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## Selective Genome Packaging Mechanisms of Influenza A Viruses

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The genome of influenza A virus (IAV) comprises eight segmented, single-stranded, negative-sense RNAs. The genome packaging mechanism of IAV was a long-standing enigma, but it is now widely accepted that IAV packages one copy of each of the eight viral RNA (vRNA) segments in a selective manner. Accumulating evidence over the last decade suggests that the eight unique vRNAs are selected via intersegment interactions mediated by their segment-specific genome packaging signals; however, the characteristics of these RNA-based interactions largely remain unknown. This review summarizes our current knowledge of IAV selective genome packaging and the possible mechanisms underlying the selection of the eight unique vRNAs.

Influenza A virus (IAV) is a member of the Orthomyxoviridae, which is a family of enveloped viruses with a segmented, single-stranded, negative-sense RNA genome. Each of the eight viral RNA (vRNA) segments of IAV, which encode distinct viral protein(s) essential for efficient virus replication, associates with multiple viral nucleoproteins (NPs) and a heterotrimeric RNA-dependent RNA polymerase composed of PB2, PB1, and PA to form a ribonucleoprotein (vRNP) complex. The eight vRNPs are independently responsible for the transcription and replication of the vRNA. The segmented nature of the IAV genome is advantageous in that it allows IAV to evolve rapidly through the exchange of the vRNA segments, a process termed reassortment, in which two or more different IAV strains co-infect the same cell to generate genetically different viruses from the parental viruses.

IAV is classified into 18 hemagglutinin (HA) subtypes and 11 neuraminidase (NA) subtypes on the basis of their antigenic properties. All subtypes except for the H17N10 and H18N11 subtypes circulate in their natural reservoir of wild aquatic birds (Tong et al. 2012, 2013). Since the twentieth century, we have experienced four pandemics: H1N1 Spanish influenza in 1918, H2N2 Asian influenza in 1957, H3N2 Hong Kong influenza in 1968, and H1N1 pandemic influenza in 2009. Although the origin of the H1N1 Spanish IAV remains controversial (Taubenberger et al. 2005; Worobey et al. 2014), the H2N2 Asian IAV was generated by the introduction of the HA, NA, and PB1 vRNA segments of an H2N2 avian virus into the circulating human H1N1 virus through reassortment (Kawaoka et al. 1989; Kilbourne 2006). Reassortment between the human H2N2 virus and avian H3



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viruses led to the generation of the H3N2 Hong Kong IAV, in which HA and PB1 vRNA segments were introduced from the avian H3 viruses into the human H2N2 virus background. Multiple reassortment events among avian, swine, and human IAVs in swine resulted in the generation of the 2009 H1N1 pandemic virus (Garten et al. 2009; Neumann et al. 2009; Smith et al. 2009). Thus, the introduction and exchange of vRNA segments between different IAVs through reassortment plays an important role in the emergence of pandemic strains, which acquire an HA with novel antigenic properties and the ability to transmit efficiently among humans, allowing the rapid evolution of IAVs.

Despite these evolutionary advantages, the reassortment event must surmount substantial difficulties in order to produce infectious IAVs with adequate viral fitness (Villa and Lässig 2017). One such difficulty is overcoming RNA-based incompatibility among vRNA segments. Reassortment is tightly associated with the selective genome packaging process in which eight distinct vRNAs are selected from a large pool of viral and host genetic material in the virus-infected cell and are packaged into each virion to be replicated (Hutchinson et al. 2010; Noda and Kawaoka 2010; Gerber et al. 2014). During this process, the eight vRNA segments are thought to interact with each other. Correct intersegment interactions would ensure the integrity of the genome packaging process and produce novel viruses with pandemic potential within a co-infected cell. Although a large body of recent research clearly supports the idea that IAV packages eight distinct vRNAs through their interactions, the underlying mechanism remains largely unknown. In this review, we summarize our current understanding of selective genome packaging and the possible mechanism by which the eight unique vRNAs are selected.

