



Packaging of the Influenza Virus Genome Is Governed by a Plastic Network of RNA- and Nucleoprotein-Mediated Interactions

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ABSTRACT The genome of influenza A virus is organized into eight ribonucleoproteins, each composed of a distinct RNA segment bound by the viral polymerase and oligomeric viral nucleoprotein. Packaging sequences unique to each RNA segment together with specific nucleoprotein amino acids are thought to ensure the precise incorporation of these eight ribonucleoproteins into single virus particles, and yet the underlying interaction network remains largely unexplored. We show here that the genome packaging mechanism of an H7N7 subtype influenza A virus widely tolerates the mutation of individual packaging sequences in three different RNA segments. However, combinations of these modified RNA segments cause distinct genome packaging defects, marked by the absence of specific RNA segment subsets from the viral particles. Furthermore, we find that combining a single mutated packaging sequence with sets of specific nucleoprotein amino acid substitutions greatly impairs the viral genome packaging process. Along with previous reports, our data propose that influenza A virus uses a redundant and plastic network of RNA-RNA and potentially RNA-nucleoprotein interactions to coordinately incorporate its segmented genome into virions.

IMPORTANCE The genome of influenza A virus is organized into eight viral ribonucleoproteins (vRNPs); this provides evolutionary advantages but complicates genome packaging. Although it has been shown that RNA packaging sequences and specific amino acids in the viral nucleoprotein (NP), both components of each vRNP, ensure selective packaging of one copy of each vRNP per virus particle, the required RNA-RNA and RNA-NP interactions remain largely elusive. We identified that the genome packaging mechanism tolerates the mutation of certain individual RNA packaging sequences, while their combined mutation provokes distinct genome packaging defects. Moreover, we found that seven specific amino acid substitutions in NP impair the function of RNA packaging sequences and that this defect is partially restored by another NP amino acid change. Collectively, our data indicate that packaging of the influenza A virus genome is controlled by a redundant and plastic network of RNA/protein interactions, which may facilitate natural reassortment processes.

KEYWORDS nucleoprotein, complexity, influenza, packaging, vRNPs

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Zoonotic transmissions of influenza A viruses (IAVs) from the animal world can cause devastating pandemics in the human population, as exemplified by the 1918 pandemic virus that claimed about fifty million victims one century ago (1, 2). To become pandemic, animal IAVs have to cross the human species barrier, usually by acquiring new viral gene variants. The IAV genome, which consists of eight distinct single-stranded viral RNA (vRNA) segments, greatly facilitates the acquisition of such novel gene variants, since it allows the exchange of cognate vRNA segments between virus strains that coinfect the same cell, a process known as genetic reassortment (3–6).

Although the segmented IAV genome thus facilitates virus evolution, it requires a sophisticated genome packaging mechanism (7–9). Each vRNA segment forms an individual viral ribonucleoprotein (vRNP) complex, in which the conserved and partially complementary 3' and 5' termini bind to the viral polymerase, while the remaining vRNA associates with oligomeric viral nucleoprotein (NP) (10–12). Electron tomography studies could visualize that during the genome incorporation process into viral particles, these eight vRNPs adopt a typical “7 + 1” arrangement with seven vRNPs surrounding a central one (13–15). In addition, fluorescence *in situ* hybridization experiments showed that exactly one copy of each vRNA segment is present in the vast majority of viral particles (16), manifesting the selective nature of the genome packaging process.

A growing body of experimental data indicates that specific RNA-RNA interactions among the eight vRNPs coordinate viral genome packaging. For example, all eight vRNA segments contain unique packaging sequences, which drive the incorporation of the complete vRNP set into viral particles (17–24). Consequently, some recombinant IAV mutants harboring synonymous nucleotide changes in a single packaging sequence develop packaging defects, characterized by the preferential production of virions that lack either a specific subset of vRNA segments (22, 24–26) or the whole genome (27). Importantly, such a deficiency can be restored through the introduction of *trans*-complementary mutations into the formerly interacting vRNA segment (27), providing proof of principle that specific RNA-RNA interactions are crucial for viral genome packaging.

In addition to these specific vRNA sequences, distinct amino acids in NP are essential for coordinated genome packaging (28). In particular, a combination of seven amino acid substitutions in the NP head domain (NP7) selectively impairs the incorporation of four of the eight vRNPs into virions (28). Intriguingly, this genome packaging defect is restored through a single additional amino acid substitution at position 31 (NP7-R31G) (28). These findings suggest that NP amino acid residues support packaging-relevant interactions among the eight vRNPs, either by facilitating RNA-RNA or NP-RNA contacts between heterologous vRNPs.

While these studies indicate that single vRNA packaging sequences, together with specific NP amino acids, mediate vRNP interactions essential for genome packaging, little is known about the complexity, redundancy, and plasticity of the network of interacting packaging sequences.

Here, we addressed this issue by testing the effect of single versus multiple mutated packaging sequences, located in different vRNA segments, on viral genome packaging. We show that an individually mutated packaging sequence in either vRNA segment 1, 2, or 3 has no or only minor impact on this process. In sharp contrast, certain combinations of these mutated vRNA segments cause the preferential production of noninfectious virus particles that lack distinct subsets of the eight vRNA segments. Moreover, we present evidence that combining either of these mutated vRNA segments with NP7, and surprisingly also NP7-R31G, provokes similar genome incorporation defects. Overall, our data suggest that IAV genome packaging is orchestrated by a complex vRNP interaction network, which involves multiple functionally redundant and plastic vRNA packaging sequences and specific NP amino acids.

