FEBS *Letters*

Review

Structure and assembly of t
complex

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ABSTRACT

The genome of influenza A viruses consists of eight segments of single-stranded, negative-sense RNA that are encapsidated as individual rod-shaped ribonucleoprotein complexes (RNPs). Each RNP contains a viral RNA, a viral polymerase and multiple copies of the viral nucleoprotein (NP). Influenza A virus RNPs play important roles during virus infection by directing viral RNA replication and transcription, intracellular transport of the viral RNA, gene reassortment as well as viral genome packaging into progeny particles. As a unique genomic entity, the influenza A virus RNP has been extensively studied since the 1960s. Recently, exciting progress has been made in studying the RNP structure and its assembly, leading to a better understanding of the structural basis of various RNP functions.

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1. Introduction

Influenza A viruses, the causative agents of both epidemic and pandemic flu, are enveloped, single-stranded, negative-sense RNA viruses [1]. The genome of the influenza A virus is segmented into eight RNA molecules, each folded into a rod-shaped, double-helical ribonucleoprotein complex (RNP). Each RNP contains a viral RNA, a heterotrimeric viral polymerase (consisting of PA, PB1, and PB2) and multiple copies of the viral-encoded nucleoprotein (NP) that bind viral RNA in a stoichiometric manner [2–5].

As shown in Fig. 1, the RNPs of the influenza A virus play a crucial role during the virus infection cycle. Influenza A virus replication takes place in the cell nucleus. During infection, influenza A virus enters the host cell by clathrin-mediated endocytosis, and after viral membrane fusion occurs in the endosome, releases viral RNPs into the cytosol. Viral RNPs enter the host nucleus by active transport. In the nucleus, the RNPs from the infecting virus serve as active templates for the synthesis of viral mRNA as well as anti-genomic, complementary RNAs (cRNA). The cRNAs are replication intermediates that direct the synthesis of nascent virion RNAs (vRNAs). Newly translated NP, PB1, PB2 and PA are imported back to the nucleus. Nascent cRNAs and vRNA are both encapsidated into RNP structures but viral mRNAs are not. Two other

influenza virus proteins, M1 and NEP, facilitate RNP nuclear export. In the cytosol, influenza virus RNPs are transported to the cytoplasmic membrane where they are selectively packaged into budding virions.

As a complex genomic entity with unique structure and function, the influenza A virus RNP has been a subject of extensive study since the 1960s. It is clear that the structure of the RNP directly impacts our understanding of influenza virus biology, including RNA replication and transcription; intracellular trafficking of the viral genome; selective packaging of the vRNPs; and gene reassortment, etc. Recently the influenza virus field has witnessed exciting progresses from studies of the RNP, and a coherent model for RNP structure and assembly is now emerging, as discussed below.

2. Overall structure and properties of the RNP

RNPs purified from virions have been examined by electron microscopy (EM) in great detail [2,6]. Back in the 60s and 70s, it was found that isolated RNPs were rod-shaped structures that were about 10 nm in width. Statistically, purified RNPs could be categorized into three length groups: 90–110 nm, 60–90 nm, and 30–50 nm. Considering that RNPs have a uniform diameter, the length of an RNP likely correlates with the size of its associating vRNA. The rod-shaped RNPs are structurally flexible and appeared to adopt a right-handed, double-helical structure in negative-staining EM