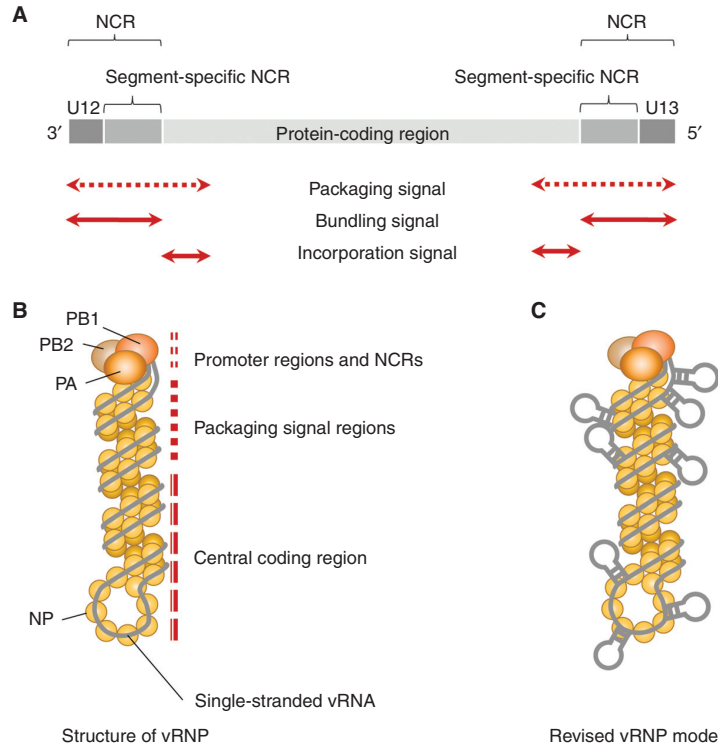
### STRUCTURE OF THE IAV vRNP

The eight single-stranded negative-sense vRNA segments of IAV (PB2, PB1, PA, HA, NP, NA, M, and NS) vary in length from 2341 to 890 bases, but share the same structure: a central

coding region in the antisense orientation flanked by segment-specific noncoding regions (NCRs) (19–58 nucleotides) and the terminal promoter regions U12 and U13 (12 and 13 nucleotides from the 3' and 5' ends, respectively), which are highly conserved among all eight vRNA segments (Fig. 1A). The sequences of the terminal regions containing U12 and U13 are partially complementary to each other (Robertson 1979) and form a corkscrew structure through base-pairing (Fodor et al. 1994; Flick et al. 1996; Tomescu et al. 2014), where the heterotrimeric RNA-dependent RNA polymerase complex is associated (Klumpp et al. 1997). The rest of the single-stranded vRNA is bound by multiple NPs through its phosphate-sugar backbone in a sequence-independent manner (Baudin et al. 1994). In this way, each vRNA forms a twisted rod-like vRNP complex (Fig. 1B), which is about 12 nm in diameter but varies in length from about 30 to 120 nm, which correlates with the nucleotide length of each vRNA (Pons et al. 1969; Compans et al. 1972; Murti et al. 1988; Sugita et al. 2013). Recent cryo-electron microscopy (cryo-EM) has yielded a more detailed structure of the twisted rod-like vRNP, in which a single strand of a multiple NP-vRNA complex is folded back to form a loop at the opposite end to the polymerase-bound end and coils back on itself to form an antiparallel double-stranded helix (Arranz et al. 2012; Moeller et al. 2012).

One of the most important findings regarding the vRNP structure in recent years came from the RNA sequencing technique CLIP (UV-cross-linking and immunoprecipitation). Previously, it had been believed that NPs uniformly bind to the entire length of the single-stranded vRNA, where secondary and tertiary structures of the vRNA are completely melted by the binding of the NPs (Baudin et al. 1994), although there is a report that a bacteriophage PP7 RNA tag inserted into the NA vRNA maintains the hairpin structure in the context of the vRNP (York et al. 2013). Recently, Lakdawala and her colleagues performed a HITS-CLIP (high-throughput sequencing of RNA isolated by CLIP) analysis to determine the NP-binding sites of vRNAs within virion-derived vRNPs

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**Figure 1.** Structure of influenza A virus (IAV) viral RNA (vRNA) and ribonucleoprotein (vRNP). (A) A protein-coding region in the antisense orientation is flanked by segment-specific noncoding regions (NCRs) and terminal U12 and U13 promoter regions. The bipartite conventional packaging signal, bundling signal, and incorporation signal regions are indicated by arrows. (B) Conventional model of vRNP. Predicted promoter regions, NCRs, packaging signal regions, and central coding regions in the rod-like vRNP are shown. (C) Revised model of vRNP. Secondary and/or tertiary structures composed of nucleoprotein (NP)-free vRNAs protrude from the vRNP at multiple positions.