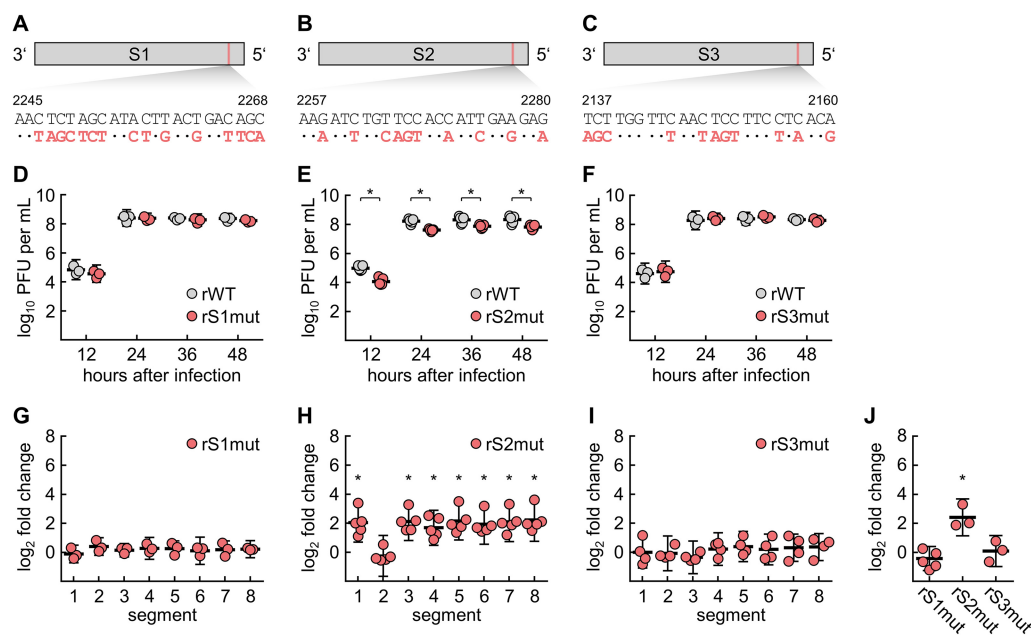


FIG 1 SC35M viruses with a single mutated packaging sequence show no or minor genome packaging defects. (A to C) Schematic representation of the SC35M vRNA segments (S) 1, 2, and 3. Wild-type and mutated nucleotide sequences are shown in black and red, respectively, and numbered in the positive sense. (D to F) Multicycle replication kinetics of the indicated mutant viruses compared to wild-type SC35M (rWT) ($n = 3$ to 5). (G to I) Relative quantification of the vRNA segment levels between mutant and wild-type virus particles by RT-qPCR. RNA levels were normalized to identical PFU numbers ($n = 3$ to 5). (J) Relative quantification of HAU-to-PFU ratios between mutant and wild-type virus particles ($n = 3$ to 5). In panels D to J, data are presented as means $\pm$ 95% confidence intervals. *, $P < 0.05$ (Student t test).

RESULTS AND DISCUSSION

A single mutated packaging sequence causes no or only a minor genome packaging defect. To compare the impact of single versus multiple mutated packaging sequences on viral genome packaging, we used the prototype H7N7 influenza strain A/SC35M (SC35M) and inserted up to eight synonymous codons into the conserved 5' terminal coding region of either vRNA segment 1, 2, or 3 (Fig. 1A to C). For this, we chose mutation sets that have been previously described to cause specific genome packaging defects when individually introduced into these vRNA segments of the H1N1 subtype strain A/WSN/1933 (WSN) (25).

First, we generated recombinant SC35M viruses that harbored a single mutated genome segment, designated rS1mut, rS2mut, and rS3mut. To monitor the production of infectious virus particles (i.e., PFU) over time, we performed multicycle replication kinetics in MDCK-II cell cultures. Surprisingly, while the propagation of rS2mut was slightly impaired in relation to wild-type (wt) SC35M, replication of rS1mut and rS3mut was not affected (Fig. 1D to F). To test the genome packaging capacity of these mutant viruses in comparison to wt SC35M, we quantified by quantitative reverse transcription-PCR (RT-qPCR) the levels of all vRNA segments derived from virion preparations containing identical PFU numbers. This analysis revealed similar vRNA segment levels for wt SC35M, rS1mut, and rS3mut, whereas rS2mut showed deficient incorporation of vRNA 2 (Fig. 1G to I). By determining the ratio of hemagglutination units (HAU) per PFU of the same virus particle preparations, we estimated to which extent these mutants produced noninfectious virus particles relative to wt SC35M. While rS1mut, rS3mut and rWT showed similar HAU/PFU ratios, the HAU/PFU ratio of rS2mut was 5-fold elevated (Fig. 1J). These data demonstrate that, although rS2mut produced noninfectious viral particles lacking vRNA segment 2, genome packaging of both other SC35M single vRNA mutants was not impaired.

Combinations of mutated packaging sequences in different vRNA segments cause distinct genome packaging defects. Next, we characterized recombinant

