sucrose cushion and then analyzed by Western blotting using anti-VP24 and anti-myc antibodies. Both VP30-AA and VP30-DD mutants were found to be associated with viral particles (Fig. 2D). These data are in agreement with a recent study (6) showing that recruitment of VP30 into VLPs is dependent on the C-terminal part of the protein, while our

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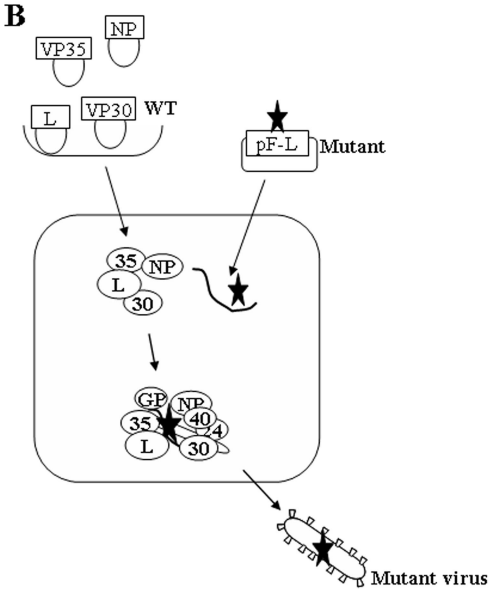
▽ Published ahead of print on 1 October 2008.

A

EBOV VP30 wild-type RAR**S**$_{29}$**S**$_{30}$**S**$_{31}$RENYRGEYRQ**S**$_{42}$R**S**$_{44}$A**S**$_{46}$QVRVPTVFH			
Substitution	**First cluster**	**Second cluster**	**Nomenclature**
S29,30,31→ A S42,44,46→ A	AAA	AAA	VP30-AA
S29,30,31→ D S42,44,46→ D	DDD	DDD	VP30-DD
S29,30,31→ A S42,44,46→ D	AAA	DDD	VP30-AD
S29,30,31→ D S42,44,46→ A	DDD	AAA	VP30-DA

B



VP30 gene	Rescue
WT	+
AA	-
DD	-
AD	-
DA	-

FIG. 1. (A) Nomenclature of VP30 mutants. Two clusters of serine residues (S29, S30, and S31; S42, S44, and S46) constitute the protein phosphorylation sites. Several cellular protein kinases (e.g., PKA, CKII, and cAMKII) are predicted to recognize VP30 phosphorylation sites. Amino acid substitutions and the nomenclature of the constructed mutants are indicated. (B) Recovery of recombinant viruses. (Left) Schematic representation of the reverse genetics system. Mutations in the VP30 gene are introduced in a plasmid carrying a full-length cDNA of Zaire EBOV. The recovery of the recombinant virus is then supported by four plasmids encoding wild-type nucleocapsid proteins (NP, VP35, VP30, and L). (Right) Results of the recovery experiment. Successful recovery of the virus is indicated by a plus. Lack of virus recovery is indicated by a minus.

mutations affected the N terminus. Moreover, our results suggest that the intracellular colocalization of VP30 and NP described in an earlier publication (10) does not reflect the ability of VP30 to be incorporated into budding VLPs. It should be mentioned that the smaller amount of VP30-DD in pelleted virions compared with that of VP30-WT and VP30-AA was not expected based on our results with VLPs. However, this is explained by the negative effect of VP30-DD on viral replication. Since only approximately 10 to 15% of cells were transfected and expressed mutated VP30, this effect is not seen at the level of the VP24 expressed in all virus-infected cells. To confirm the effect of VP30-DD on viral replication, 293T cells, which have much higher transfection efficiency, were used (Fig. 2E).