(Lee et al. 2017; Le Sage et al. 2018). They found that NPs associate with vRNAs in a nonuniform and nonrandom manner, in which NPs preferentially bind to guanine-rich and uracil-poor regions, and that there are NP-free regions on vRNAs. Similarly, Boon and colleagues performed a PAR-CLIP (photoactivatable ribonucleoside-enhanced CLIP) analysis to assess NP-vRNA interactions in virus-infected cells. They demonstrated that NPs bind to about 12 nucleotides of vRNA with a mean distance of 25 nucleotides between adjacent NP binding sites without apparent sequence specificity (Williams et al. 2018). Interestingly, ~10% of the viral genome comprises low-NP binding regions and

the vRNAs of low-NP binding regions are predicted by use of in silico analysis to form secondary and/or tertiary structures. Collectively, these studies led to a revised vRNP model, in which certain vRNA regions are free of NP and form secondary and/or tertiary structures that protrude from the twisted rod-like body of the vRNP (Fig. 1C). According to the vRNP structure revealed by cryo-EM (Arranz et al. 2012; Moeller et al. 2012), vRNA nucleotides located in the spaces between two neighboring NP molecules are “naked” (i.e., not associated with NP) and potentially form secondary and/or tertiary structures on the vRNP. In agreement with this revised vRNP model, several bioinformatics

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studies have suggested the importance of predicted stem-loop and pseudoknot structures in the HA and NP vRNAs for viral fitness (Gulyaev et al. 2014, 2016). In addition, Kobayashi et al. predicted several stem-loop structures in the M vRNA that are highly conserved in IAVs and they experimentally demonstrated that disruption of one of the stem-loop structures consisting of nucleotide positions 219–240 (throughout, nucleotide numbering refers to positive-sense cRNA) attenuates virus replication (Kobayashi et al. 2016). Taken together, it is now believed that vRNPs display multiple protruding secondary and/or tertiary structures, some of which likely play important roles in virus replication and selective genome packaging as described later.

#### PACKAGING MODELS OF THE SEGMENTED IAV GENOME

Genome packaging of IAV occurs during the late stage of the infection cycle, when eight-segmented vRNAs are incorporated into progeny virions at the surface of virus-infected cells (Hirst 1962; McGeoch et al. 1976). Historically, two conflicting models had been proposed to explain the mechanism: the random packaging model and the selective packaging model (Hutchinson et al. 2010; Noda and Kawaoka 2010). In the random packaging model, any number and combination of vRNAs are packaged randomly into progeny virions through a genome packaging signal common to all eight vRNAs, which theoretically differentiates between vRNAs and cellular RNAs but not among the eight vRNAs, resulting in the generation of considerable numbers of noninfectious and semi-infectious particles (Enami et al. 1991; Bancroft and Parslow 2002). In contrast, in the selective packaging model, one copy of each of the eight vRNAs is selectively packaged into each progeny virion through distinct packaging signals specific to each vRNA (Duhaut and McCauley 1996; Odagiri and Tashiro 1997). Although a substantial proportion of released virus particles fails to express one or more viral gene product(s) in virus-infected cells (Martin and Heleniust 1991; Brooke et al. 2013), which

seemingly supports the random packaging model, these observations do not necessarily refute the selective packaging model (Diefenbacher et al. 2018), because these phenomena can be explained by failures of uncoating or nuclear import of vRNPs, or lethal mutations or deletions in the vRNAs for transcription, replication, and/or viral protein synthesis, or by incomplete genome packaging of less than eight vRNA segments (Nakatsu et al. 2018). Rather, almost all of the evidence obtained over the past 15 years strongly supports the selective packaging model and indicates that the eight distinct vRNAs are selectively packaged into most, but not all, progeny virions.