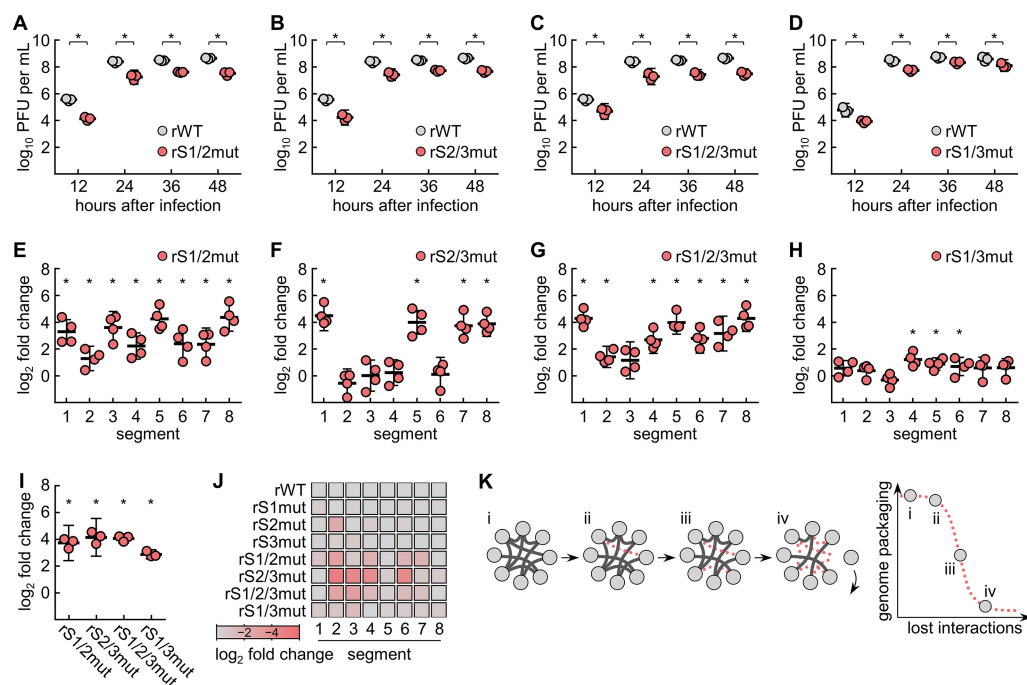


FIG 2 SC35M viruses with multiple mutated packaging sequences in different vRNA segments show distinct genome packaging defects. (A to D) Multicycle replication kinetics of the indicated mutant viruses compared to wild-type SC35M (rWT) ($n = 3$). (E to H) Relative quantification of the vRNA segment levels between mutant and wild-type virus particles by RT-qPCR. RNA levels were normalized to identical PFU numbers ($n = 4$). (I) Relative quantification of HAU/PFU ratios between mutant and wild-type virus particles ($n = 3$). (J) Heat map summarizing the packaging phenotypes of the indicated single and combinatorial vRNA mutant viruses. The incorporation efficiency of each vRNA segment was calculated as $\log_2$ -fold change compared to rWT. For each mutant virus, the most frequently packaged vRNA segment was set to $\log_2 = 0$. (K, left) Schematic model depicting the concept of a redundant vRNP interaction network. The eight vRNPs are shown as gray circles and interactions are drawn as dark connections. Note that this graph does not show specific vRNP-vRNP interactions. The sequential mutation of these interactions (i to iv) is indicated by dashed red lines. (Right) This hypothetical network tolerates the mutation of a single interaction site; however, the loss of more interactions impairs coordinated genome packaging, finally causing the breakdown of the network. In panels A to I, data are presented as means $\pm$ 95% confidence intervals. *, $P < 0.05$ (Student *t* test).

SC35M viruses comprising either two (rS1/2mut, rS1/3mut, rS2/3mut) or three (rS1/2/3mut) of these vRNA segments with a mutated packaging sequence. These combinatorial vRNA mutants showed pronounced growth defects in MDCK-II cell cultures compared to wt SC35M (Fig. 2A to D). While rS1/3mut replicated to 3-fold reduced PFU titers at 36 h postinfection (p.i.), rS1/2mut, rS2/3mut, and rS1/2/3mut reached only 10-fold-decreased infectious virus titers. RT-qPCR analyses of the viral particle content revealed substantial genome packaging defects for rS1/2mut, rS2/3mut, and rS1/2/3mut. These were characterized by the preferential incorporation of distinct subsets of the eight genome segments varying with the set of mutated vRNAs (Fig. 2E to G). In addition, these deficiencies were accompanied by an increased production of noninfectious viral particles, as judged by about 15-fold-higher HAU/PFU ratios compared to wt SC35M (Fig. 2I). In contrast, the combinatorial vRNA mutant rS1/3mut showed only a minor packaging preference for vRNAs 4, 5, and 6 (Fig. 2H) and a 5-fold-higher HAU/PFU ratio (Fig. 2I) relative to wt SC35M. These data demonstrate that the combination of mutated packaging sequences in different vRNA segments of SC35M results in pronounced and distinct genome packaging defects.

Overall, our data reveal that the effect of a mutated packaging sequence on viral genome packaging depends on the state of other packaging sequences in the viral genome. For example, the exclusive mutation of the vRNA 3 packaging sequence did not impair coordinated genome packaging (rS3mut, Fig. 1F). Likewise, this mutated vRNA segment caused only a minor defect when combined with the mutated segment 1 packaging sequence (rS1/3mut, Fig. 2H). However, joining it with the mutated vRNA

2 provoked a severe packaging deficiency (rS2/3mut, Fig. 2F). Importantly, the composition of the preferentially incorporated vRNA subset of this mutant virus was further changed when the mutated segment 1 was added (rS1/2/3mut, Fig. 2G). Intriguingly, we noticed these nonlinear changes in the packaging phenotypes throughout all our tested combinations (Fig. 2J). Based on this observation, we propose that a complex network of interacting packaging sequences coordinates the incorporation of the segmented IAV genome into virions.

Since our single vRNA mutants rS1mut and rS3mut (Fig. 1G and I), in contrast to the combinatorial mutants rS1/2mut and rS2/3mut (Fig. 2E and F), showed wild-type-like genome packaging, we speculate that the mutated packaging sequences in segments 1 and 3 can be partially compensated by other packaging sequences in the intersegment network (Fig. 2K). Indeed, a recent biochemical study revealed that most vRNA segments have multiple RNA contact sites with other genome segments, thereby creating a redundant web of RNA-RNA interactions (29). Importantly, the individual disruption of some of these interaction sites by synonymous mutation did not provoke obvious genome packaging defects (29). In line with this concept, we further speculate that such a redundant set of packaging sequences would allow multiple interaction “solutions” to the specific incorporation of the eight genome segments into virions. Indeed, electron tomography studies revealed that the “7 + 1” arrangement of the eight vRNPs within budding viral particles is not fixed but rather flexible (13, 14), supporting the plastic nature of the genome incorporation process.

