RESEARCH ARTICLE

Single Nucleoprotein Residue Modulates Arenavirus Replication Complex Formation

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ABSTRACT **The *Arenaviridae* are enveloped, negative-sense RNA viruses with several family members that cause hemorrhagic fevers. This work provides immunofluorescence evidence that, unlike those of New World arenaviruses, the replication and transcription complexes (RTC) of lymphocytic choriomeningitis virus (LCMV) colocalize with eukaryotic initiation factor 4E (eIF4E) and that eIF4E may participate in the translation of LCMV mRNA. Additionally, we identify two residues in the LCMV nucleoprotein (NP) that are conserved in every mammalian arenavirus and are required for recombinant LCMV recovery. One of these sites, Y125, was confirmed to be phosphorylated by using liquid chromatography-tandem mass spectrometry (LC-MS/MS). NP Y125 is located in the N-terminal region of NP that is disordered when RNA is bound. The other site, NP T206, was predicted to be a phosphorylation site. Immunofluorescence analysis demonstrated that NP T206 is required for the formation of the punctate RTC that are typically observed during LCMV infection. A minigenome reporter assay using NP mutants, as well as Northern blot analysis, demonstrated that although NP T206A does not form punctate RTC, it can transcribe and replicate a minigenome. However, in the presence of matrix protein (Z) and glycoprotein (GP), translation of the minigenome message with NP T206A was inhibited, suggesting that punctate RTC formation is required to regulate viral replication. Together, these results highlight a significant difference between New and Old World arenaviruses and demonstrate the importance of RTC formation and translation priming in RTC for Old World arenaviruses.**

IMPORTANCE **Several members of the *Arenaviridae* cause hemorrhagic fevers and are classified as category A pathogens. Arenavirus replication-transcription complexes (RTC) are nucleated by the viral nucleoprotein. This study demonstrates that the formation of these complexes is required for virus viability and suggests that RTC nucleation is regulated by the phosphorylation of a single nucleoprotein residue. This work adds to the body of knowledge about how these key viral structures are formed and participate in virus replication. Additionally, the fact that Old World arenavirus complexes colocalize with the eukaryotic initiation factor 4E, while New World arenaviruses do not, is only the second notable difference observed between New and Old World arenaviruses, the first being the difference in the glycoprotein receptor.**

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The *Arenaviridae* are a family of pathogenic RNA viruses with several members capable of causing hemorrhagic fevers (1). The arenaviruses are divided into two groups, Old World and New World, on the basis of their geographical distribution (2). Lymphocytic choriomeningitis virus (LCMV), an Old World arenavirus with worldwide distribution, is the prototypic member of this family (3). Arenavirus genomes are bipartite, with each segment encoding two open reading frames (ORFs) in ambisense orientation (2, 4). The large genome segment encodes the matrix protein (Z) and the RNA-dependent RNA polymerase (L) (5, 6), while the small segment encodes the glycoprotein (GP) and the nucleoprotein (NP) (7).

Protein phosphorylation is one of the most prevalent post-translational modifications (PTM) in eukaryotic cells (8–10). It is estimated that approximately one third of all cellular proteins are targets of phosphorylation and that 30% of these are phosphorylated at any given time within the cell (8, 9, 11). Because phosphorylation is reversible, a primary function of this PTM is to act as a switch, rapidly changing a protein's activity (12). Phosphorylation plays a vital role in a number of cellular processes, including but certainly not limited to signal transduction, development, differentiation, metabolism, and cell cycle regulation (9).

Because phosphorylation is broadly employed by host cells and provides such rapid flexibility in protein function, it is not surprising that viruses would use this PTM in their carefully regulated life cycles (10). The temporal regulation of viral transcription and genome replication is critical to viral life cycles and, in many negative-sense RNA viruses, these are regulated by phosphorylation (13–21). All negative-sense RNA viruses encode nucleoproteins to protect their genomes. Because of the intimate relation-

ship between viral genome RNAs and NP, the phosphorylation state of the NP is often the regulatory switch in transcription and translation (13–16). Recently, protein kinase activities were determined to be required for arenaviruses to establish infection and for genome transcription (22, 23). Moreover, an unidentified virion-associated serine/threonine kinase can phosphorylate LCMV NP after being released from purified virions (24). However, whether NP is phosphorylated within infected cells and how phosphorylation affects the function of NP remain to be determined.

L and NP are the only arenavirus proteins required for viral RNA transcription and replication, and NP is sufficient to nucleate formation of replication transcription complexes (RTC) (25, 26). Studies of RTC in Tacaribe virus (TACV), a New World arenavirus, have demonstrated that they contain phosphatidylinositol 4-phosphate (PI4P) and associate with subunits eukaryotic initiation factor 4E (eIF4E) and eIF4G of the eIF4F complex (25). Interestingly, TACV RTC do not associate with eIF4E (25, 27). The viral genome is contained within RTC, while viral transcripts appear to be transported out into the cytosol (25). Additionally, studies of another New World arenavirus, Junin virus (JUNV), demonstrate that NP coimmunoprecipitates with subunits eIF4A and eIF4G but not with eIF4E and that eIF4E is dispensable for JUNV translation (27). Together, these studies suggest that NP may replace eIF4E in the eIF4F complex and that eIF4A and eIF4G may associate with RTC to prime viral transcript translation.

In this study, we sought to identify sites of phosphorylation on NP and determine their roles in LCMV replication. We have identified NP Y125 as a phosphorylation site that is required for the rescue of recombinant LCMV (rLCMV). A conservative mutation at this site to phenylalanine, which cannot be phosphorylated, results in the inability to recover rLCMV. We also demonstrate that an additional site in NP, namely, T206, is a candidate for regulatory phosphorylation. Mutation of T206 to alanine, which cannot be phosphorylated, results in the loss of both the normal punctate RTC that are typically observed during LCMV infection and viral mRNA translation when Z and GP are present. This loss of translation in the absence of normal RTC provides additional evidence that translation priming occurs within RTC. Additionally, NP T206 is required for the recovery of rLCMV. We also demonstrate that LCMV RTC, unlike RTC of Old World arenaviruses, associate with each of the three proteins that comprise the eIF4F complex, suggesting that eIF4E may be required for LCMV mRNA translation.

RESULTS

Predicted phosphorylation at three serines and one threonine in NP. To identify potential NP phosphorylation sites, we generated a multiple sequence alignment (MSA) containing all arenavirus NP sequences in the UniProt database. Using Jalview (28), we identified residues in NP that are conserved across all arenaviruses, and we identified residues that are predicted to be phosphorylated by using NetPhos2.0 (29) and NetPhosK (30). Mapping residues that are both conserved and predicted to be phosphorylated onto an alignment of two PDB structures of NP (3MWP and 3T5N) identified S9, S82, T206, and S233 as solvent exposed and, therefore, available to be phosphorylated in the folded protein.

MS identified five NP phosphorylation sites A prior study reported that serine and threonine residues of NP are phosphorylated by a virion-associated kinase (24). We purified LCMV virions from infected BHK-21 cells and used liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify specific phosphorylated residues. This approach identified phosphorylation at NP residues S116, S122, S343, and T330. Interestingly, MSA analysis of these sites in Jalview showed NP residue S116 to be conserved at a level of 74%, S122 at 30%, T330 at 100%, and S343 at 32% across all mammalian arenaviruses. Unexpectedly, tyrosine phosphorylation was also observed, namely, at Y125. This residue is conserved in all Old and New World arenaviruses.

