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The parts are greater than the whole: the role of semi-infectious particles in influenza A virus biology

Meghan Diefenbacher[1], Jiayi Sun[1] and Christopher B Brooke[1,2]

The influenza A virus (IAV) genome is incorporated into newly produced virions through a tightly orchestrated process that is one of the best studied examples of genome packaging by a segmented virus. Despite the remarkable selectivity and efficiency of this process, it is clear that the vast majority of IAV virions are unable to express the full set of essential viral gene products and are thus incapable of productive replication in the absence of complementation. Here, we attempt to reconcile the widespread production of these semi-infectious particles (SIPs) with the high efficiency and selectivity of IAV genome packaging. We also cover what is known and what remains unknown about the consequences of SIP production for the replication and evolution of viral populations.

Addresses
[1] Department of Microbiology, University of Illinois, Urbana, IL 61801, USA
[2] Carl R. Woese Institute for Genomic Biology, University of Illinois, Urbana, IL 61801, USA

Corresponding author: Brooke, Christopher B (cbrooke@illinois.edu)

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Introduction

Influenza A virus (IAV) is a respiratory pathogen that remains a serious threat to public health. Seasonal epidemics of IAV continue to cause significant morbidity and mortality worldwide, causing hundreds of thousands of deaths annually, while the threat of pandemic strains entering the population and causing millions of excess deaths continually looms [1]. IAV populations exhibit high levels of diversity both in terms of nucleotide sequence (genetic diversity) and in the specific viral gene products expressed by individual particles (genomic diversity) [2]. While the consequences of genetic diversity have been explored to some extent in a number of other studies, the role that genomic diversity plays in IAV biology is just beginning to be appreciated [2,3].

Like many viruses, IAV has long been known to have a high particle to PFU ratio [4]. Only a small fraction of IAV particles (~1–30% depending on the strain examined) are capable of initiating a productive replication cycle, with the remaining bulk of the population consisting of defective interfering particles (DIPs) and semi-infectious particles (SIPs) [2,5–8,9••]. While DIPs are defined by a specific molecular signature (large internal deletions in one or more gene segments), SIPs lack such a signature and are instead defined by a functional phenotype (failure to express one or more gene products) [2]. Here, we attempt to reconcile the existence of SIPs with the highly selective packaging mechanism of IAV, and highlight outstanding questions regarding the role of SIPs in IAV biology.

IAV genome packaging

The organization and packaging scheme of the IAV genome is central to the biology of SIPs, and to understanding broader patterns of viral genomic diversity. The IAV genome is divided into eight negative sense, single-stranded RNA segments that each encode one or more gene products, and expression from all eight gene segments is required to initiate a productive infection [10]. Each individual segment is generally maintained as a viral nucleoprotein complex (vRNP), in which the viral genomic RNA is bound by multiple copies of NP and associates with a single copy of the viral RNA-dependent RNA polymerase complex [10].

Genome segmentation imposes the challenge of packaging the full set of IAV gene segments required for the next round of replication. While many specific details of the influenza packaging mechanism remain a mystery, a multitude of studies using a variety of approaches have clearly demonstrated the existence of a highly selective and efficient genome packaging mechanism [11••]. Structural analyses using electron microscopy revealed that vRNPs are arranged in a highly ordered structure within the virion. Cross sections of budding particles demonstrated that the 8 vRNPs are organized into a '7+1' array, with 7 vRNPs surrounding 1 central vRNP [12•,13,14]. The formation of this array likely depends upon specific physical interactions between the viral genomic RNAs that occur prior to and during the packaging process, likely sometime between nuclear export and viral budding [15–18]. Interestingly, the formation of the 7+1 array appears to be crucial for virion assembly, as recombinant viruses lacking a single gene segment recruit host ribosomal RNAs to complete the array [19•]. Further,

influenza C and D viruses, which only have 7 genome segments in total, also form a 7+1 array [20]. These EM studies were complemented by the work of Chou et al., which used single molecule fluorescent *in situ* hybridization (smFISH) to demonstrate that most IAV particles contain a single copy of each of the 8 distinct vRNAs [21••].