Moreover, in combination with previous data (25), we found that the effect of an individually mutated packaging sequence is virus strain dependent. While the mutated segment 1 packaging sequence had no effect on SC35M (rS1mut, Fig. 1G), it provoked large growth and packaging defects in WSN. Similarly, mutations in vRNA segment 3 did not reveal such defects in SC35M (rS3mut, Fig. 1I) but did reveal such effects in WSN. These phenotypic variations between these IAV strains cannot be explained by differences in the mutated packaging sequences, as we introduced identical nucleotide changes into conserved vRNA regions (25, 30). Rather, we speculate that the internal organization of the intersegment network is partially strain dependent (14, 27, 31, 32), so that identical mutations can have various effects. However, some mutated packaging sequences might cause similar packaging defects in different IAV strains. In support of this, we find that the mutated segment 2 packaging sequence provoked the reduced incorporation of vRNA segment 2 in both SC35M (Fig. 1H) and WSN (25).

NP7 impairs the function of genome packaging sequences. Consistent with our previous findings (28), an SC35M variant (rNP7), encoding NP protein with seven specific amino acid substitutions in the head domain (Fig. 3A and B), showed impaired viral replication (Fig. 3C), preferential incorporation of four out of eight vRNA segments (Fig. 3D) and a 7-fold-increased HAU/PFU ratio (Fig. 3E) in comparison to wt SC35M. Interestingly, the genome packaging defect of rNP7 resembled the phenotype of our SC35M viruses harboring combinations of mutated packaging sequences (compare Fig. 3D and 2E–G). Based on this, we speculated that the mutant NP7 protein fails to support the function of multiple vRNA packaging sequences to establish a fully operative vRNP interaction network, either by influencing RNA-RNA or NP-RNA interactions between heterologous vRNPs. In any case, if the NP7 protein provoked such changes in the vRNP network, we would expect that adding a single mutated vRNA packaging sequence to this context alters the pattern of preferentially incorporated vRNA segments. To test this hypothesis, we analyzed SC35M viruses harboring the mutant NP7 protein in combination with a single mutated packaging sequence in either vRNA segment 1 (rNP7/S1mut), 2 (rNP7/S2mut), or 3 (rNP7/S3mut). Although we repeatedly failed to recover rNP7/S1mut and rNP7/S2mut, we could generate rNP7/S3mut. This virus showed impaired replication kinetics compared to wt SC35M, reaching 6-fold-lower PFU titers at 36 h p.i. (Fig. 3F). This defect was associated with an increased production of noninfectious virions, as suggested by a 6-fold-increased HAU/PFU ratio compared to wt SC35M (Fig. 3E). Intriguingly, rNP7/S3mut and rNP7

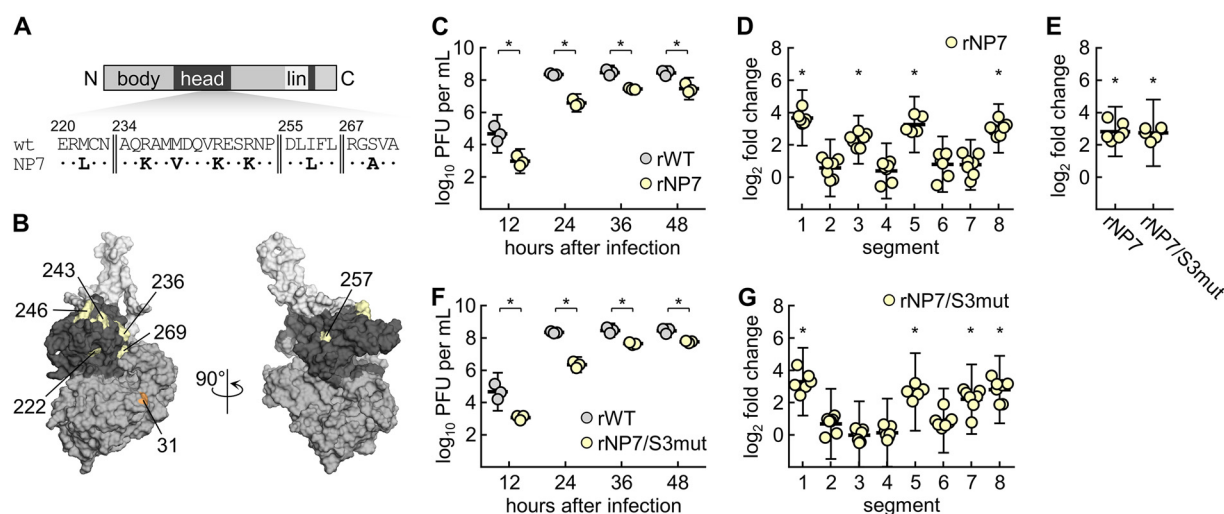


FIG 3 rNP7 and rNP7/S3mut show distinct genome packaging defects. (A) Schematic representation of the NP primary structure. The NP7 amino acid substitutions are highlighted in boldface. body, body domain; C, C terminus; head, head domain; lin, tail loop/linker region; N, N terminus. (B) Schematic representation of the three-dimensional NP structure. NP7 amino acid residues (yellow) and R31G (orange) are highlighted. Note that position 239 is not surface exposed. NP regions are colored as in panel A. (C and F) Multicycle replication kinetics of the indicated mutant viruses compared to wild-type SC35M (rWT) ($n = 3$). (D and G) Relative quantification of the vRNA segment levels between mutant and wild-type virus particles by RT-qPCR. RNA levels were normalized to identical PFU numbers ($n = 7$). (E) Relative quantification of HAU-to-PFU ratios between mutant and wild-type virus particles ($n = 6$). Data are presented as means $\pm$ 95% confidence intervals. *, $P < 0.05$ (Student t test).

packaged different subsets of the eight genome segments into their virions. While rNP7 favored incorporation of vRNAs 1, 3, 5, and 8 (Fig. 3D), rNP7/S3mut particles mainly contained vRNAs 1, 5, 7, and 8 (Fig. 3G), indicating a swap between vRNAs 3 and 7. These results suggest that NP7 interferes with the function of multiple packaging sequences to establish a fully viable vRNP interaction network.