MG expression of CAT with NP T206A is significantly reduced in the presence of Z and GP. Site-directed mutagenesis of NP yielded mutants with the mutations S9A, S82A, S116A, S122A, Y125A, T206A, S233A, T330A, and S343A. Western blot analysis demonstrated the expression of all NP mutants in HEK293T cells (Fig. 1A). Viral gene expression was quantified in a reporter assay using a minigenome (MG) in which the GP gene is deleted and the NP gene is replaced by the chloramphenicol acetyltransferase (CAT) gene (26, 31). L and NP were previously shown, using this assay, to be the only viral proteins required to transcribe and replicate the arenavirus genome (26). Additionally, this assay has been used to investigate the effects on viral replication of mutations in different viral proteins (31, 32). To this end, we constructed point mutations in NP corresponding to the predicted (NP S9A, S82A, T206A, and S233A) and confirmed (NP S116A, S122A, Y125F, T330A, and S343A) phosphorylation sites and used these mutants in the MG reporter assay (Fig. 1). In the absence of Z and GP, all mutants having predicted phosphorylation sites, with the exception of NP S82A, supported CAT expression levels comparable to that obtained with wild-type (WT) NP. With NP S82A, the CAT expression levels were 57% of the level with WT NP ($P = 0.0014$) (Fig. 1B). Of the mutants with mutations that ablated known phosphorylation sites, mutants NP S122A and NP T330A expressed significantly less CAT protein than was obtained with WT NP ($P = 0.0012$ and $P < 0.0001$, respectively) (Fig. 1C).

The results of previous studies demonstrate that CAT expression in the MG reporter assay is lower in the presence of Z and GP (26, 31). We therefore assessed the effects of Z and GP in combination with each of the NP mutants that we generated. Because of the substantial reduction of CAT expression with WT NP in the presence of Z and GP (1.7%) (Fig. 1B), we normalized all MG assay results using NP mutants with Z and GP to the CAT expression levels when using WT NP in the presence of Z and GP (Fig. 1D and E). Among the predicted phosphorylation mutants (Fig. 1D), NP T206A and NP S82A supported significantly lower levels of CAT expression than did WT NP in the presence of Z and GP ($P < 0.0001$). Among the confirmed phosphorylation site mutants (Fig. 1E), NP S122A and NP T330A supported substantially lower levels of CAT expression than did WT NP, beyond their effects in the absence of Z and GP (Fig. 1C). Interestingly, in the presence of Z and GP, NP Y125F supported significantly increased levels of CAT expression (141% of the level with WT NP; $P = 0.0166$) (Fig. 1E).

NP T206A does not form typical NP punctae. The expression of WT NP in BHK-21 cells (Fig. 2A) demonstrated punctae of NP in the cytosol that are typical of RTC formed during LCMV infec-

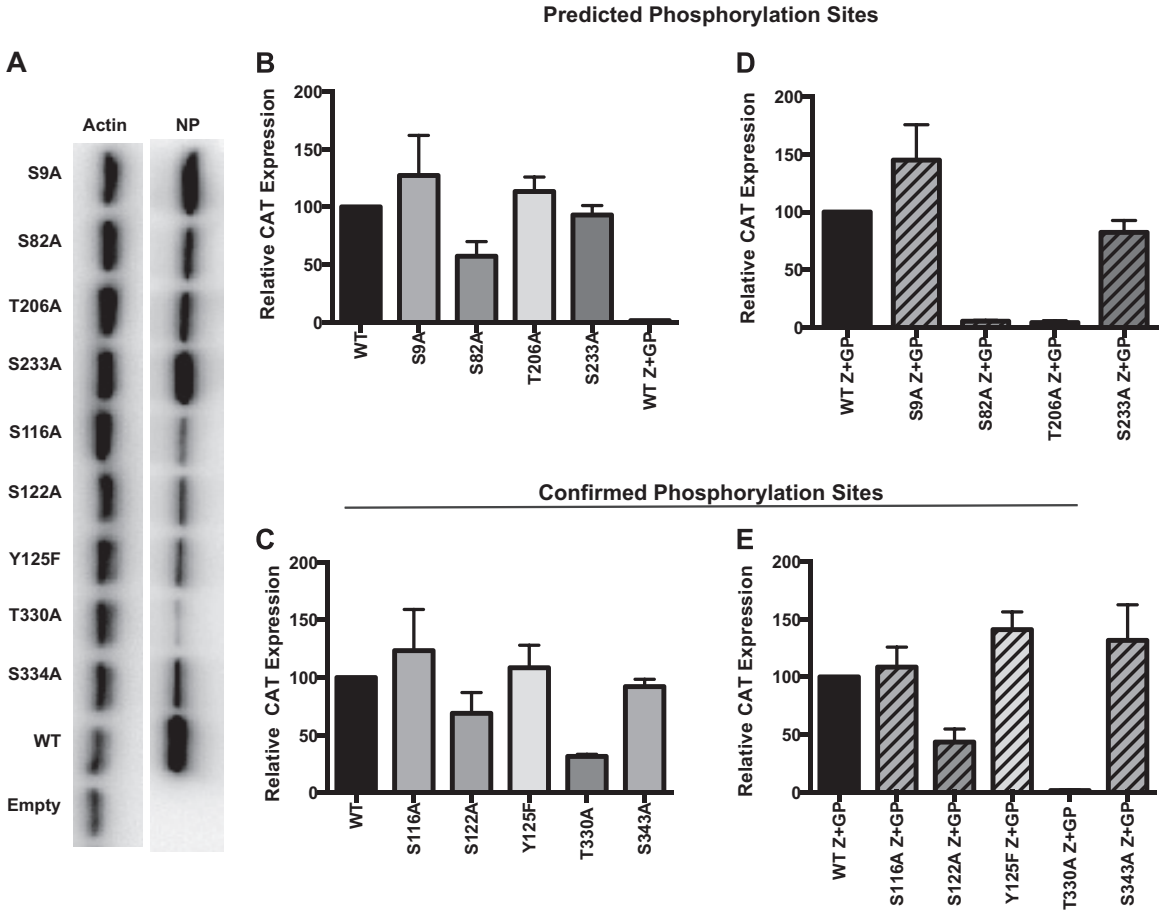

FIG 1 MG CAT expression with various NP point mutants. (A) HEK293T cells were transfected for expression of the NP point mutants, and Western blot analysis was performed for both NP and actin. (B to E) For the MG CAT expression assays, HEK293T cells were transfected, with and without Z and GP, with various NP point mutants. Forty-eight hours posttransfection, cell lysates were collected and CAT ELISAs were performed to quantify CAT protein. Results for assays that included Z and GP have "Z+GP" in the *x*-axis labels. (B and D) CAT expression levels when NP mutants had mutations at predicted phosphorylation sites. (C and E) CAT expression levels when NP mutants had mutations at confirmed phosphorylation sites. (B and C) Results are normalized to CAT expression with WT NP. (D and E) Results are normalized to CAT expression with WT NP in the presence of Z and GP. Each bar represents the results of a minimum of three separate experiments. All error bars represent standard deviations.

tion. All NP point mutants formed characteristic NP punctae (data not shown), except for the T206A mutant. The results for cells transfected with NP T206A showed that this mutant formed atypical NP aggregations that appeared to diffuse into the cytoplasm (Fig. 2B).