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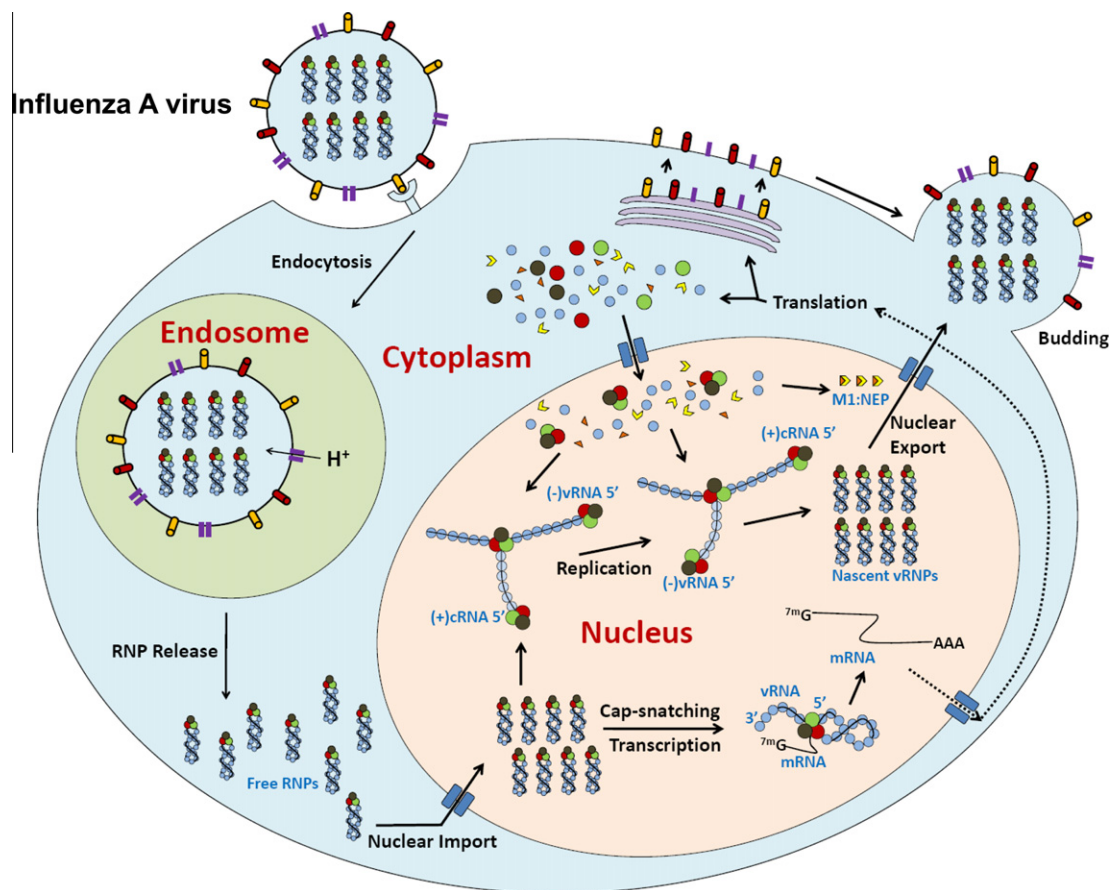
Fig. 1. The influenza A virus life cycle. The RNPs are represented by helical hairpins, with the polymerase subunits (red, brown, and green) and NP (cyan) shown in different colors. In the nucleus, the viral transcription and replication processes are depicted according to model proposed by Jorba et al. [62]. The figure is modified from Das et al. [43].

[2]. Immuno-EM indicated that the viral polymerase complex was bound at one end of the helical rod [7]. Interestingly, RNPs were able to maintain an intact structure even in the absence of vRNA, suggesting that NP plays a major role in the overall structural organization and stabilization of the RNP [5].

Over the years various biochemical and biophysical techniques have been used to probe the structure of the influenza RNP. The 5′ and 3′ end of each vRNA contain partially complementary sequences that are 12–13 nts in length [8,9]. These sequences, which are highly conserved among the eight viral gene segments, provide specific binding sites for the influenza heterotrimeric polymerase complex [10–16]. The influenza A virus RNPs unwind under high/low salt conditions, giving rise to closed circular structures [9]. Chemical probing experiments indicate that vRNA binds to NP with its phosphate backbone and that the nucleotide bases are exposed to solvent [17]. vRNAs associated with RNPs can be displaced by polyvinylsufate (PVS), a negatively charged polymer [18]. Furthermore, the vRNAs in the influenza virus RNPs were readily digested by RNase treatment [19], suggesting that very little protection was provided by the bound NP. These findings indicate that the RNPs of the influenza A virus adopt a unique structure compared to the nucleocapsids from the non-segmented, negative-sense RNA viruses.

3. Structures of the RNP protein components

Due to its inherent structural flexibility, high resolution structural analysis of intact RNPs is challenging. Nevertheless, X-ray structures of some of the RNP protein components have become available in recent years: NP and a number of protein fragments

derived from the viral polymerase complex. The influenza A virus NP is a multifunctional protein that has been shown to interact with a number of viral (e.g. PB1, PB2, M1, etc.) and host proteins (e.g. RAF-2p48/UAP56 and Tat-SF1, etc.) [20]. One of the NP's primary functions is to coat viral RNA to facilitate its folding into a double-helical RNP structure. To date, crystal structures of three influenza virus NPs are available [21–23], all forming ring structures in the absence of RNA. Although their oligomers vary in size, all three NP proteins assume the overall shape of a crescent with a head and a body domain (Fig. 2a). In between the two domains is a deep groove enriched for basic amino acid residues and thus may function as the RNA-binding site. It has been shown the mutation of several arginine residues from the two flexible loops within the groove resulted in dramatic reduction of the RNA binding affinity of influenza A virus NP [22]. Oligomerization of the NP is mediated by an extended tail-loop structure (*i.e.* aa402–428 in the influenza A virus NP) located at the back of each NP molecule [21,22]. Loss of NP oligomerization due to amino acid substitutions in the tail loop resulted in NP mutants unable to support viral gene expression in mini-genome assays [24]. Drug compounds that promote aberrant NP aggregation can effectively inhibit influenza A virus replication in cell cultures, suggesting that NP is a valid target for anti-influenza therapy [25,26].