The compensatory amino acid mutation R31G partially restores the NP7-mediated defect. Through passaging of rNP7 in cell culture, we have previously (28) identified the compensatory amino acid substitution R31G in the NP body domain (Fig. 3B), which restores viral growth (Fig. 4A), genome packaging (Fig. 4B) and the HAU/PFU ratio (Fig. 4C) to wild-type-like levels. We wondered whether the vRNP interaction network established in the context of NP7-R31G is a genuine redundant wild-type network and consequently able to compensate a single mutated vRNA packaging sequence, or whether the network is changed so that an additional mutated vRNA packaging sequence causes a genome packaging defect. To explore this, we characterized rNP7-R31G variants harboring either the mutated vRNA segment 1 (rNP7-R31G/S1mut), 2 (rNP7-R31G/S2mut), or 3 (rNP7-R31G/S3mut). All of these mutant viruses could be generated. However, and in sharp contrast to rNP7-R31G, their propagation was substantially attenuated in MDCK-II cell cultures compared to wt SC35M (Fig. 4D to F). We observed the most pronounced growth defect at 36 h p.i. for rNP7-R31G/S2mut (80-fold-reduced PFU titers relative to wt SC35M), followed by rNP7-R31G/S3mut (40-fold reduced) and rNP7-R31G/S1mut (10-fold reduced). RT-qPCR analyses of the viral particle content revealed that these replication defects resulted from a preferential incorporation of distinct vRNP subsets into viral particles (Fig. 4G to I). Moreover, these deficiencies were accompanied by elevated levels of noninfectious virions, as judged from increased HAU/PFU ratios relative to wt SC35M (Fig. 4C). Interestingly, we observed similar patterns of preferentially incorporated genome segments among the NP7-R31G and NP7 variants (Fig. 4J). While rNP7 and rNP7-R31G/S1mut favored the incorporation of vRNAs 1, 3, 5, and 8, rNP7/S3mut and rNP7-R31G/S3mut preferred packaging of vRNAs 1, 5, 7, and 8. Overall, these data suggest that the R31G substitution only partially restores the defects in the vRNP network provoked by NP7 (Fig. 4K).

How the NP7 and R31G amino acid substitutions affect the vRNP interaction network remains elusive. However, two recent biochemical studies revealed that all eight