#### SEGMENT-SPECIFIC GENOME PACKAGING SIGNALS OF THE IAV GENOME

Although several early studies suggested that the genome packaging process was not purely random (Laver and Downie 1976; Nakajima and Sugiura 1977; Lubeck et al. 1979; Duhaut and McCauley 1996; Odagiri and Tashiro 1997; Duhaut and Dimmock 2000, 2002), the first direct evidence in support of selective genome packaging was established by our group. Using a reverse genetics approach, Fujii et al. systematically searched for the genome packaging signal of the NA vRNA and demonstrated that, in addition to its NCRs (Luytjes et al. 1989; Bancroft and Parslow 2002), about 180 and 160 nucleotides from the 3' and 5' ends of the coding region, respectively, are required for efficient and stable packaging of the NA vRNA into virus particles, indicating that the bipartite regions act as the packaging signal of NA vRNA (Fujii et al. 2003). Because the regions contain protein-coding sequences unique to NA vRNA, this finding indicates that NA vRNA is packaged into progeny virions in a selective manner. Subsequently, the genome packaging signals unique to each of the other seven vRNA segments have been identified (Watanabe et al. 2003; Dos Santos Afonso et al. 2005; Fujii et al. 2005; Liang et al. 2005; de Wit et al. 2006; Muramoto et al. 2006; Ozawa et al. 2007, 2009); all of the segment-specific packaging signals are bipartite, being located in both NCRs and adjacent terminal coding re-



gions (within 100–300 nucleotides from each end of the coding region, depending on the vRNA) (Fig. 1A). Collectively, the existence of the segment-specific packaging signal sequences in all eight vRNAs indicates that eight unique vRNAs are selectively packaged into progeny virions. Because there seem to be no boundaries in the packaging signals that separate whether they are functional or nonfunctional, the packaging signals appear to comprise multiple short functional regions, and therefore the minimal packaging signal sequences have not been determined. In line with this assumption, the introduction of synonymous point mutations into the packaging signal region sometimes can cause a significant reduction in the genome packaging efficiency of mutated vRNA, although no significant impact on the genome packaging efficiency may be observed with other synonymous point mutations (Fujii et al. 2005; Gog et al. 2007; Marsh et al. 2007, 2008; Hutchinson et al. 2008, 2009; Liang et al. 2008). Therefore, it is thought that each segment-specific packaging signal is a cluster that comprises multiple, discontinuous, short-nucleotide elements (Gog et al. 2007). This idea is compatible with the revised vRNP model, in which multiple vRNA regions are free of NP and form secondary and/or tertiary structures on the surface of the vRNP; such structured short vRNA regions would cooperatively and redundantly act as packaging signals.