CAT expression levels with NP T206S and NP T206E, along with distribution in BHK-21 cells, suggest that NP T206 is transiently phosphorylated. Because NP T206A supported WT levels of CAT in the absence of Z and GP but only 4.3% of the WT levels in the presence of Z and GP (Fig. 1B and D), we hypothesized that T206 may be a site of regulatory phosphorylation. To test this, we constructed an NP T206S mutant, which has the potential to be phosphorylated by the same serine/threonine kinase that may phosphorylate residue T206, and tested it using the MG assay in HEK293T cells. In the absence of Z and GP, NP T206S produced CAT protein at 141% of the level obtained with WT NP ($P = 0.0077$) (Fig. 3A). When coexpressed with Z and GP in the MG assay, NP T206S also showed no defects (Fig. 3B). Additionally, we made a phosphomimetic mutant, NP T206E, to mimic constitutive phosphorylation at this site. In the absence of Z and GP, NP T206E produced CAT protein at 64% of the level obtained with WT NP ($P = 0.0008$) (Fig. 3A). No CAT protein was expressed when NP T206E was coexpressed with Z and GP in the MG assay (Fig. 3B). The expression of NP T206S in BHK-21 cells had a WT NP-like punctate appearance (not shown). However, the phosphomimetic NP T206E had a phenotype that was intermediate between those of WT NP and NP T206A, with NP both being diffuse in the cytoplasm and forming large punctae (Fig. 3C).

Recombinant LCMV containing NP Y125F or NP T206A were not recovered. To study NP Y125F and NP T206A in the context of LCMV infection, we sought to generate rLCMV containing these mutant NPs (33). While we were able to recover rLCMV with WT NP and most of the predicted and confirmed NP phosphorylation mutants (NP S9A, S82A, T206S, S233A, S116A, and S122A), after four independent attempts, we were unable to recover rLCMV that contained the NP T206A or NP Y125F mutations. Moreover, we were unable to recover a tripartite rLCMV (34) that contained NP T206A on the same segment with GP and WT NP on a segment that lacked GP (Fig. 4A).

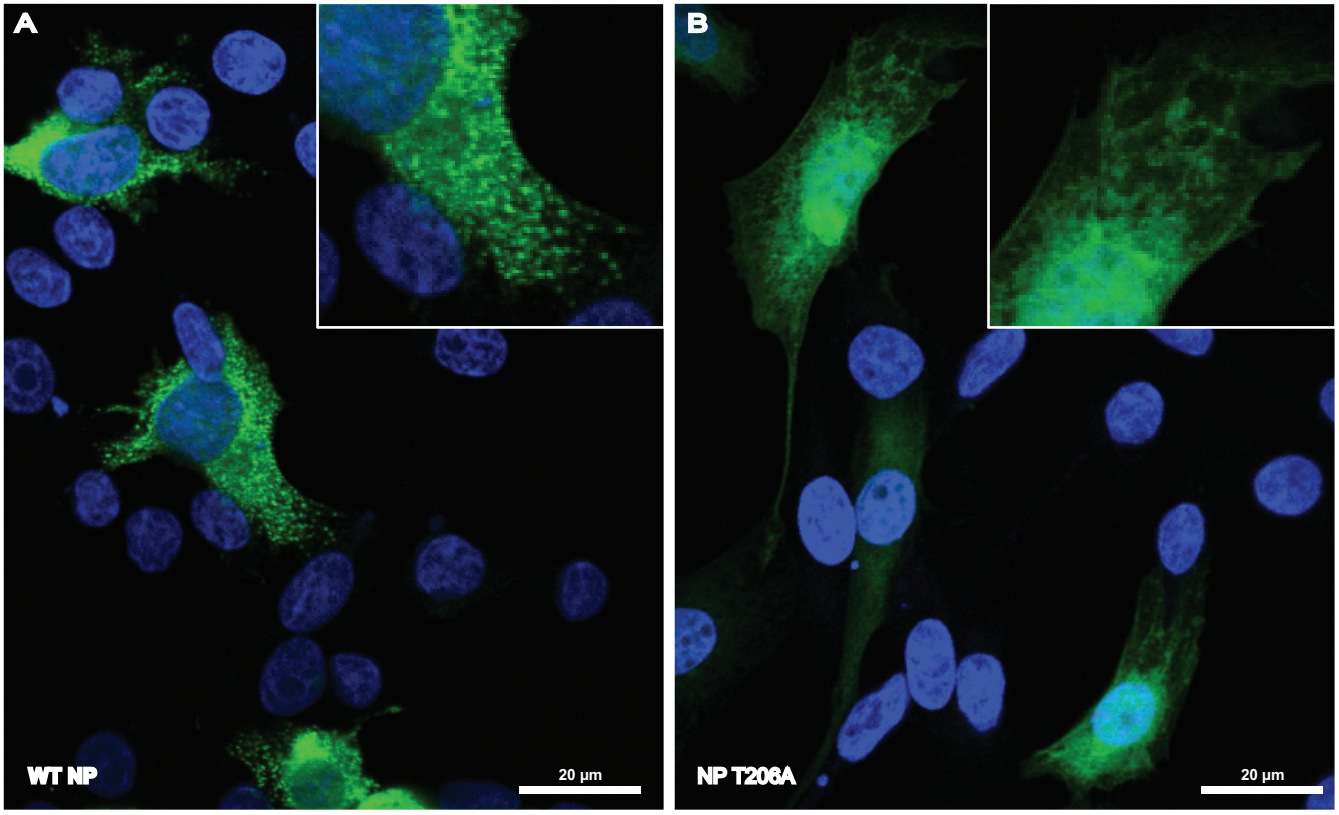

FIG 2 Cellular distribution of WT NP and NP T206A. BHK-21 cells were fixed 48 h posttransfection with plasmids expressing either WT NP (A) or NP T206A (B). NP was detected using anti-LCMV NP antibody and Alexa Fluor 488 (green). Nuclei were stained with DAPI (blue).

Because rLCMV were not recovered with NP T206A or NP Y125F, we investigated whether these NPs and RNAs were packaged into virus-like particles (VLP). For this study, we isolated the VLP that were produced during the MG assay from the cell culture supernatants and performed Western blot analysis to detect NP and reverse transcription followed by quantitative PCR (RT-qPCR) to assess the packaging of MG RNA in VLP. The Western blot analysis confirmed that both NP Y125F and NP T206A were packed into VLP (Fig. 4B). Additionally, RT-qPCR of the VLP demonstrated that MG RNA was packaged into VLP produced using both NP mutants (Fig. 4C). However, VLP produced with NP T206A packaged an amount of MG RNA that was 0.45-fold the amount that was packaged using WT NP ($P = 0.0002$) (Fig. 4C).

