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Interaction network linking the human H3N2 influenza A virus genomic RNA segments

Emilie Fournier[a,1], Vincent Moules[b,1], Boris Essere[b], Jean-Christophe Paillart[a], Jean-Daniel Sirbat[a], Annie Cavalier[c], Jean-Paul Rolland[c], Daniel Thomas[c], Bruno Lina[b], Catherine Isel[a,*], Roland Marquet[a,**]

[a] Architecture et Réactivité de l'ARN, Université de Strasbourg, CNRS, IBMC, 15 rue René Descartes, 67084 Strasbourg, France
[b] Virologie et Pathologie Humaine, Université Lyon 1, FRE 3011 CNRS, Faculté de Médecine RTH Laennec, 69008 Lyon, France
[c] Interactions Cellulaires et Moléculaires, Université Rennes 1, UMR 6026 CNRS, Campus de Beaulieu, bâtiment 13, 35042 Rennes, France

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ABSTRACT

The genome of influenza A viruses is comprised of eight negative-sense viral RNAs (vRNAs) that form viral ribonucleoproteins (vRNPs). In order to be infectious, an influenza A viral particle must encapsidate at least one copy of each of the vRNAs. Thus, even though genome segmentation is evolutionary advantageous, it undeniably complicates viral assembly, which is believed to occur through a selective mechanism that still remains to be understood. Using electron tomography 3D-reconstructions, we show that the eight vRNPs of an influenza A Moscow/10/99 (H3N2) virus are interconnected within a star-like structure as they emerge from a unique "transition zone" at the budding tip of the virions. Notably, this "transition zone" is thick enough to accommodate all described packaging signals. We also report that, *in vitro*, each vRNA segment is involved in a direct contact with at least one other vRNA partner, in a single network of intermolecular interactions. We show that in several cases, the regions involved in vRNA/vRNA interactions overlap with previously identified packaging signals. Our results thus provide support for the involvement of RNA/RNA interactions in the selection and specific packaging of influenza A genomic RNAs, which appear embedded into an organised supramolecular complex likely held together by direct base-pairings between packaging signals.

1. Introduction

Influenza A viruses are enveloped negative-strand RNA viruses that belong to the *Orthomyxoviridae* family and infect birds and many species of mammals, including humans, swine and horses. Influenza A viruses are responsible for the recurrent seasonal flu epidemics which are a significant cause of morbidity and mortality, but also for the emergence of occasional severe pandemics [1] and therefore remain a major threat to public health [2]. A specific feature of these viruses is the segmented nature of their genome that consists of eight negative-sense viral RNAs (vRNAs), ranging from 890 to 2341 nucleotides (nts) in length. All influenza A genomic vRNAs also share a common organisation and structure: they consist of a central open-reading frame (in antisense orientation) flanked at both ends by two short untranslated regions (UTRs) that range from 19 to 58 nts. Within these UTRs, the distal unique 12 and 13 non-coding bases (U12 and U13) that form the extreme 3′ and 5′ termini, respectively, of each segment are highly conserved amongst both the viral strains and the eight segments themselves. They contain the promoter elements necessary for initiation of transcription and replication and are partially complementary, allowing the vRNAs to fold back on themselves to form a so-called "panhandle" structure [3,4]. The base-paired 5′ and 3′ ends of the vRNAs are recognised by the heterotrimeric viral RNA-dependant RNA polymerase complex consisting of the PB1, PB2 and PA proteins [5], whilst the rest of the vRNA is covered by nucleoprotein (NP) at the ratio of one monomer per 24 nts [6]. Each viral ribonucleoprotein (vRNP), which acts as an independent unit for transcription and replication in the nuclei of infected cells [7], resembles a twisted rod that is folded back and coiled on itself ([8–10] and [11] for a review).

Abbreviations: vRNA, viral RNA; vRNP, viral ribonucleoprotein; nt(s), nucleotide(s); U12, unique 12 nucleotide-long sequence at the 3′ end; U13, 13 nucleotide-long sequence at the 5′ end; NP, nucleoprotein.

* Corresponding author. Tel.: +33 388417040; fax: +33 388602218.
** Corresponding author. Tel.: +33 388417054; fax: +33 388602218.
E-mail addresses: emifou@gmail.com (E. Fournier), vincent.moules@univ-lyon1.fr (V. Moules), JC.Paillart@ibmc-cnrs.unistra.fr (J.-C. Paillart), jean-daniel.sirbat@etu.unistra.fr (J.-D. Sirbat), annie.cavalier@univ-rennes1.fr (A. Cavalier), jean-paul.rolland@univ-rennes1.fr (J.-P. Rolland), daniel.thomas@univ-rennes1.fr (D. Thomas), Bruno.Lina@univ-lyon1.fr (B. Lina), C.Isel@ibmc-cnrs.unistra.fr (C. Isel), R.Marquet@ibmc-cnrs.unistra.fr (R. Marquet).
[1] These two authors equally contributed to this work.