#### ORGANIZATION OF vRNPs WITHIN THE IAV VIRION

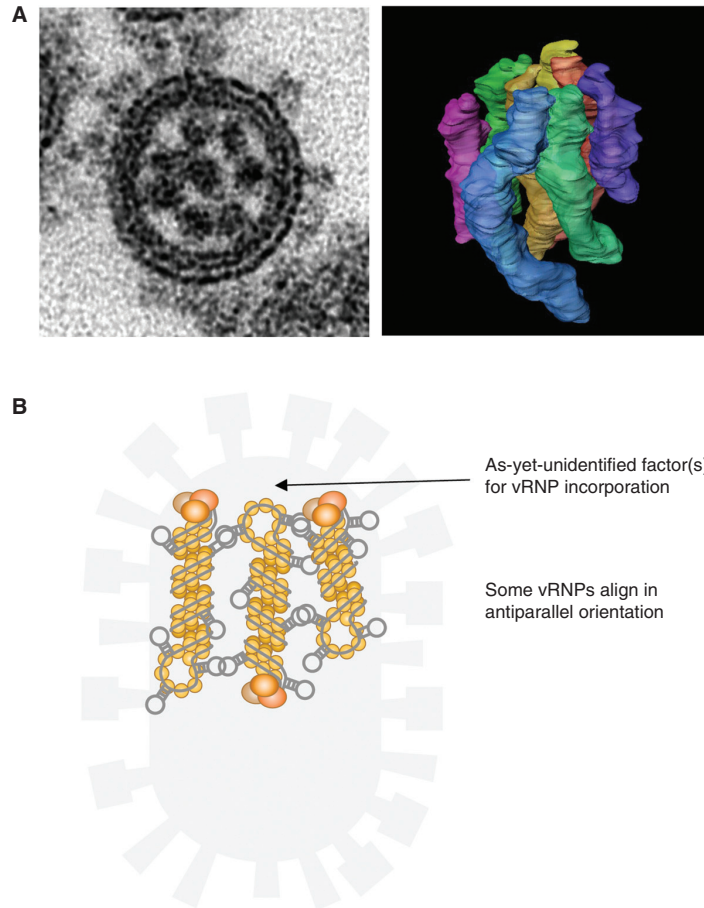
In addition to the reverse genetics studies described above, multicolor single-molecule fluorescence in situ hybridization (FISH) analysis has provided additional evidence in support of selective packaging. Chou et al. (2012) examined colocalization of various combinations of two different vRNAs within individual virus particles and quantitatively showed, by using a photobleaching technique, that most virus particles likely contain one copy of each of the eight different vRNAs. However, it had remained unclear how many vRNPs are exactly packaged into each virion and how they are arranged within a virion. By using thin-section electron microscopy, we

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revealed that each budding virion packages eight vRNPs arranged in a specific “1 + 7” pattern, in which a central vRNP is surrounded by seven vRNPs (Fig. 2A; Noda et al. 2006). The specific arrangement of the eight vRNPs is commonly observed in virions of various IAV strains (Harris et al. 2006; Noda et al. 2006) and also in influenza B viruses (Nakatsu et al. 2016). These observations provided strong evidence that eight vRNPs are packaged into each virion in a selective manner. Interestingly, we also revealed that a mutant virus that possessed only seven vRNAs and lacked the HA vRNA also packaged eight vRNPs arranged in a specific “1 + 7” arrangement (Noda et al. 2018). This is consistent with the finding that influenza C and D viruses, which naturally possess a seven-segmented RNA genome, package eight vRNPs arranged in the same configuration (Nakatsu et al. 2018) and suggests that assembly of eight vRNPs into the specific arrangement is an important step for the selective genome packaging process. When budding virions are longitudinally sectioned, a set of eight vRNPs arranged in the specific pattern is always found at the top of each budding virion, even in huge filamentous virions of ~1 μm in length (Noda et al. 2006), suggesting that there are as-yet-unidentified viral and/or host factors at the budding tip that are responsible for the incorporation of the set of eight vRNPs (Fig. 2B). Regarding the directionality of the eight vRNPs within the virions, our immunoelectron microscopic analysis using antipolymerase antibodies revealed that some of the eight vRNPs direct their polymerase end to the budding tip, but others direct their loop ends to the budding tip (Sugita et al. 2013), indicating antiparallel alignment of some of the vRNPs (Fig. 2B). Because both of the bipartite packaging signal sequences are located close to the polymerase end of the respective rod-like vRNPs (Fig. 1A), this finding suggests that the respective packaging signals, in the context of the vRNPs, are not always close to each other within a virion (Fig. 2B). It remains unclear which vRNAs direct their polymerase ends to the budding tip and whether the directionality of the eight vRNAs is always the same.