In the presence of Z and GP, WT NP and NP T206A support comparable levels of MG RNA production. When Z and GP were included in the MG assay, NP T206A supported the production of significantly smaller amounts of CAT protein than WT NP (Fig. 1B and D). To determine whether the smaller quantities of CAT produced in the presence of the NP T206 mutants were due to differences in MG RNA levels, we quantified the MG RNA produced from the MG assay using RT-qPCR. For WT NP, Z and GP led to a 5.9-fold decrease in MG RNA levels (Fig. 5A). In the absence of Z and GP, NP T206A and T206S produced 1.2- and 1.7-fold more MG RNA, respectively, than did WT NP ($P = 0.0005$ and $P = 0.0016$, respectively) (Fig. 5A). When Z and GP were included in the MG assay, there was no significant difference in MG RNA levels between NP T206A and WT NP (Fig. 5B). However, NP T206S produced 1.6-fold more ($P = 0.0056$) MG RNA than did WT NP when Z and GP were included (Fig. 5B).

Translation is inhibited during the MG assay with NP T206A in the presence of Z and GP. Because RT-qPCR is unable to distinguish between transcript, genome, and antigenome, we used Northern blot analysis to determine whether the reduced CAT expression in the presence of NP T206A resulted from changes in transcription or translation. In the absence of Z and GP, NP T206S displayed an increased level of CAT mRNA, while the levels were unchanged between WT NP and NP T206A (Fig. 5C). In cells cotransfected with Z and GP, the CAT mRNA levels were similar for all NPs (Fig. 5C), demonstrating that transcription was not inhibited and, therefore, casting a spotlight on translation. The Northern blot of MG antigenome/CAT mRNA also shows concatemer RNA, also referred to as homodimer RNA, as has been observed previously (Fig. 5C) (26, 35). Additionally, as has been seen previously, L had a low level of activity in the absence of NP, which was observed in the empty vector control lanes (Fig. 5C) (26).

LCMV NP colocalizes with PI4P and all eIF4F proteins. Studies with Tacaribe virus (TACV), a New World arenavirus, have shown colocalization of phosphatidylinositol 4-phosphate (PI4P) with NP at RTC, both during infection and transfection (25). To determine whether NP T206A associated with PI4P, we infected BHK-21 cells with LCMV or transfected them with either WT NP or NP T206A. PI4P was clearly associated with WT NP punctae during infection and transfection (Fig. 6). NP

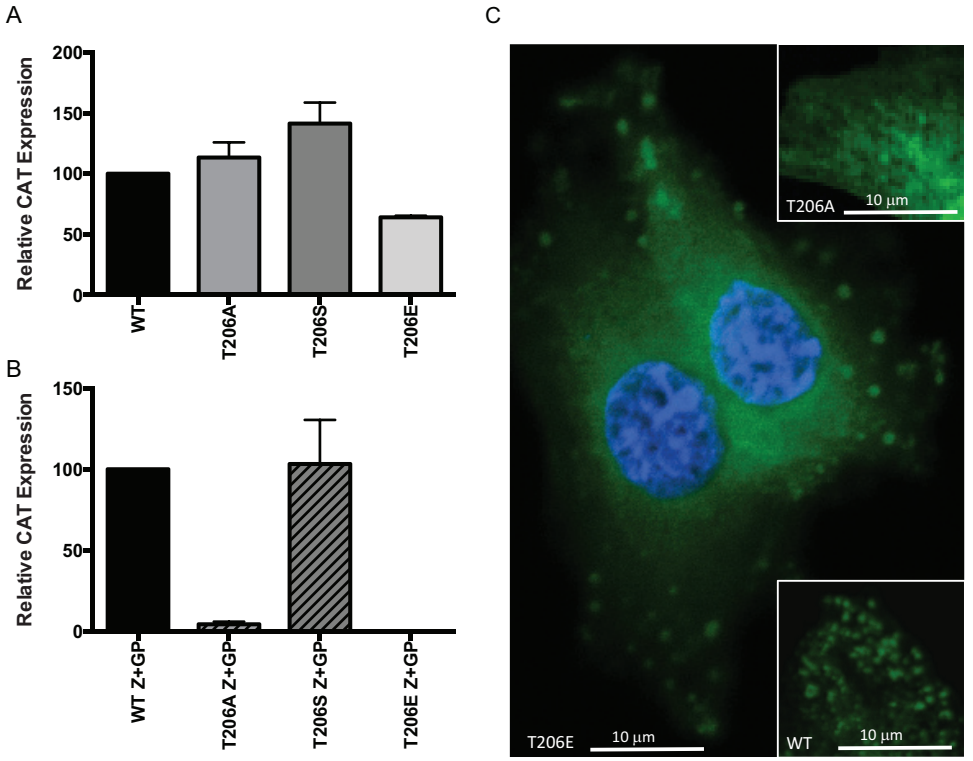

FIG 3 Levels of MG CAT expression with NP T206S and NP T206A, and NP T206E cellular distribution. HEK293T cells used for the MG reporter assay were transfected with various NP point mutants with and without Z and GP. Forty-eight hours posttransfection, cell lysates were collected and CAT ELISAs were performed to quantify CAT protein. Results for assays that included Z and GP have "Z+GP" in their *x*-axis labels. (A) Comparison of CAT expression levels using WT NP, NP T206A, or T206S without Z and GP. Results are normalized to the CAT expression level with WT NP. (B) Comparison of CAT expression levels using WT NP, NP T206A, or T206S with Z and GP. Results are normalized to the CAT expression level with WT NP in the presence of Z and GP. Each bar represents the results of a minimum of three separate experiments. All error bars represent standard deviations. (C) BHK-21 cells were fixed 48 h after transfection with a plasmid expressing NP T206E. (Insets) NP T206A (top) and WT NP (bottom), both at the same magnification as NP T206E. NP was detected using anti-LCMV NP antibody and Alexa Fluor 488 (green). Nuclei were stained with DAPI (blue).

T206A was also associated with PI4P, although because of the diffuse distribution of T206A in the cytoplasm, it was not as distinct (Fig. 6).

The NPs of TACV and another New World arenavirus, Junin virus (JUNV), colocalize with eIF4A and eIF4G but not with eIF4E during infection, with eIF4E being dispensable for JUNV translation (25, 27). Because Z interacts with eIF4E to block cap binding (36), we hypothesized that NP T206A may require eIF4E for translation. Immunofluorescence analysis of BHK-21 cells infected with LCMV demonstrated the colocalization of all three eIF4F proteins (eIF4A, eIF4G, and eIF4E) with NP at RTC (Fig. 7).

LCMV NP does not coimmunoprecipitate with eIF4F proteins. The NP of JUNV coimmunoprecipitates with eIF4A and eIF4G (27). This provides support for the hypothesis that JUNV NP may replace eIF4E within translation initiation complexes (27). Coimmunoprecipitation of NP from LCMV-infected cells was performed to determine whether LCMV NP coimmunoprecipitates with any of the eIF4F proteins. While all three eIF4F proteins could be detected in Western blots of cell lysates, none of these proteins coimmunoprecipitated with LCMV NP (not shown), suggesting that LCMV NP does not replace eIF4E in translation initiation complexes.