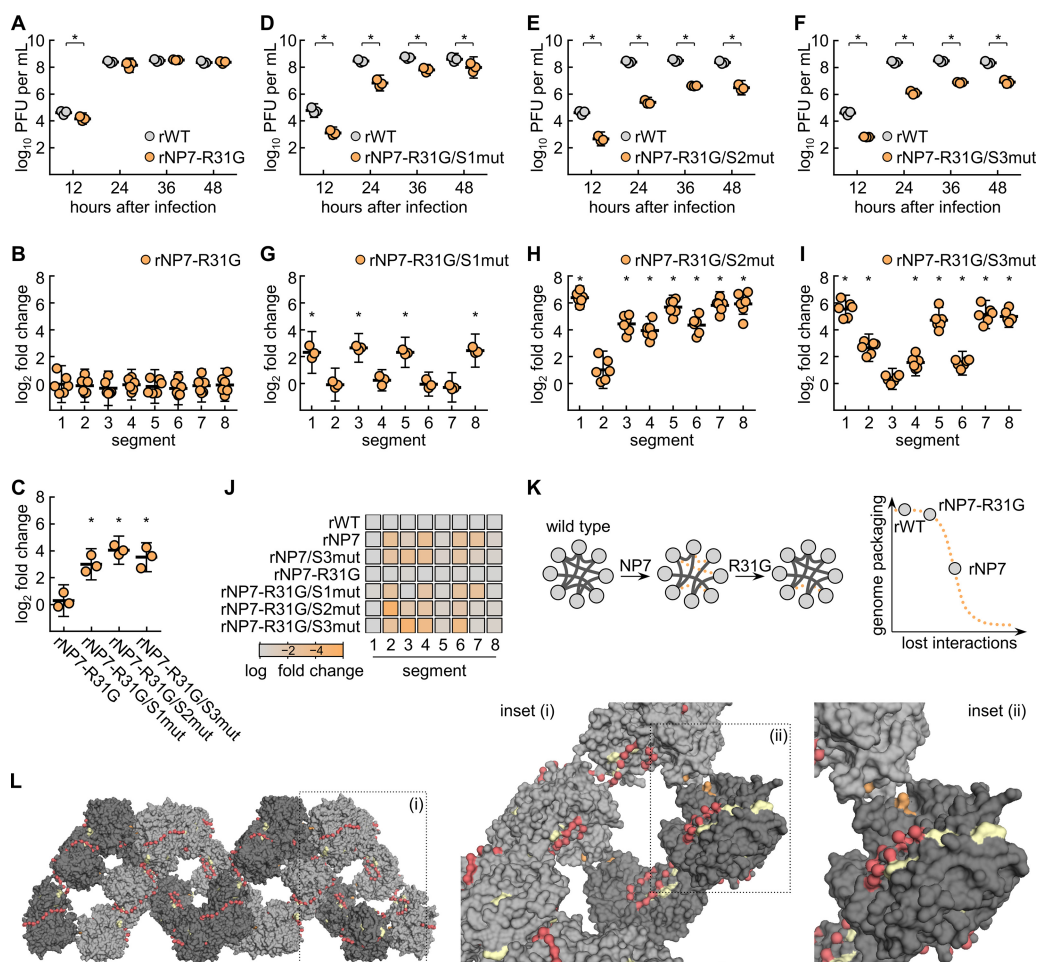


FIG 4 R31G partially restores the NP7-mediated defects in the vRNP interaction network. (A and D to F) Multicycle replication kinetics of the indicated mutant viruses compared to wild-type SC35M (rWT) ($n = 3$). (B and G to I) Relative quantification of the vRNA segment levels between mutant and wild-type virus particles by RT-qPCR. RNA levels were normalized to identical PFU numbers ($n = 3$ to 6). (C) Relative quantification of HAU/PFU ratios between mutant and wild-type virus particles ($n = 3$). (J) Heat map summarizing the packaging phenotypes of the indicated NP mutant viruses. The incorporation efficiency of each vRNA segment was calculated as log₂-fold change compared to rWT. For each mutant virus, the most frequently packaged vRNA segment was set to log₂ = 0. (K, left) Schematic model of potential changes in the vRNP interaction network mediated by NP7 and NP7-R31G. The eight vRNPs are shown as gray circles and interactions are drawn as dark connections. Lost interactions are indicated by dashed orange lines. Note that this graph does not show specific vRNP-vRNP interactions. (Right) Effect of these hypothetical changes on coordinated genome packaging. (L) Schematic representation of the three-dimensional vRNP structure. Opposing NP strands are colored in dark and light gray for clarity. NP7 amino acid residues (yellow), R31G (orange), and the vRNA (red) are highlighted. Inset i shows a closeup of the NP7 amino acid residues in proximity to the vRNA. Inset ii shows a closeup of position 31 at the NP-NP interstrand interface. In panels A to I, data are presented as means $\pm$ 95% confidence intervals. *, $P < 0.05$ (Student *t* test).

genome segments have unique NP-binding patterns composed of NP-bound and NP-free vRNA regions (33, 34), the latter of which comprising packaging sequences (34). Based on this, we speculate that NP7 and NP7-R31G change the RNA binding properties of NP and thereby influence the function of multiple packaging sequences to establish RNA-RNA interactions between the eight vRNPs. Indeed, within the vRNP structure (11), several NP7 amino acid residues are located close to the putative RNA binding groove (35, 36) (Fig. 4L). In addition, several amino acid residues in this NP region have been previously shown to be crucial for coordinated genome packaging (35), supporting this hypothesis. Amino acid position 31 is located at the interstrand NP-NP interface within the vRNP structure (Fig. 4L), and yet its compensatory mechanism remains unclear. Alternatively, the NP7 and R31G amino acid mutations could influence NP domains, which act as binding sites for vRNA packaging sequences located in heterologous vRNPs, thereby affecting the vRNP interaction network.

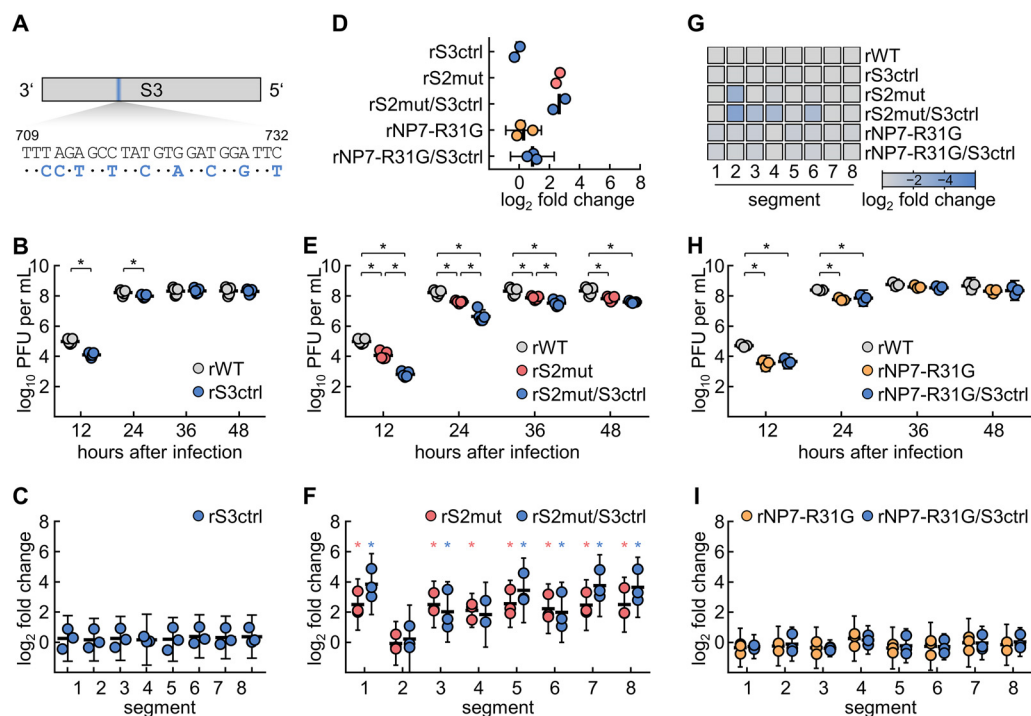


FIG 5 Synonymous mutations in a central vRNA segment 3 region do not severely affect genome packaging. (A) Schematic representation of the SC35M vRNA segment 3. Wild-type and mutated nucleotide sequences are shown in black and blue, respectively, and numbered in the positive sense. (B and E and H) Multicycle replication kinetics of the indicated mutant viruses compared to wild-type SC35M (rWT) ($n = 3$ to 5). (C, F, and I) Relative quantification of the vRNA segment levels between mutant and wild-type virus particles by RT-qPCR. RNA levels were normalized to identical PFU numbers ($n = 3$). (D) Relative quantification of HAU/PFU ratios between mutant and wild-type virus particles ($n = 2$ to 3). (G) Heat map summarizing the packaging phenotypes of the indicated viruses. The incorporation efficiency of each vRNA segment was calculated as the log₂-fold change compared to rWT. For each mutant virus, the most frequently packaged vRNA segment was set to log₂ = 0. In panels B to F and H and I, data are presented as means $\pm$ 95% confidence intervals. *, $P < 0.05$ (Student *t* test or one-way ANOVA, including Tukey's multiple-comparison test).