Three-dimensional (3D) analysis of the eight vRNPs within budding virions by use of

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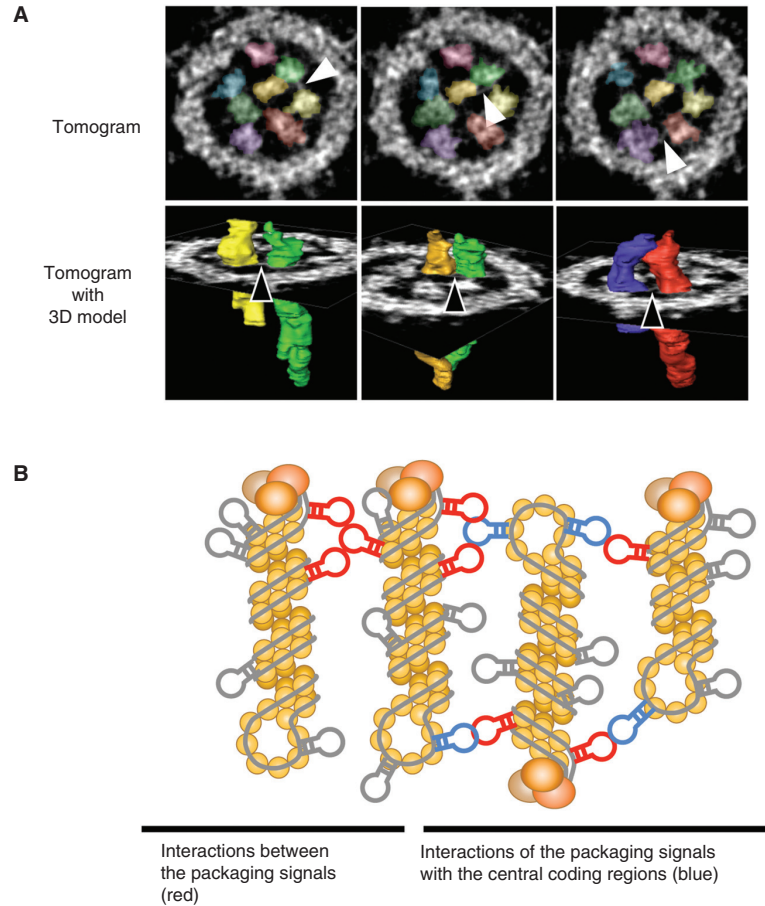


**Figure 2.** Eight vRNPs within a virion. (A) (Left) Transverse section of a budding virion (left side of A is reprinted from Noda et al. [2006] with permission from the authors); (right) three-dimensional model of eight vRNPs within a virion reconstructed by electron tomography (right side of A is reprinted from Noda et al. [2012] under a Creative Commons Attribution-NonCommercial-Share Alike 3.0 Unported License). Each of the eight vRNPs is colored differently. (B) A single bundle of eight vRNPs arranged in a “1 + 7” pattern, in which some vRNPs align in antiparallel orientation, associates with the tip of the budding virion, even in huge filamentous virions. Note that only three vRNPs are depicted here for clarity.

electron tomography showed that the eight vRNPs are different in length and that their positions within the specific “1 + 7” arrangement are not purely random, although their exact, assigned positions have not yet been determined because of technical difficulties (Fournier et al. 2012a, 2012b; Noda et al. 2012). Importantly, in our 3D analysis, we found multiple nucleic acid-like string structures connecting neighboring

vRNPs; such interactions were observed across the entire length of the rod-like vRNPs (Fig. 3A; Noda et al. 2012). Although the identity of the nucleic acid-like string structures was unclear at that time, we now believe that they may be NP-free, secondary, and/or tertiary structures of vRNA protruding from the surface of the rod-like vRNPs. These structural findings suggest that the eight vRNPs are assembled into the spe-

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**Figure 3.** Interactions among vRNPs. (A) Nucleic acid–like string structures connect vRNPs at multiple positions. Note that the string structures are not restricted to the top of the rod-like vRNPs. Arrowheads indicate nucleic acid–like string structures. (B) Intersegment interactions. Interactions between the genome packaging signals (red) as well as interactions of the packaging signals with nonpackaging signals (blue) are indicated. (A, reprinted from Noda et al. [2012] under a Creative Commons Attribution-NonCommercial-Share Alike 3.0 Unported License.)

cific “1 + 7” pattern through multiple interactions via NP-free vRNAs. Because the interconnections through the string-like structures are observed at multiple positions spanning the entire length, it is feasible that the interactions do not only occur between the packaging signals. Rather, given that the eight vRNPs align in antiparallel orientation within budding virions (Sugita et al. 2013), it has been suggested that certain packaging signals may interact not only

with the packaging signals of other vRNAs but also with secondary or tertiary RNA structures located in the protein-coding regions (Fig. 3B).