DISCUSSION

One of the more interesting and unanticipated findings described herein is the association of all three eIF4F proteins with LCMV RTC (Fig. 7), suggesting that eIF4E is required for LCMV translation. This discovery contrasts with studies of two New World arenaviruses, TACV and JUNV, which only associate with eIF4A and eIF4G and not with eIF4E, and of JUNV, which does not require eIF4E for viral message translation (25, 27). Because the NPs of New World arenaviruses coimmunoprecipitate with eIF4A and eIF4G, NP may interact with the 7-methylguanylate cap of mRNA to replace eIF4E within translation initiation complexes (25, 27, 37). However, crystal structures and biochemical studies of an Old World arenavirus, Lassa virus (LASV), strongly suggest that LASV NP does not bind the 7-methylguanylate cap (38, 39); this is supported by our coimmunoprecipitation studies, which demonstrate that LCMV NP does not pull down with eIF4A or eIF4G. The observation that Old World arenavirus RTC associate with eIF4E, while New World arenaviruses do not require eIF4E, defines an important difference between these two arenavirus lineages in how viral mRNAs are translated.

Here, we also demonstrate the importance of NP residue T206 in the LCMV life cycle and provide evidence that T206 is a site of regulatory phosphorylation. All mammalian arenaviruses con-

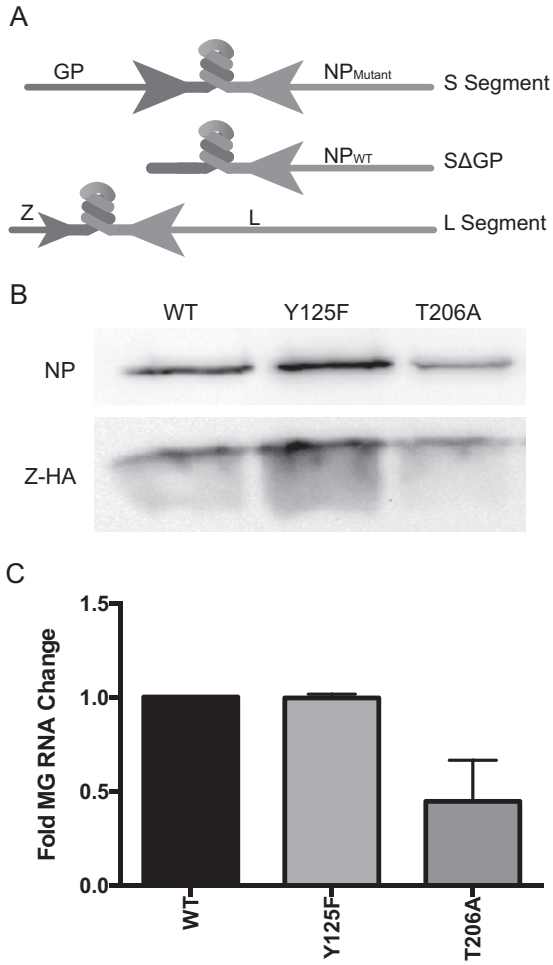

FIG 4 Tripartite rLCMV production and VLP packaging with NP Y125F and NP T206A. (A) Schematic of RNA segments produced during tripartite rLCMV production. (B and C) HEK293T cells were transfected for expression of the MG with NP Y125F or NP T206A in the presence of Z and GP. Forty-eight hours posttransfection, VLP were isolated from cell culture supernatants by centrifugation. (B) Western blot analysis of NP and Z-HA packaged in VLP using anti-LCMV NP antibody or anti-HA antibody. (C) MG RNAs packaged in VLP were quantified by RT-qPCR. The fold change from the amount of MG RNA packaged in VLP when WT NP was used is shown for each mutant NP. Each bar represents a minimum of three separate experiments. All error bars represent standard deviations.

serve NP T206, and we could not recover rLCMV containing NP T206A. Additionally, NP T206A had a dominant-negative effect over WT NP, and a tripartite rLCMV with WT NP supplemented on a third segment also could not be recovered. Immunofluorescence analysis of NP in cells infected by LCMV showed that NP accumulated in cytoplasmic punctae. These punctae are the RTC, which are nucleated by NP (25). Immunofluorescence analysis of cells expressing WT NP showed typical LCMV NP punctae (Fig. 2A), while cells expressing NP T206A showed loose, poorly defined aggregations of NP in the cytoplasm (Fig. 2B) and, therefore, an atypical form of RTC. At this time, the mechanism by which NP nucleates RTC is unknown, albeit our immunofluorescence analysis data demonstrate that NP T206 is critical to the process. In contrast to NP T206A, which was unable to form normal RTC, NP T206S, which could be phosphorylated, retained a punctate distribution in cells and was indistinguishable from WT NP (not shown). NP T206E, whose substitution mimics constitutive phosphorylation at this site, showed a phenotype intermediate between those of WT NP and NP T206A, being diffuse and also forming punctae (Fig. 3C). It is noteworthy that the NP T206E punctae were larger than the WT NP punctae. The intermediate phenotype of NP T206E suggested either a need for NP to be transiently phosphorylated at T206 for normal RTC formation or an incomplete recapitulation of the normal phenotype by the glutamate substitution. Our studies suggest that transient phosphorylation of NP T206 may contribute to the formation of the punctate RTC that are typical in cells infected by LCMV.

LCMV NP T206 corresponds to Lassa virus (LASV) NP T210, which is solvent exposed and maps to α-helix 8 in the N-terminal domain (39). This residue does not change position when NP interacts with RNA (39). While it is solvent exposed, NP T206 is conserved in all mammalian arenaviruses, demonstrating the importance of this site in the function of NP. To demonstrate intracellular phosphorylation of NP T206, we isolated NP from infected BHK-21 cells and performed LC-MS/MS to identify sites of phosphorylation. We did not observe phosphorylation of T206, suggesting that phosphorylation at this site may be transitory, which is consistent with the known transient nature of some viral regulatory phosphorylation sites (14). When we infected BHK-21 cells with LCMV and transfected those cells with a plasmid expressing NP T206A that contained a C-terminal HA tag, T206A associated with normal RTC punctae (not shown). This suggests that, while NP T206A cannot mediate the formation of typical RTC on its own, when supported by preexisting WT NP, it can participate in normal RTC. This supports the hypothesis that NP T206 may be transiently phosphorylated to form normal RTC but phosphorylation may not be required to participate in RTC.

While NP T206A is deficient in rLCMV production and RTC formation, our results demonstrate that NP T206A is competent in two activities that occur in RTC, replication (Fig. 5) and transcription (Fig. 1B, 3A, and 5). The MG assay expressed wild-type levels of CAT reporter protein when the point mutant NP T206A was included (Fig. 1B and 3A). Because cumulative studies of NP strongly support the fact that NP must be complete and properly folded to transcribe RNA (32, 39, 40), the results of the MG assay used here demonstrate than NP T206A is folding properly. Z and GP were associated with a greater than 50-fold reduction in CAT protein expression from the MG (Fig. 1B). This decrease is likely due to the binding of Z to L and the locking of L onto arenavirus promoters, thereby inhibiting further transcription and replication (41). However, in the presence of Z and GP, WT NP mediated CAT expression in the MG assay (Fig. 1B and D), while CAT expression with NP T206A in the presence of Z and GP was significantly reduced (Fig. 1D). This loss of CAT expression was shown to be a result of loss of translation (Fig. 5B and C). In the MG assay that included Z and GP, NP T206S restored CAT expression to a level similar to that obtained with WT NP (Fig. 3B). In the MG assay, CAT expression with NP T206E was reduced compared to that obtained with WT NP (Fig. 3A), further indicating that phosphorylation at this residue may be transitory.