Taken together, our results showed that VP30-WT, VP30-AA, VP30-AD, and VP30-DA were similar in terms of their association with the nucleocapsid and transcription of EBOV minigenomes, but they differed in their capacities to rescue recombinant viruses. One possible explanation of their differences is that in the minigenome system, a monocistronic template is transcribed, whereas rescue of full-length viral RNA needs transcription of seven individual genes in a consecutive manner. At every gene end, transcription stops and is reinitiated at the start of the following gene (11). We therefore analyzed the capacities of the VP30 mutants to reinitiate transcription. The plasmid encoding VP30-WT was replaced by others encoding VP30-DD, VP30-AA, VP30-AD, or VP30-DA in the full-length rescue system in combination with the wild-type EBOV genome (Fig. 3). If the mutants of VP30 are able to support transcription of all viral genes, VP30, which is encoded by the fifth gene of the viral genome, will be synthesized and will compensate for putative missing functions of the mutant VP30. Here, we demonstrate that VP30-DD, VP30-AD, and VP30-DA do not support the recovery of the wild-type

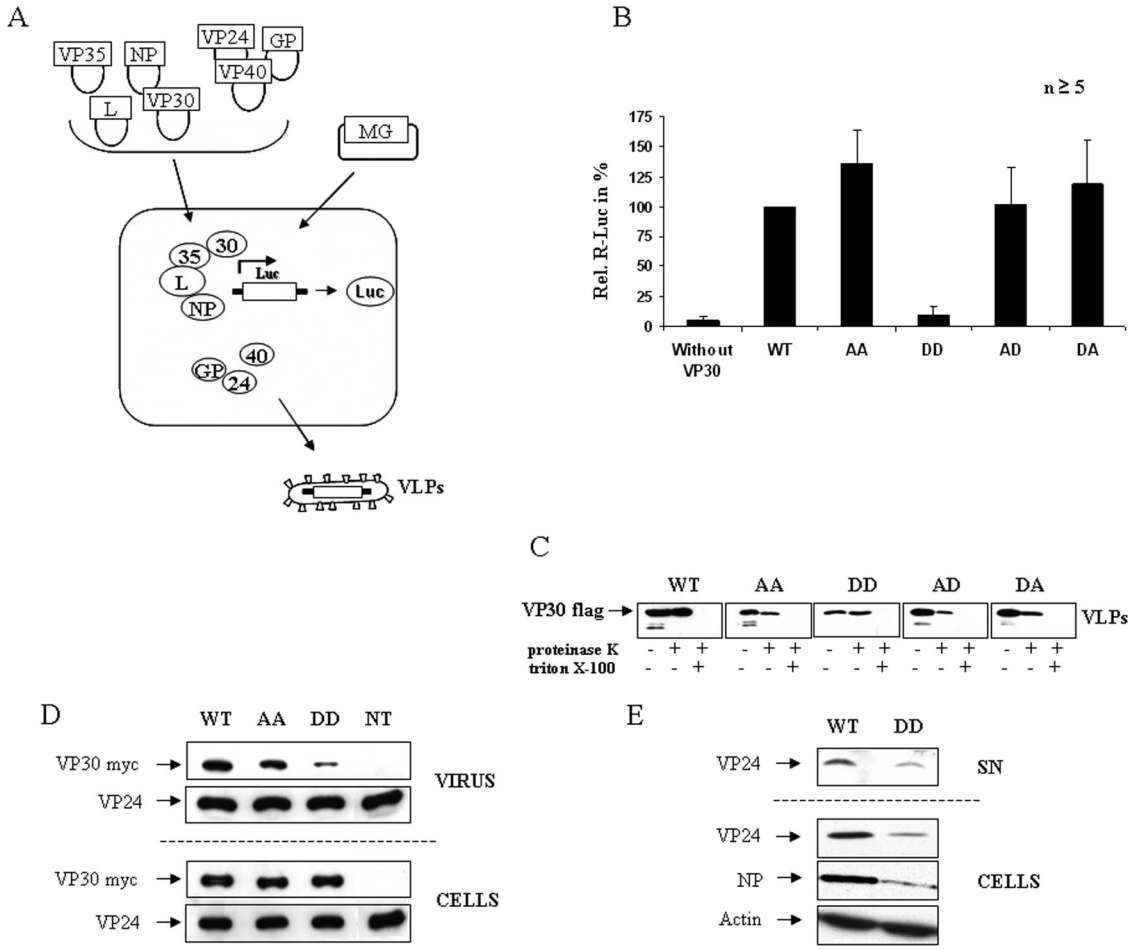
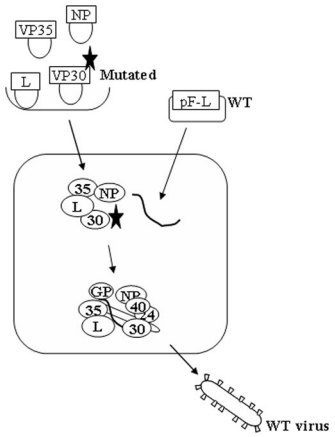