### FUNCTIONAL vRNA–vRNA INTERACTIONS DURING GENOME PACKAGING

An attractive model to explain the mechanisms underlying the selective genome packaging of eight unique vRNAs is that specific interactions

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take place among the eight distinct vRNAs through their genome packaging signals. The initial evidence suggesting vRNA–vRNA interactions came from our group. Muramoto et al. (2006) searched for the genome packaging signals of the PB2, PB1, and PA vRNAs and showed that those packaging signals are necessary not only for the packaging of the respective residential vRNAs, but that they also affect the packaging efficiencies of the other vRNAs. Subsequently, similar findings were reported for the other vRNAs, in which mutations in the packaging signal of a certain vRNA were shown to affect the packaging efficiency of one or more other vRNAs (Gog et al. 2007; Marsh et al. 2007, 2008; Hutchinson et al. 2008, 2009). It appears that the eight vRNAs are not independently, but rather cooperatively, packaged into virions and interact with each other during the genome packaging process. Importantly, Muramoto et al. (2006) also demonstrated that the *trans*-acting effect on the packaging efficiency of the other vRNAs is most prominent with PB2 vRNA, indicating that there is a hierarchy among the eight vRNAs for the selective genome packaging. Similarly, Gao et al. (2012) used a different IAV strain to show that all eight vRNAs are not equally important for the packaging of the other vRNAs, and that in addition to the PB2 vRNA, the PA, NP, and M vRNAs play important roles. Thus, selective genome packaging likely involves intersegment associations among the eight unique vRNAs in a hierarchal process, in which certain vRNA(s) (e.g., the PB2 vRNA) may act as a master segment for assembling multiple vRNAs through their interactions.

Our group has also identified important regions in the packaging signal for intersegment association. Goto et al. (2013) showed that the NCRs of a vRNA, comprising the promoter regions and segment-specific NCRs, are sufficient for its efficient packaging into virus particles and that the terminal protein-coding regions of the packaging signal are essential for the packaging of other vRNAs. The finding indicates that the conventional packaging signal, which is composed of the conserved promoter region, segment-specific NCRs, and adjacent terminal protein-coding regions, can be divided into two

regions based on their different roles: the NCRs (i.e., the promoter regions and the segment-specific NCRs) of vRNAs serve as the “incorporation signal” that is responsible for the packaging of the residential vRNA, whereas the terminal coding regions serve as the “bundling signal” that ensures the packaging of a complete set of eight vRNAs (Fig. 1A). In this scenario, the bundling signal regions would be important for intersegment associations during selective genome packaging. This is partially consistent with a bioinformatics-based study by Gog et al. (2007), which showed that codons in the terminal protein-coding regions of the vRNAs are more conserved than those in the central protein-coding regions. Intriguingly, our group also showed that the bundling signals could have another role in selective genome packaging. Inagaki et al. (2012) demonstrated that two artificial vRNAs that had identical bundling signals but encoded different reporter proteins competed with each other for packaging into virions, leading to the packaging of a single copy of the vRNA. Such self-repulsion of identical vRNAs through the bundling signals would strengthen the selectivity of a single copy of each of the eight unique vRNAs (Venev and Zeldovich 2013).

Although intersegment associations are predicted to be mediated by the genome packaging signals through base-pairing, NP proteins are also involved in selective genome packaging. Moreira et al. (2016) introduced mutations into certain NP residues and showed that such mutations altered the packaging efficiencies of specific vRNA subsets. In addition, a defect in packaging efficiency could be partially restored by mutations in specific residues of NP (Bolte et al. 2019). These results strongly suggest that intersegment associations are regulated not only by vRNA–vRNA interactions but also by vRNA–NP interactions. The manner of vRNA–NP interaction would affect the formation of stable secondary and/or tertiary structures of vRNA protruding from the vRNP, and such local structural changes caused by mutations in NP as well as in the vRNA would affect the vRNA–vRNA interactions and subsequent packaging of specific vRNAs (Takizawa et al. 2019).