The loss of translation observed with NP T206A in the presence of Z and GP demonstrates the potential importance of proper RTC formation for Old World arenaviruses. Prior studies suggest that ribosomal proteins, along with eIF4A and eIF4G, are present in RTC to prime translation before being transported out of the

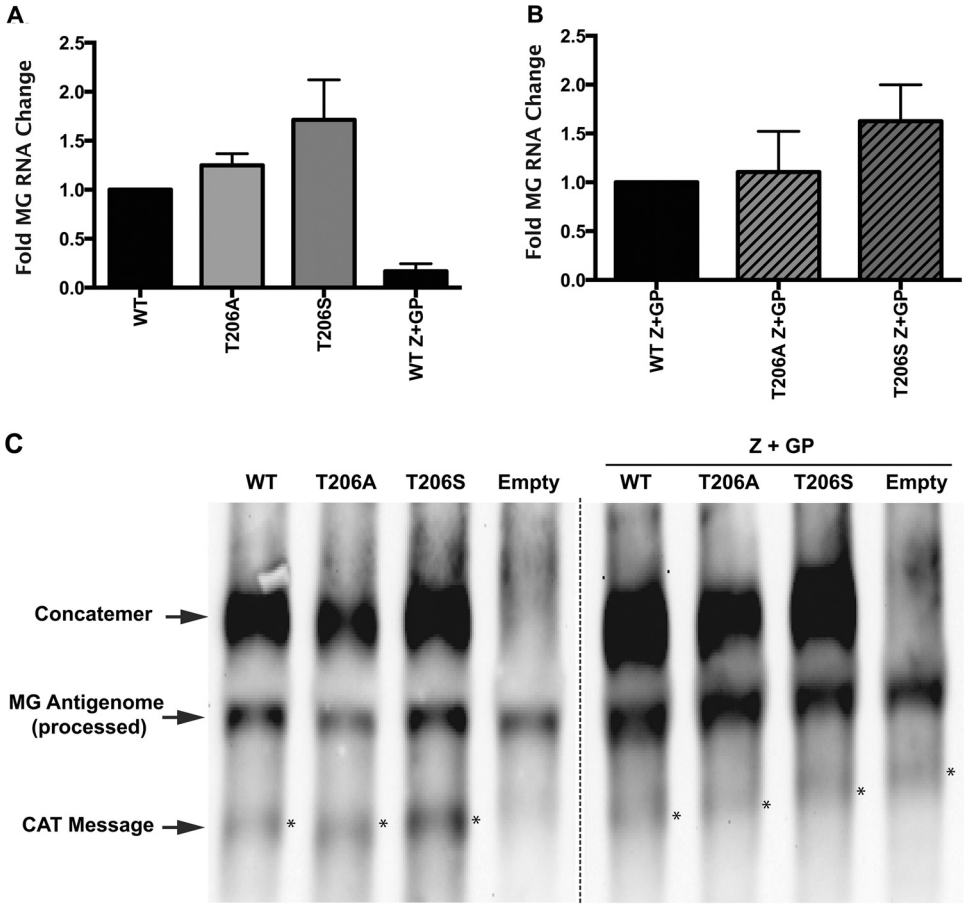

FIG 5 Expression of MG RNA by WT NP, NP T206A, or NP T206S. HEK293T cells used for the MG assay were transfected with WT NP, NP T206A, or NP T206S. The results for assays that included Z and GP have "Z+GP" in the *x*-axis labels. (A and B) The fold change from the amount of MG RNA produced in assays containing WT NP without Z and GP (A) or with Z and GP (B) was determined by RT-qPCR for each mutant NP. Each bar represents the results of a minimum of three separate experiments. All error bars represent standard deviations. (C) Northern blot analysis of RNA isolated from MG assays and probed with a strand-specific probe for CAT message and antigenome. (Left) RNA from an experiment that did not include Z and GP; (right) RNA from an assay with Z and GP. Arrows denote concatemer MG RNA, MG antigenome RNA, and CAT message transcribed from the MG by the viral polymerase and NP. Additionally, asterisks denote CAT mRNAs.

RTC (25). Our results suggest that all of the eIF4F complex proteins are involved in priming translation within LCMV RTC. Arenavirus Z protein binds to eIF4E, changing its conformation so it cannot bind to the 7-methylguanylate cap of mRNA (36). Because NP T206A did not form normal RTC (Fig. 2B), the priming of translation that is predicted to occur in RTC would not have taken place. Without normal RTC, Z would have more access to eIF4E before translation priming occured, allowing a greater impact on the inhibition of cap binding by eIF4E.

Because PI4P is a lipid associated with New World arenavirus RTC (25), we sought to determine whether LCMV NP and NP T206A associated with PI4P. Immunofluorescence analysis of BHK-21 cells infected with LCMV showed that NP colocalized with PI4P (Fig. 6). This association was also observed in BHK-21 cells transfected with a plasmid expressing WT NP (Fig. 6). While close inspection of confocal images of BHK-21 cells transfected with NP T206A showed association between NP T206A and PI4P, the association was not as distinct as it was for WT NP (Fig. 6). This lack of overt association between NP T206A and PI4P is likely a result of the more diffuse distribution of NP T206A, as opposed to the concentrations of protein and lipid at WT NP punctae shown by immunofluorescence analysis.

Using LC-MS/MS, we demonstrated that NP Y125 is phosphorylated in LCMV virions, suggesting a regulatory role for this site. Because kinase activity associated with the virion acts on serine/threonine residues but not tyrosine, NP Y125 was likely phosphorylated in the host cell. However, our mass spectroscopy studies did not show phosphorylation of NP isolated from infected BHK-21 cells. As with NP T206A, NP Y125 phosphorylation may be transient and difficult to detect in cell lysates. Like NP T206, NP Y125 is conserved across all mammalian arenaviruses, and we did not recover rLCMV containing NP Y125F. However, unlike NP T206A, NP Y125F was not dominant negative over WT NP, and a tripartite rLCMV (Fig. 4A) was recovered. NP Y125 lies within the region of NP that is disordered when NP binds RNA (39). CAT protein expression from the MG assay with NP Y125F in the presence of Z and GP results in higher levels of reporter protein than when WT NP is used (Fig. 1E). Because of the potential role of NP phosphorylation in viral replication, studies of the phosphorylation and function of NP Y125 in the arenavirus life cycle are ongoing.