The IAV packaging process depends upon segment-specific packaging signals located at both the 5′ and 3′ ends of each vRNA [11••,22]. Specifically, the promoter sequences and non-coding regions together serve as incorporation signals that ensure the incorporation of that segment, while the terminal coding regions serve as bundling signals that ensure the incorporation of the full set of 8 distinct segments [23]. Additional sequence elements outside of the canonical packaging signals also appear to be important for achieving maximum packaging efficiency [17,24]. Mutations in packaging signals can decrease the incorporation frequency of the mutated segment, as well as other segments due to the essential role of intersegment interactions during packaging [11••].

Mechanisms of SIP production

Given the vast multitude of data demonstrating the efficiency and specificity of the IAV packaging process, why do so few virions actually encode the full set of essential viral genes? Below, we review the most likely mechanisms behind the generation of SIPs and their predominance in IAV populations. Please note that these mechanisms are not mutually exclusive.

Imperfect genome packaging

Despite being incredibly selective and efficient, the IAV genome packaging process is not perfect. EM-based examination of a collection of IAV and IBV strains revealed that up to 20% of virions contained fewer than eight RNA segments, suggesting a greater degree of flexibility in packaging fidelity than was previously thought [25•]. This number is likely an underestimation, given the observation from the same group that non-viral RNAs can be packaged into the 7 +1 array within virions in some cases [19•]. In addition, we found that mutations within NP that emerge during host adaptation can selectively modulate the packaging efficiency of individual segments, likely through changes in the overall intracellular abundance of the genomic RNAs [26•].

This flexibility in IAV packaging fidelity does not fully account for the ten-or-more-fold higher proportion of non-infectious to fully infectious particles, however. It is likely that many SIPs arise from the failure to complete the early steps in the viral life cycle that are necessary for successful gene segment expression.

Failure of gene segment transport

IAV vRNPs must successfully enter the nucleus to initiate gene expression. This entails transit across the cytosol from the point of fusion to the nuclear pore, as well as successful translocation through the nuclear pore complex. The success rates of these individual steps, and the specific factors that may influence them, remain poorly defined. Numerous studies have indicated that cytosolic trafficking of IAV vRNPs and the nuclear import of model cargo molecules are far from perfect [27–29]. Further, interferon inducible antiviral effectors such as Mx1 and PLSCR1 may actively interfere with vRNP import [30,31]. In the only study to date to specifically examine the co-trafficking of individual IAV vRNPs, Chou *et al.* used smFISH to quantify the degree of co-localization between vRNPs during early infection [32•]. By comparing segment co-localization within the cytosol following inhibition of membrane fusion or nuclear export, they concluded that the vRNPs enter the nucleus as a complex and that dissociation of individual vRNPs prior to nuclear entry was minimal. This suggests that degradation or loss of vRNPs prior to nuclear entry is a minor contributor to the SIP phenotype. By contrast, Heldt *et al.* used an experimentally parameterized computational model to predict that 38% of infections were non-productive as a result of vRNA degradation [[32•]]. More work is clearly needed to reconcile these results and to determine whether the success rates of these steps vary under different conditions, in different cell types, and for different viral genotypes.

Failure of gene segment expression

Incomplete viral gene expression patterns may also arise through the failure to initiate vRNA transcription and replication in the nucleus or produce a functional gene product. Russell et al. used single cell mRNA sequencing to estimate that ~50–65% of IAV infected cells fail to express one or more viral mRNAs as under low MOI conditions [33]. The high mutation rate of influenza viruses (predicted to be as high as 1.8×10^{-4}, or ~2–3 mutations per replicated genome), is likely to contribute to viral gene expression failure [34]. For instance, an incoming vRNA may carry a mutation in a critical *cis*-acting regulatory sequence that prevents replication and transcription of the gene segment or mRNA. Additionally, non-synonymous mutations in coding sequences may result in the production of truncated or misfolded proteins that will not be detected by antibody staining.

Measurements of IAV mutant ***frequencies*** are generally much lower than the above rate estimate, around 1.5×10^{-5} to 2×10^{-6} mutations per nucleotide per infectious cycle, not high enough to explain observed SIP frequencies [35–39]. Further, if random mutations were the main mechanism underlying the SIP phenotype, the gene expression frequencies would be roughly inversely proportional to segment length. Instead, what is observed