FIG. 2. Influence of permanent charges at the phosphorylation sites of VP30 on viral transcription and incorporation of VP30 into virus particles. (A) Schematic representation of the minigenome and VLP assay. HEK293 cells were transfected with plasmids encoding all viral proteins, the T7 polymerase, and the EBOV-specific minigenome (MG) containing a *Renilla* luciferase reporter gene. Additionally, the vector pGL4 carrying the firefly luciferase reporter gene was transfected and used for normalization of the results. Nucleocapsid proteins provided in *trans* direct transcription/replication of the EBOV minigenome, which is subsequently encapsidated into VLPs. ⌐→, transcription initiation of the minigenome containing the luciferase reporter. (B) Transcription activation by VP30-WT and VP30 mutants. At 72 h posttransfection, cells were harvested and analyzed for reporter activity using the Dual-Luciferase assay (Promega). *Renilla* luciferase activity (R-Luc), reflecting minigenome replication and transcription, was normalized by firefly luciferase activity. Transcription activation by VP30-WT was set to 100%. Replacement of VP30-WT by the respective mutants is indicated. Rel., relative. (C) Incorporation of flag-tagged VP30 into VLPs. VLPs from culture supernatants collected at 72 h posttransfection were purified by ultracentrifugation through a 20% sucrose cushion. Incorporation of VP30-WT and mutants of VP30 was analyzed by a protease protection assay. Samples were treated with proteinase K in the presence or absence of Triton X-100, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then detected by Western blotting using antiflag antibodies. Proteins inside VLPs were protected from proteinase K digestion and are visible in the middle columns. (D) Incorporation of myc-tagged VP30 proteins in EBOV particles. Vero E6 cells were transfected with plasmids encoding myc-tagged VP30 (VP30-WT [WT], VP30-AA [AA], or VP30-DD [DD]) and then infected with EBOV at an MOI of 1 PFU/cell. Virus particles from culture supernatants were purified by ultracentrifugation through a 20% sucrose cushion. NT, nontransfected cells. (E) Expression of VP30-DD negatively affects EBOV replication. 293T cells were transfected with plasmids expressing either VP30-WT or VP30-DD and then infected with EBOV at an MOI of 1 PFU per cell. Samples of culture supernatants (SN) and lysed cells were analyzed by Western blotting using anti-VP24, anti-NP, and antiactin antibodies.

EBOV. In contrast, VP30-AA supported the recovery of the wild-type EBOV genome, indicating that the nonphosphorylated form of VP30 activates transcription of all viral genes, including the gene encoding VP30. Since VP30-AD and VP30-DA showed an activity comparable to that of VP30-AA in supporting transcription of the reporter gene, their failure to recover the wild-type virus pointed to a difference in supporting transcription of downstream genes.