## EVIDENCE FOR DIRECT vRNA–vRNA ASSOCIATIONS

Early evidence for direct intersegment associations was obtained by using *in vitro* electrophoretic mobility shift assays and *in vitro*–synthesized naked vRNAs. In both human H3N2 and avian H5N2 viruses, all eight vRNAs interact with one or more vRNAs and form single networks of intersegment associations (Fournier et al. 2012a, 2012b; Gavazzi et al. 2013a, 2013b). Similar findings have been reported for human H1N1 virus, in which each vRNA interacts with at least one vRNA through terminal coding regions in many cases (Miyamoto and Noda 2020). Curiously, the networks of intersegment interactions are different among these three strains, and only a few combinations are conserved. Because vRNA–vRNA associations are partially regulated by vRNA–NP interactions (Moreira et al. 2016), such *in vitro* interaction networks do not necessarily reflect actual networks in the context of vRNPs and could include false-positive associations while missing actual associations because of the *in vitro* experimental conditions and the absence of NPs and host proteins. However, despite the limited efficacy, such an *in vitro* study identified a direct interaction between the PB1 and NS vRNAs of an H5N2 virus, which was mediated by stem-loop structures composed of nucleotides 257–277 of the NS vRNA and nucleotides 289–309 of the PB1 vRNA; the study also showed the importance of this interaction for efficient virus replication (Gavazzi et al. 2013b). In addition, a direct interaction between the PB1 and NA vRNAs of a human H3N2 virus has also been shown to play an important role in selective genome packaging, in which preferential co-packaging of the PB1 vRNA with the NA vRNA into progeny virions was shown to be mediated by an RNA-based direct interaction through nucleotides 1776–2070 of the PB1 vRNA (Cobbin et al. 2014; Gilbertson et al. 2016). To date, these two reports are the only experimental demonstrations of direct intersegment interactions being important for co-packaging. However, these regions are located slightly outside the bundling signals (more than 250 nucleotides from the ter-

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mini of the coding regions). Direct interactions through the bundling signals (or conventional packaging signal) have not yet been experimentally proven to be essential for co-packaging.

Recently, Dadonaite et al. (2019) employed a SPLASH (sequencing of psoralen cross-linked, ligated, and selected hybrids) approach to examine direct vRNA–vRNA interactions in the context of vRNPs *in vivo*. They found that most vRNAs establish multiple and redundant RNA-mediated intersegment interactions at both the terminal and central protein-coding regions. The interaction of the PB1 vRNA with the NA vRNA through nucleotides 1776–2070 (Gilbertson et al. 2016) was detected by using SPLASH, and the association was experimentally shown to be important for efficient co-packaging, confirming the importance of direct vRNA–vRNA interactions for selective genome packaging. However, many of the intersegment interactions detected by use of SPLASH and electrophoretic mobility shift assays have not yet been evaluated in the context of virus replication, and the contributions of the respective vRNA–vRNA interactions to co-packaging remain uncertain. In addition, because of the lack of ultrastructural information, it is not clear how the vRNAs are interconnected in the context of the vRNPs to form complexes of multiple rod-like vRNPs.

## CONCLUDING REMARKS

Accumulating evidence indicates that intersegment interactions among the eight unique vRNAs of IAVs are involved in the selective genome packaging process and that these interactions are likely mediated by secondary and/or tertiary structures of NP-free vRNA regions protruding from the vRNPs. In addition to packaging signals (i.e., the incorporation signal and the bundling signal), other regions (i.e., central protein-coding regions) are likely involved in the intersegment interactions. Although many reverse genetics studies have shown the importance of the bundling signals for the co-packaging of multiple vRNAs, no direct interaction sites between two vRNAs have yet been identified in the bundling signals. Accordingly, there

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is a long way to go before the overall picture of the interactive network is revealed. Further studies are needed to understand the precise mechanisms by which the eight unique vRNPs are specifically interconnected and form the specific “1 + 7” arrangement during selective genome packaging.

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