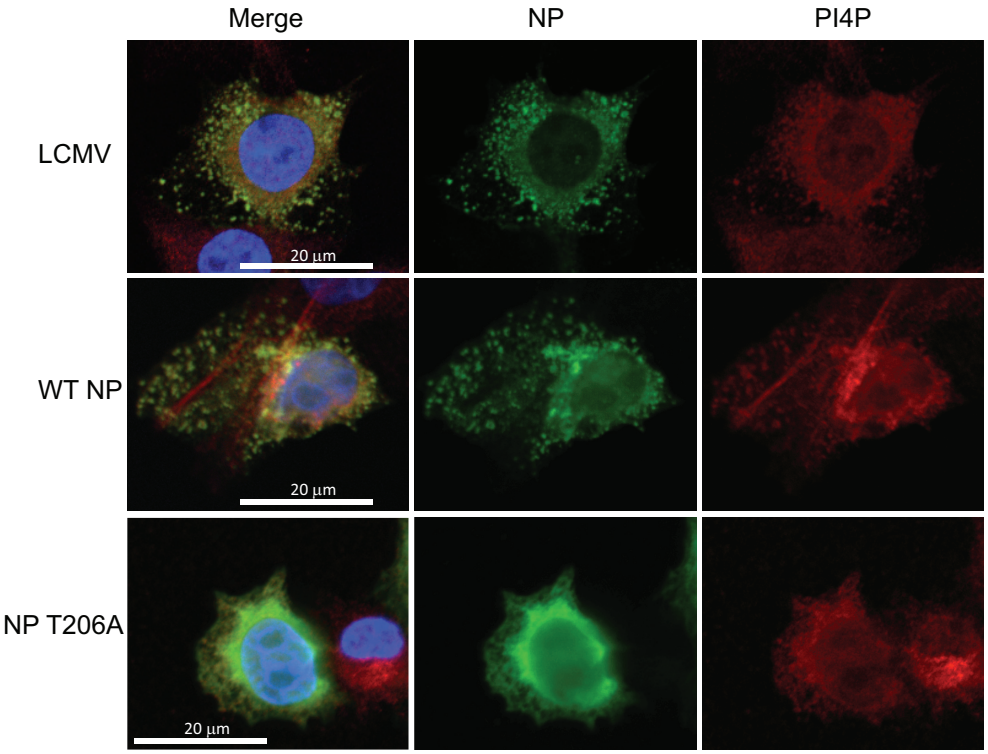

FIG 6 Colocalization of PI4P with NP. (Top) LCMV-infected BHK-21 cells were fixed 24 h postinfection; (middle and bottom) BHK-21 cells were transfected with plasmids expressing either WT NP or NP T206A and fixed 48 h posttransfection. NP was visualized using anti-LCMV NP and Alexa Fluor 488 (green). PI4P was stained using anti-PI4P IgM and Alexa Fluor 594 (red). Nuclei were stained with DAPI (blue).

In summary, we demonstrated that LCMV NP colocalized with all eIF4F proteins and suggest that, unlike New World arenaviruses, Old World arenaviruses may require eIF4E for cap-dependent translation. We also identified two phosphorylation sites in LCMV NP that are required for rLCMV recovery, NP T206 and Y125, one of which is predicted and one confirmed by mass spectrometry studies of isolated virions. NP T206A is a dominant-negative mutant that was unable to form normal RTC, and in the

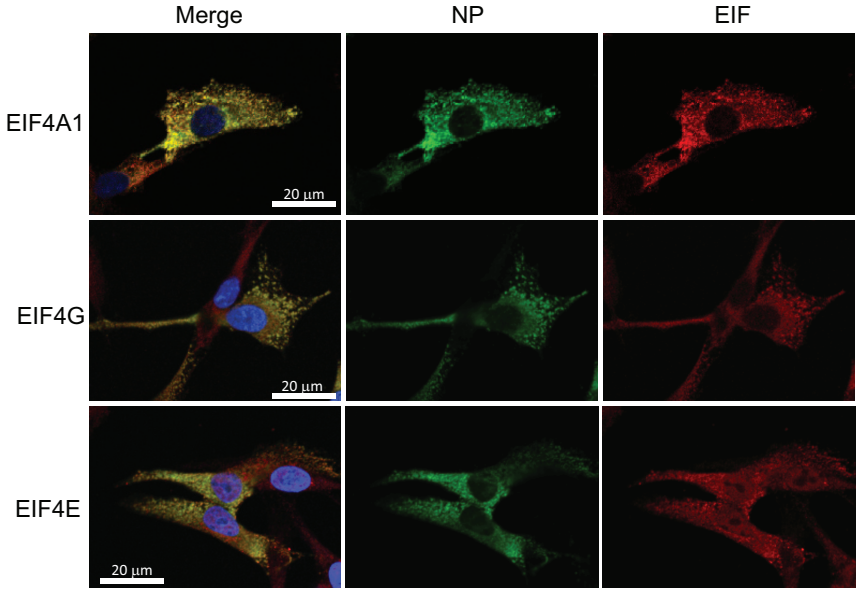

FIG 7 Colocalization of eIF4F complex proteins with NP. LCMV-infected BHK-21 cells were fixed 24 h postinfection and stained for NP and eIF4F proteins. LCMV NP was visualized by using anti-LCMV NP antibody and Alexa Fluor 488 (green). eIF4F complex proteins were observed using anti-eIF4A1, anti-eIF4G, or anti-eIF4E antibody and Alexa Fluor 594 (red). Nuclei were stained with DAPI (blue).

MG assay, in the presence of Z and GP, it was deficient in translation. We propose that this deficiency in translation results from the loss of translation priming at RTC due to NP T206A's loss of normal RTC.

MATERIALS AND METHODS

Bioinformatic analysis. All arenavirus nucleoprotein sequences were obtained from the UniProt database and aligned using MUSCLE (42). The MSA produced using MUSCLE was viewed using Jalview (28, 43). Jalview identified residues conserved above a threshold of 98%. Additionally, the alignment was manually viewed for positions where serine and threonine residues were mixed. The sequence for NP of LCMV strain Armstrong (Swiss-Prot P09992) was submitted to NetPhos2.0 (29) and NetPhosK (30) for phosphorylation prediction. Solvent-exposed residues predicted to be phosphorylated were identified by aligning PDB files 3T5N (39) and 3MWP (37) using MacPyMol (The PyMOL Molecular Graphics System, version 1.5.0.4, Schrödinger, LLC).

Cells and transfections. BHK-21 and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin. All DNA was prepared using Promega PureYield plasmid purification reagents (Promega Corporation, Madison, WI). HEK293T cells were transfected in 12-well or 6-well plates using Lipofectamine 2000 (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. BHK-21 cells were transfected in 12-well or 6-well plates using JetPRIME (Polyplus-transfection, Inc., New York, NY) according to the manufacturer's instructions.

Plasmids. Several plasmids were constructed in the pCAGGS (pC) backbone (44) and have been described previously. pC-NP, pC-L, pC-T7, pC-Z, and pC-GP were described by Lee et al. (45). pC-T7 was designed for cytoplasmic expression of T7 polymerase. Plasmid pMG#7Δ2G contains an LCMV MG utilizing a CAT reporter, which is further bounded by T7 RNA polymerase promoter, hairpin ribozyme, and terminator sequences to mediate transcription by T7 polymerase and produce a precise 3′ terminus (46, 47). All NP constructs were made in pTarget (Promega Corporation, Madison, WI) by subcloning NP from pC-NP into pTarget to produce pTarget-LCMV-NP. pMG/S-CAT/GFP expresses an additional green fluorescent protein (GFP) reporter gene replacing the GP open reading frame (ORF) (35).