Next, we assessed the abilities of VP30-AD and VP30-DA to support transcription reinitiation. We generated a plasmid carrying a full-length cDNA of EBOV containing the green fluorescent protein (GFP) gene as an additional transcription unit between the genes encoding VP35 and VP40 (the second and third genes). When VP30-AA was used to rescue this virus, GFP-expressing cells appeared 2 days posttransfection and gradually increased in number, corresponding to the spread of the recombinant virus (Fig. 4A). In contrast, no GFP-expressing cells were observed in the presence of VP30-AD or VP30-DA, suggesting that these mutants did not support transcription of the GFP gene. These results strongly support the idea



VP30 construct	Rescue
WT	+
AA	+
DD	-
AD	-
DA	-

FIG. 3. Rescue of wild-type EBOV using mutants of VP30. (Left) Schematic representation of the experimental setup. The recovery of a wild-type recombinant EBOV is supported by plasmids expressing the nucleocapsid proteins NP, VP35, and L together with mutants of VP30. (Right) Results of the recovery experiment. Successful recovery of the virus is indicated by a plus. Lack of virus recovery is indicated by a minus.

that the AD and DA substitutions in VP30 affected the ability of the polymerase complex to reinitiate transcription of viral genes downstream of the second gene.

To confirm that VP30-AA was able to reinitiate transcription without the expression of VP30-WT, we mutated the full-length cDNA of EBOV containing the GFP gene by introducing the AA mutation in the VP30 gene and employed this construct in the rescue system by expressing the different mutants of VP30 in *trans*. Individual cells expressing GFP were observed when VP30-AA was employed, whereas no expression of GFP was detected when VP30-AD or VP30-DA was employed (Fig. 4B). This result confirmed that VP30-AA was able to support viral transcription of the first gene as well as consecutive genes. In contrast, the mutants VP30-AD and VP30-DA, while capable of initiating transcription of the first gene, were unable to reinitiate transcription of downstream genes. Besides, these data suggest that a cluster of negative charges mimicking phosphorylation could inhibit VP30's transcription reinitiation function.

Our experiments demonstrate that the serine clusters at the N terminus of VP30 represent a critical region for the activity of the protein. A combination of alanine and aspartate residues (VP30-AD and VP30-DA) resulted in a VP30 protein that was able to support transcription of an EBOV-specific monocistronic minigenome but unable to reinitiate transcription in the full-length genome. Permanently uncharged amino acids at the phosphorylated region (VP30-AA) resulted in VP30 molecules that were able to activate transcription of a minigenome and all the genes of the EBOV genome in a consecutive manner. However, a recombinant EBOV encoding VP30-AA could not be generated, indicating that this mutant lacks another function needed to produce fully infectious virions. The step at which phosphorylated VP30 is essential in the viral life cycle is currently unknown. Since VP30-DD blocks EBOV transcription, the phosphorylation of VP30 might play a role in switching off the transcription favoring replication of the viral RNA. In this regard, the permanently nonphosphorylated VP30-AA would impair the regulation of the transcription/replication processes. In addition, since a phosphorylated form of VP30 is present in EBOV virions (3), phosphorylated VP30 may be necessary for a very early step in the viral life cycle. Our results thus suggest that the dynamic phosphorylation of VP30 is essential for the function of the protein in EBOV infection.

It is presumed that transcription in viruses from the order *Mononegavirales* follows a sequential stop-start mechanism in which transcription of downstream genes is dependent on termination of the synthesis of the upstream gene. While the majority of studies have focused on the influence of *cis*-acting sequences in transcription regulation (1, 2, 19), in our work, we provide experimental evidence of the participation of a structural protein other than the viral polymerase in transcription reinitiation. The function of VP30 in reinitiation of transcription expands on results from a previous report showing that a specific stem-loop structure at the beginning of the NP gene is involved in VP30-dependent transcription and that only transcription initiation of the NP gene requires VP30 (18). In the artificial bicistronic EBOV minigenome, transcription of the second gene did not require the activity of VP30. Using a full-length EBOV genome, we obtained results that partially