The influenza virus polymerase is a heterotrimeric complex consisting of PA, PB1 and PB2, with multiple enzymatic and ligand binding activities that allow the synthesis of capped, poly-adenylated mRNAs during transcription as well as full-length genomic/anti-genomic RNAs during replication [1]. The known X-ray structures of the influenza A virus polymerase include: the 25 kD N-terminal PA domain which displays the endonuclease activity needed
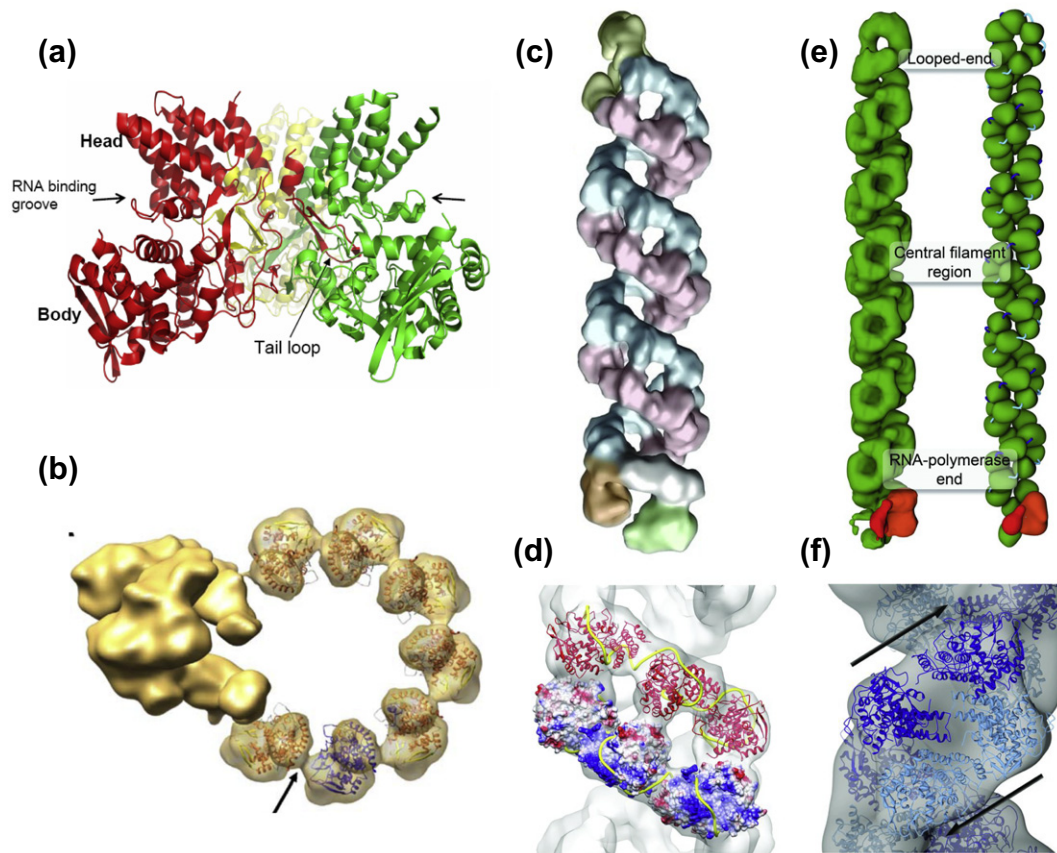
Fig. 2. The influenza A virus RNP structure. (a) Crystal structure of the influenza A virus NP. The three subunits of the NP trimer are colored differently [21]. (b) Cryo-EM reconstruction of the mini-RNP [48,50]. The NP crystal structure from (a) is fitted into the electron density. The arrow points to adjoining densities that are presumably made of vRNA and the NP oligomerization tail loop. (c) Cryo-EM reconstruction of a double-helical RNP by Arranz et al. [54]. The viral polymerase complex is located at the bottom end of the RNP and is shown in green and orange. The two opposite-running NP-RNA strands are colored differently in blue and pink. The NP-RNA turning loop on the top end of the RNP is highlighted in dark green. (d) Helical stem of the RNP from (c) fitted with NP crystal structure and modeled with RNA (in yellow). (e) Cryo-EM reconstruction of a RNP by Moeller et al. [55]. On the right is a model showing the RNP organization. The viral polymerase is highlighted in red. (f) Central filament region from (e) fitted with NP crystal structure protomers. Arrows indicate RNA polarity. (b), (c-d) and (e-f) are taken/modified with permission from Coloma et al., Arranz et al., and Moeller et al., respectively [50,54,55].