Synonymous mutations in the central coding region of vRNA segment 3 have only minor impact on genome packaging. Finally, we wanted to test whether synonymous mutations in other conserved viral genome regions apart from known packaging sequences affect genome incorporation. To this end, we introduced eight thus-far-undescribed consecutive synonymous codons into a central part of segment 3, the only conserved internal vRNA region present in segment 1 to 3 (25, 30) (Fig. 5A). To assess the effect of these mutations on genome packaging, we then rescued recombinant SC35M viruses harboring this mutated genome segment, either exclusively (rS3ctrl), in combination with the mutated packaging sequence in segment 2 (rS2mut/S3ctrl) or in combination with NP7-R31G (rNP7-R31G/S3ctrl). Compared to wt SC35M, rS3ctrl exhibited a slight replication defect at 12 and 24 h p.i., which was not observed at later time points (Fig. 5B). Moreover, this mutant virus neither displayed a genome packaging defect (Fig. 5C) nor an increased HAU/PFU ratio relative to wt SC35M (Fig. 5D).

The combinatorial vRNA mutant rS2mut/S3ctrl showed decreased infectious viral titers compared to wt SC35M, as well as slightly reduced PFU titers in relation to the control virus rS2mut between 12 and 36 h p.i. (Fig. 5E). However, both mutant viruses reached similar infectious titers at 48 h p.i. and produced similarly increased numbers of noninfectious virions in comparison to wt SC35M, as evidenced by ~ 5 -fold-higher HAU/PFU ratios (Fig. 5D). RT-qPCR analyses of the viral particle content revealed only slight differences in the packaging phenotypes between rS2mut/S3ctrl and rS2mut (Fig. 5F and G). While both virus mutants showed deficient incorporation of genome segment 2 compared to wt SC35M, rS2mut/S3ctrl also packaged slightly lower levels of vRNAs 3, 4, and 6 than rS2mut.

In contrast, both rNP7-R31G and its combinatorial variant rNP7-R31G/S3ctrl, harboring the internal vRNA segment 3 mutations, showed similar replication kinetics in MDCK-II cell cultures (Fig. 5H), exhibited similar HAU/PFU ratios compared to wt SC35M (Fig. 5D) and displayed no genome packaging defect (Fig. 5G and I). Overall, these data suggest that synonymous mutations in this central vRNA 3 region do not affect major packaging-relevant vRNP interactions. However, we cannot exclude that these mutations impair coordinated genome packaging when combined with other additional mutated packaging sequences, since we observed minor differences between the packaging phenotypes of rS2mut and rS2mut/S3ctrl (Fig. 5F).

In conclusion, we provide evidence that the segmented IAV genome, consisting of eight distinct vRNPs, is coordinately assembled and packaged into viral particles through the action of multiple vRNA packaging sequences and specific amino acids in NP. We identified that the vRNP interaction network of the H7N7 subtype IAV SC35M largely tolerates the mutation of single packaging sequences in three different vRNA segments. However, their combined mutation caused packaging defects characterized by the preferential absence of distinct vRNP subsets from the virions. We speculate that this mutational robustness of the vRNP network is inherently coupled to its redundant architecture, especially through the involvement of a large number of possible RNA-RNA interactions between the eight viral genome segments (29). Such complex systems are not only known to be robust to genetic perturbations but also highly plastic and adaptable (37–39), features which would greatly facilitate the acquisition of new viral genome segments during genetic reassortment.

MATERIALS AND METHODS

Cell lines. 293T cells (ATCC, CRL-3216) and MDCK-II cells (ECACC, 00062107) were maintained at 37°C and 5% CO₂ in Dulbecco modified Eagle medium (DMEM; Thermo Fisher Scientific, catalog no. 41966029) containing 10% fetal calf serum (Merck, F7524) and penicillin-streptomycin (Thermo Fisher Scientific, catalog no. 15140122).

Plasmids. The pHW2000 plasmids encoding wild-type SC35M-derived vRNA segments (40), as well as NP7 and NP7-R31G (28), were previously described. The pHW2000 plasmids encoding the modified SC35M vRNA segments 1, 2, and 3 were generated using overlap extension PCR with Phusion polymerase (Thermo Fisher Scientific, F530S) and mismatched primers (see Table SA1 in the supplemental material). Synonymous mutations introduced into the 5'-terminal coding region of vRNA 1, 2, and 3 were adapted from (25) and correspond to mutation sets PB2-53, PB1-51, and PA-53, respectively.

Generation of recombinant influenza A viruses. Recombinant SC35M viruses were generated using the eight-plasmid transfection system (41). Subconfluent 293T cells in 6-well plates were transfected with 300 ng of each pHW2000 plasmid and Lipofectamine 2000 (Thermo Fisher Scientific, catalog no. 11668027). Six hours later, the cells were further maintained in DMEM containing 0.2% bovine serum albumin (AppliChem, A1391,0100) and penicillin-streptomycin. After 48 h, cell culture supernatants were collected and cleared by centrifugation for 5 min at 500 × *g*. Successful recovery of infectious viruses was assessed by plaque assay in MDCK-II cells. To produce clonal virus stocks, isolated virus plaques were propagated for 48 h on confluent MDCK-II cells in T75 flasks. Mutations in the modified vRNA segments were confirmed using Sanger sequencing. Briefly, 200 μl of a virus stock was mixed with 500 μl of peqGOLD TriFAST reagent (PEQLAB Biotechnologie, catalog no. 30-2020), and RNA was extracted using a Direct-zol RNA MiniPrep kit (Zymo Research, R2052). Then, 5 μl of RNA was reverse transcribed and amplified using a OneStep RT-PCR kit (Qiagen, catalog no. 210212) with segment-specific primer sets (Table SA2). DNA products were then sequenced by GATC Biotech.