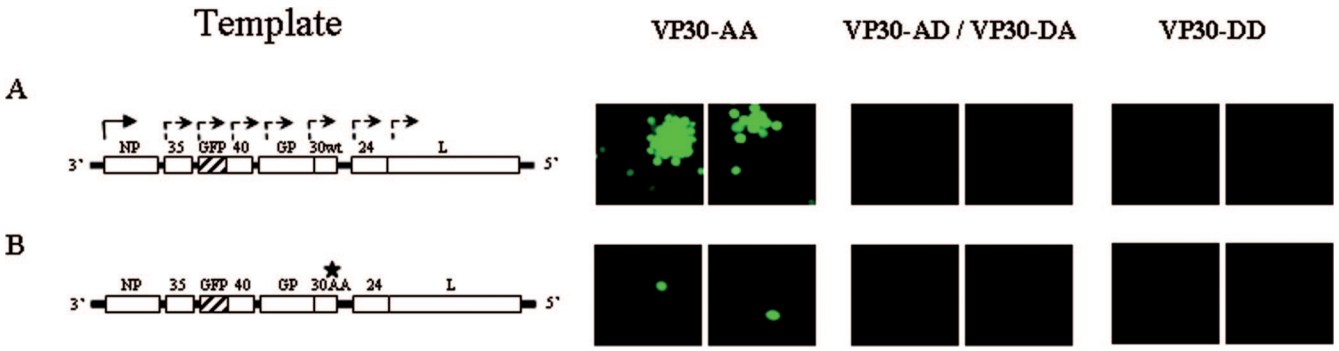


FIG. 4. Recovery of recombinant EBOV expressing GFP. The GFP gene was introduced into the full-length cDNA of either wild-type EBOV (A) or EBOV/VP30-AA (B). Recombinant virus recovery was performed in BHK T7 cells using standard protocols and different VP30-expressing plasmids as indicated. The presence of groups of green cells indicated recovery and replication of the recombinant viruses. The appearance of single green cells indicates the ability of mutated VP30 to support both transcription initiation and reinitiation but with subsequent failure of virus replication. ⌐→, transcription initiation; ⌐→, transcription reinitiation.

contradict this earlier report. This, however, only highlights the need to complement minigenome studies with the analysis of full-length genome expression systems. Recently, EBOV VP30 was shown to be an RNA-binding protein (9). Notably, VP30 binds to the sequence in the NP gene's stem-loop structure that includes the transcriptional start signal and its complementary sequence. Sequences complementary to the respective transcription start signal are present in close proximity to the start signal of all EBOV genes, suggesting that the stem-loop structures also play a role in the transcription of other genes (11). Interestingly, analysis of these structures using the mfold program (20) showed that their energetic stability (ΔG) is increased in a 3′→5′ order (NP, −7.40; VP35, −8.6; VP40, −17.6; GP, −14.6; VP30, −19.1; VP24, −27.9; L, −23.9). Given that VP30 is involved in transcription reinitiation, it is reasonable to speculate that there is a gradient along the viral genome that controls the efficiency of transcription, especially at the 5′ genome end.

In conclusion, our data indicate, for the first time, that activity of VP30 is required for reinitiation of gene transcription and that mutations at the phosphorylation sites of VP30 affect this activity. While the mechanism of VP30 action is not yet clearly understood, our results suggest that VP30 could be an important target for antiviral therapy.

All experiments involving live EBOV were carried out in the INSERM BSL-4 laboratory Jean Merieux in Lyon, France. We thank the biosafety team members for their support and assistance in conducting experiments and Ulla Thiesen for expert technical assistance. We are also grateful to Robin Buckland for his helpful comments on the manuscript.

This work was supported by INSERM, the French Ministère de la Recherche (grant 04G537), the Agence National de la Recherche (ANR), the National Institutes of Health (grant AI059536), and the Deutsche Forschungsgemeinschaft (SFB 593 to V.V. and SFB 535 to S.B.). M.J.M. was partially supported by the Sociedad Española de Enfermedades Infecciosas y Microbiologia Clinica (SEIMC). N.B. is supported by the Ernst Schering Foundation.

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