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Replicated vRNPs are subsequently transported into the cytoplasm of infected cells where packaging occurs [7,12]. Although genome segmentation confers evolutionary advantages to the virus, it undoubtedly complicates the packaging process. Indeed, an influenza A viral particle must incorporate at least one copy of each vRNA, each encoding at least one essential gene product, in order to be infectious [13,14]. In a random packaging model, all genomic vRNAs would carry a common packaging signal and would as a whole be discriminated against all the other RNAs. In that case, the mathematical models suggest that the odds of assembling a complete set of eight genomic viral RNAs are around 0.24% [15,16] but such numbers do not account for measured infectivity figures reaching 1–10% [17] in influenza viral stocks. Several lines of evidence now strongly support the existence of a specific packaging mechanism that implies an additional level of discrimination between the eight vRNAs, each of them bearing a unique packaging signal. First, genetic evidence indicated that the influenza viral genome is haploïd [16,18,19] and that eight different vRNA segments must be present for efficient virion formation [20]. Secondly, naturally occurring defective interfering RNAs (DI RNAs) derived from every influenza A vRNA segment have been observed, under high multiplicity of infection, in cell culture or/and in natural influenza infections (for a review, see [21]). These defective RNAs carry large deletions in their open reading frames whilst preserving the 5′ and 3′ termini of their progenitor RNA segments. Importantly, they are capable of specifically competing for packaging with their larger parental vRNAs, suggesting the presence of specific *cis*-acting packaging sequences at both ends of all vRNA segments. The usage of modern biological tools (*i.e.* introduction of directed mutations and reverse genetics) on the laboratory adapted H1N1/WSN and H1N1/PR8 influenza strains further allowed to map the packaging sequences on all eight vRNAs (Fig. 1) [20,22–30]. These segment-specific sequences were usually found to be *bipartite* and located at the 3′ and 5′ ends of the vRNAs, in the UTRs and the extremities of the coding sequences (Fig. 1). Deletions and point mutational analysis, in particular of highly conserved codons that tend to accumulate in the terminal packaging regions (Fig. 1), suggested that within the packaging sequences, the signal is discontinuous and that its strength decreases as the distance from the termini increases [31–34]. The concept of a hierarchical organisation of vRNPs further arose from experiments showing that the loss of incorporation of one segment also affected incorporation of other segments, in *trans* [25,26,32–34]. Finally, the last piece of evidence supporting a specific packaging model came from structural analysis of budding virions. Electron microscopy and tomography data repeatedly revealed the existence of a well organised internal structure, with an array of 7 vRNPs surrounding a central segment [35,36], all closely aligned and suspended perpendicularly from the budding tip of the virion [11,33,37].

Despite growing evidence in favour of segment-specific packaging of influenza A vRNAs, the molecular basis underlying such a mechanism still remain elusive. The electron microscopy and virological data listed above now bring converging evidence to the hypothesis of intersegment RNA/RNA interactions which we tested in the work we describe here. First, we performed electron tomography 3D-reconstitution of the interior of H3N2 influenza A viral particles that highlighted the existence of contacts between vRNPs. We also developed an *in vitro* approach based on a native electrophoretic mobility shift assay that revealed that all genomic vRNA fragments are involved in a single interaction network. Importantly, we also showed that in some defined cases, the regions involved in RNA/RNA base pairings overlap with previously identified packaging signals. Our combined structural and biochemical data support a model in which the eight vRNPs form a supramolecular complex held together by intersegment RNA/RNA interactions.

2. Materials and methods

2.1. Electron tomography

Electron tomography experiments were performed on influenza A viruses produced from Madin-Darby canine kidney (MDCK) cells infected by the A/Moscow/10/99 (H3N2) strain. Viral preparation, image acquisition and analysis have been described in [38].

2.2. Plasmids

cDNAs for expression of the A/Moscow/10/99 (H3N2) influenza vRNAs were cloned under the control of the T7 RNA polymerase promoter, in puc19-based vectors, as described [38]. Ecl136II (for PB2, HA, M and NS vRNAs), Bsh1236I (for PB1, PA and NA vRNAs) and Eco47III (for NP vRNA) restriction sites were introduced downstream of the vRNA sequence for plasmid linearization prior to *in vitro* transcription.

2.3. In vitro transcription and native bandshift assay

DNA templates (300–500 ng) digested with the appropriate restriction enzymes (see above) were transcribed or co-transcribed *in vitro* for 3 h using previously published transcription conditions [38]. The products of the transcription reaction were analysed by 0.8% native agarose gel electrophoresis [38]. The percentage of homo- and hetero-dimers was determined by dividing the weight fraction (%) of the RNA mass for the homo- or hetero-dimer for each band by the sum of the weight fraction of all the RNA species in the lane. For oligonucleotide mapping experiments, a mixture of non-labelled and radio-labelled oligonucleotides complementary to the 5′ or 3′ ends of M vRNA were added to the *in vitro* transcription mixture. After electrophoresis, gels were visualised under UV-light and quantified.

3. Results

3.1. A model for supramolecular assembly of vRNPs inside human H3N2 influenza viral particles

We used electron tomography experiments to visualise the interior of viral particles from the contemporary human influenza A/Moscow/10/99 (H3N2) virus as they are budding from infected MDCK cells. Large fields of view allowed to identify several viral particles that were selected for further analysis. 3D rendering of a portion of such a view, in which the lower part of the viral envelope has been omitted for clarity, revealed the individual vRNPs inside the viral matrix (Fig. 2A). A longitudinal view of a viral particle still attached to the cell clearly shows the vRNPs hanging from the apical tip of the budding virion, where much more density is visible as compared to the opposite end, that only the longest vRNPs reach (Fig. 2B). We also present here five identical transversal sections of three different particles (P1–P3) (Fig. 2C) that reveal that the number of dots, corresponding to individual vRNPs, gradually increases from the bottom to the top of the virions. In section 1 of each particles, four vRNPs are visible, most likely including the four longest vRNAs, PB2, PB1, PA and HA. In section 2, six vRNPs can be distinguished whereas all eight are clearly visible and distinct in section 3. As sections become closer to the apical tip of the virions, the dots become more elongated (section 4) and less distinct. In this so-called “transition zone”, some density appears between the dots which often appear interconnected in a star-like structure that contacts the matrix layer. This data strongly suggest that intermolecular interactions between vNRPs could take place inside this “transition zone”.