Multicycle replication kinetics. Confluent MDCK-II cells in 6-well plates were infected with recombinant wild-type and mutant SC35M viruses at a multiplicity of infection (MOI) of 0.001 PFU per cell. Samples of the cell culture supernatants were collected 12, 24, 36, and 48 h later and cleared by centrifugation for 5 min at 500 × *g*. Infectious virus titers were quantified using plaque assays on MDCK-II cells.

For statistical analyses, infectious virus titers were log₁₀ transformed. For comparison of wild-type and mutant viruses, an unpaired, two-sided, two-sample Student *t* test was performed using Microsoft Excel. For comparison between three samples, one-way analysis of variance (ANOVA), including Tukey's multiple-comparison test, was performed using GraphPad Prism 6.

Relative quantification of viral RNA segments per PFU. Confluent MDCK-II cells in 6-well plates were infected with recombinant wild-type and mutant SC35M viruses at an MOI of 0.001 PFU per cell. After 24 h, cell culture supernatants were collected and cleared by centrifugation for 5 min at 500 × *g*. The PFU titers of these virion preparations were quantified using plaque assays on MDCK-II cells. In addition, RT-qPCRs were performed to quantify the relative amounts of each vRNA segment in the same virion preparations. Briefly, 200 μl of supernatant were mixed with 500 μl of peqGOLD TriFAST reagent, and RNA was extracted using a Direct-zol RNA MiniPrep kit and eluted in 50 μl of RNase-free water. The purified RNA was further diluted 1:10 in RNase-free water, and 4 μl of it was reverse transcribed with

random hexamer primers using a RevertAid first-strand cDNA synthesis kit (Thermo Fisher Scientific, K1621). The cDNA products were diluted 1:100 in Milli-Q water and used for quantitative PCR using a SensiFAST SYBR Hi-ROX kit (Bioline, BIO-92020) with segment-specific primers (Table SA3). For this, in 384-well plates (Thermo Fisher Scientific, catalog no. 4309849), 5 μ l of the diluted cDNA was mixed with 5 μ l of 2 $\times$ SensiFAST SYBR Hi-ROX mix and 200 nM concentrations of each primer in a total volume of 12.5 μ l. Quantitative PCRs were performed in technical triplicates with a QuantStudio 5 real-time PCR system (Thermo Fisher Scientific).

To compare the vRNA segment levels between wild-type and a mutant virion preparation harboring identical PFU numbers, we calculated the $\log_2$ -fold change (FC) of each vRNA segment per PFU as follows: (i) we averaged the threshold cycles (C_T) from technical triplicates; (ii) $\log_2FC_{vRNA} = C_T(WT) - C_T(\text{mutant})$; (iii) $\log_2FC_{PFU} = \log_2PFU(\text{mutant}) - \log_2PFU(WT)$; and (iv) $\log_2FC_{vRNA/PFU} = \log_2FC_{vRNA} - \log_2FC_{PFU}$. For statistical analyses, we calculated: $SE(\log_2FC_{vRNA/PFU}) = \sqrt{SE(\log_2FC_{vRNA})^2 + SE(\log_2FC_{PFU})^2}$. This standard error (SE) was then used to determine the 95% confidence interval. For comparison of wild-type and mutant viruses, a two-sided, one-sample Student *t* test was performed with the calculated mean and SE values using Microsoft Excel.

Relative quantification of hemagglutination units per PFU. Confluent MDCK-II cells in 6-well plates were infected with recombinant wild-type and mutant SC35M viruses at an MOI of 0.001 PFU per cell. After 24 or 36 h, cell culture supernatants were collected and cleared by centrifugation for 5 min at $500 \times g$. The PFU titers of these virion preparations were quantified using plaque assays on MDCK-II cells. To estimate the relative number of total virus particles in the same virion preparations, the hemagglutination units (HAU) were quantified using hemagglutination assays as previously described (28).

To compare the HAU/PFU ratios between a wild-type and a mutant virion preparation, we performed the following calculations: (i) $\log_2FC_{HAU} = \log_2HAU(\text{mutant}) - \log_2HAU(WT)$; (ii) $\log_2FC_{PFU} = \log_2PFU(\text{mutant}) - \log_2PFU(WT)$; and (iii) $\log_2FC_{HAU/PFU} = \log_2FC_{HAU} - \log_2FC_{PFU}$. For statistical analyses, we calculated: $SE(\log_2FC_{HAU/PFU}) = \sqrt{SE(\log_2FC_{PFU})^2 + SE(\log_2FC_{HAU})^2}$. This SE was then used to determine the 95% confidence interval. For comparison of wild-type and mutant viruses, a two-sided one-sample Student *t* test was performed with the calculated mean and SE values using Microsoft Excel.

Representation of three-dimensional protein structures. Three-dimensional structures of NP (PDB 2Q06 [42]; note that only one NP protomer is shown) and the vRNP complex (PDB 4BBL) (11) were obtained from the protein data bank (www.rcsb.org) and displayed using the PyMOL software (www.pymol.org).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JVI.01861-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.05 MB.

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H.B. and M.S. conceived and designed the experiments. H.B., M.E.R., and E.H. performed the experiments. H.B., M.E.R., E.H., A.G.-S., and M.S. analyzed the data. H.B., M.S., and A.G.-S. wrote the paper with input from all other authors.

The authors declare that they have no conflict of interest.

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