Point mutagenesis. The QuikChange primer design program (Agilent Technologies, Santa Clara, CA) was used to design primer pairs for targeted point mutations, and pTarget-LCMV NP was used as a template for mutagenic PCR with the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA), following the manufacturer's instructions. Sequences for each oligonucleotide are available upon request. Constructs were sequenced after mutagenesis.

LCMV MG reporter assay. HEK293T cells grown in 12-well plates were transfected to mediate the transcription and replication of the LCMV MG carrying the CAT reporter gene (46). Transfection mixtures lacking NP plasmid were prepared as negative controls, and empty pTarget was used to equalize the amount of total DNA per transfection. Forty-eight hours posttransfection, cell extracts were collected and CAT protein was quantified using the CAT ELISA (enzyme-linked immunosorbent assay) (Roche Diagnostics Corporation, Indianapolis, IN). The relative CAT expression level was calculated by averaging the results of three technical replicates, followed by subtracting the average of the results of the no-NP control and normalizing the results to those obtained using WT NP (with or without Z and GP as indicated). Each bar represents a minimum of three biological replicates.

Immunofluorescence analysis. Transfected or infected BHK-21 cells were cultured on glass coverslips and fixed using 3.7% paraformaldehyde at 48 h posttransfection or 24 h postinfection. Cells were permeabilized with 0.2% Triton X-100 and mounted with DAPI (4′,6-diamidino-2-phenylindole) FluoromountD (Southern Biotech). Confocal microscopy was performed using a Nikon Eclipse Ti with a Nikon D-Eclipse confocal laser assembly (Nikon, Melville, NY). Images were acquired using the Nikon EZ-C1 program.

Antibodies. The primary antibodies used for immunofluorescence and Western blot analyses were LCMV NP antibody 1-1.3 (48), anti-eIF4A1 antibody (ab31217, 1:1000; Abcam), anti-eIF4G antibody (2469, 1:200; Cell Signaling), anti-eIF4E antibody (131480, 1:100; Abcam), anti-PI4P IgM (Z-P004, 1:300; Echelon), antihemagglutinin (anti-HA) antibody (ab130275, 1:100; Abcam), and antiactin antibody (Millipore). Anti-mouse Alexa Fluor 488 antibody (A-11029, highly cross-absorbed; Life Technologies) and anti-rabbit Alexa Fluor 594 antibody (A-11037, highly cross-absorbed; Life Technologies) were the secondary antibodies used in immunofluorescence studies. For Western blot analysis, horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Laboratories) were used.

VLP and virion isolation. For VLP production, HEK293T cells for the MG assay were transfected with Z and GP. LCMV strain Armstrong was used for viral infections of BHK-21 cells at a multiplicity of infection (MOI) of 0.1. BHK-21 cells were cultured for 48 h before virus was collected for virion isolation. Forty-eight hours after transfection or infection, cell culture supernatants were clarified and centrifuged through a 20% sucrose cushion at 100,000 × *g* at 4°C for 60 min.

RT-qPCR and Northern blot analysis. RNA isolation was performed using the Illustra RNAspin RNA isolation minikit (GE Healthcare United Kingdom Limited, Little Chalfont, United Kingdom). Following RNA isolation, an additional DNase I (New England Biolabs, Inc., Ipswich, MA) reaction was carried out, and RNA was then precipitated. RNA was quantified using a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). qPCR was performed on RNA to confirm that all amplification from RNA was not above the background (no template control). Once RNA was confirmed to be DNA free, reverse transcription was performed using Maxima reverse transcriptase (Thermo Fisher Scientific, Wilmington, DE). qPCR was performed using a primer pair specific to CAT (MG-870, 5′ ATCCGGCCTTTATTCACATTCTTG, and MG-987, 5′ ATGGAAAACGGTGTAACAAGGGTG) and Maxima SYBR green/ROX qPCR master mix (2×) (Thermo Fisher Scientific, Wilmington, DE). Northern blot analysis was performed using the NorthernMax kit (Thermo Fisher Scientific, Wilmington, DE) with the BrightStar BioDetect kit (Thermo Fisher Scientific, Wilmington, DE). The probe to antigenome/CAT mRNA was approximately 700 base pairs in length; it was made *in vitro* using the TranscriptAid T7 high-yield transcription kit (Thermo Fisher Scientific, Wilmington, DE) and labeled with psoralen-biotin using the BrightStar psoralen-biotin kit (Thermo Fisher Scientific, Wilmington, DE). Probe sequences are available upon request.

Western blot analysis. Pellets from VLP isolation, beads from coimmunoprecipitation (CoIP), or cells transfected for NP expression were suspended in SDS-PAGE loading buffer with reducing agent and run on a 12% gel. Following electrophoresis, gels were transferred to nitrocellulose using a semidry apparatus (Bio-Rad, Hercules, CA). Blocking was done using 5% nonfat dry milk in phosphate-buffered saline (PBS) containing 0.2% Tween 20 (PBS-Tween 20). All incubations with antibody were done in 5% milk, and washes were done using PBS-Tween 20. Blots were visualized using Amersham ECL prime detection reagents (GE Healthcare United Kingdom Limited, Little Chalfont, United Kingdom) and the Chemi-Doc XRS gel documentation system (Bio-Rad, Hercules, CA).

rLCMV rescue. Recombinant viruses were made using reverse genetic technology as described previously (33, 49).

Mass spectrometry. Purified NP protein (0.3 mg) was equalized to 1 M urea–0.1 M triethylammonium bicarbonate–10 mM TCEP [tris(2-carboxyethyl)phosphine], treated with 50 mM iodoacetamide for 20 min in the dark, and then incubated with trypsin (1:100, mass/mass) overnight, followed by an additional aliquot of trypsin for an additional 2 h. The eluate from subsequent C_{18} desalting/vacuum desiccation steps was redissolved in 2 M lactic acid–50% CH_3CN (buffer A) and incubated with TiO2 beads that had been pretreated with buffer A. TiO2 beads were washed 2 times with buffer A and once with 0.1% TFA–50% CH_3CN and

then eluted with 50 mM K_3HPO_4 (pH 10.0). The eluate from a subsequent C_{18} desalting step, as described above, was subjected to nanoscale LC-MS/MS using an Orbotrap Velos Pro, via a 25-cm by 75-μm self-packed C_{18} tip, with alternating collision-induced dissociation (CID)/electron transfer dissociation (ETD) fragmentation cycles. Spectral data were compared to those in SwissProt with virus taxonomy using Mascot 2.5.1 (MatrixScience).

Coimmunoprecipitation. BHK-21 cells were infected at an MOI of 1 with rLCMV NP-V5, which is an rLCMV expressing a V5-tagged NP, or were mock infected. Twenty-four hours postinfection, cells were washed in PBS and cell lysates were collected in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% NP-40) containing complete protease inhibitors (RPI Corp., Mount Prospect, IL). Cell lysates were incubated with agarose-conjugated anti-V5 tag antibody beads (MBL, Nagoya, Japan) for 2 h at 4°C. Beads were washed three times in cold lysis buffer. Beads were run on SDS-PAGE for analysis.

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