for cap-snatching [27,28], the 55 kD C-terminal PA domain that mediates the PA-PB1 interaction [29,30], the PB2 aa318–483 domain that binds to the 5′ pre-mRNA cap [31], the PB2 aa538–676 domain involved in host adaptation [32], and the PB2 C-terminal NLS-domain (aa686–757) that binds cellular importin [33]. PB1, the largest subunit of the polymerase, hosts the polymerase catalytic active site [34–36] as well as specific binding sites for the conserved 5′ and 3′-vRNA termini [37]. To date, the only known structures of PB1 are the N-terminal fragment (aa1–25) [38,39] which interacts with PA and the C-terminal fragment which forms a three helical bundle (aa678–757) interacting with the N-terminus of PB2 [40]. The details of these structures can be found in reviews elsewhere [41–43].

The crystal structures of the influenza A virus polymerase domains/fragments allow structure-based design and optimization of new antiviral compounds. For instance, a recent structural study showed that several known endonuclease inhibitors, including four diketo compounds and a green tea catechin, bind to the endonuclease active site of the PA protein [44]. All these inhibitors chelate the two critical manganese ions in the active site of the enzyme, although some differences are noted in the overall ligand ordination of these compounds. Further optimization of such endonuclease inhibitors may lead to potent drugs targeting the cap-snatching endonuclease activity of influenza virus polymerase. Another promising approach to inhibit the influenza A virus replication is

to disrupt the assembly of the viral heterotrimeric polymerase complex. It has been shown that short peptides derived from the N-termini of PB1 and PB2, which target the PA-PB1 and PB1-PB2 interaction interface respectively, exhibited varying levels of effectiveness in blocking the viral polymerase activity and growth of the virus [45,46].

4. RNP structure

The first three-dimensional structure of the influenza A virus RNP that came to light is that of an artificial mini-RNP [47–50] (Fig. 2b). To circumvent the structural flexibility problem, a mini-RNP was generated from in vivo amplification by expressing the three polymerase subunits, NP, and a 248 nt model vRNA containing the highly conserved terminal sequences [48]. The mini-RNP is more structurally rigid compared to the native RNPs, thus allowing cryo-EM reconstruction to ~12 Å resolution. Cryo-EM reconstruction of the mini-RNP shows a closed ring structure consisting of nine NP molecules, with a copy of the viral polymerase attached to the outer edge of the otherwise symmetric ring [50]. The viral polymerase adopts a compact shape and simultaneously interacts with two adjacent NP molecules [50]. Each NP molecule shows a two-domain morphology that agrees with previously determined NP crystal structures [21,22]. vRNA cannot be readily discerned at this resolution, but it presumably constitutes some of the

adjoining densities that connect neighboring NP molecules. Due to the limited resolution, the boundaries between the three polymerase subunits were not obvious. Using engineered fusion tags and monoclonal antibodies, Area et al. was able to map the rough location of PA, PB1, and PB2 in the polymerase complex [49]. It was found that the polymerase contacts with the two NP monomers via the PB1 and PB2 subunits [48,50], consistent with previous biochemical studies [51,52]. The RNP-associated polymerase shows similarities in overall structure compared to the EM reconstruction of a free polymerase [49,53], but it is also clear that some conformational changes have taken place upon the interaction with NP and/or the vRNA template.

In an exciting development, cryo-EM reconstructions of authentic RNPs were reported by two research groups late last year (Fig. 2c–f) [54,55]. The RNP reconstruction reported by Moeller et al. used RNPs generated by in vitro expression of the four RNP proteins (i.e. PA, PB1, PB2 and NP) via transient transfection of a human cell line in the presence of their respective vRNA segments [55]. At ∼20 Å resolution, the final model confirms that the RNP adopts a double helical structure with two anti-parallel strand leading to and away from the polymerase that is located at one end of the RNP (Fig. 2c, d). The double-helical stem region shows a rise between two neighboring NP of 32.6 Å with 4.9 NP molecules per turn. The other cryo-reconstruction of the influenza A virus RNP was reported by Arranz et al., using native RNPs purified from virions [54]. The structure also shows a double-helical stem with major and minor grooves (Fig. 2e, f). The rise step between adjacent NPs is 28.4 Å with a rotational angle of 60° and six NPs per turn on each strand. In both RNP structures, the putative RNA binding groove of the NP scaffold is exposed on the outer surface of the RNP. Assuming that the positively charged groove of NP serves as the RNA binding site, a vRNA was built into the final model. By following a convoluted path, ∼120–150 nucleotides of RNA are placed in each helical turn.

To help better understand the structural arrangement of the influenza A virus RNP, it is useful to draw an analogy with the double-helical DNA duplex. Similar to the DNA duplex, the RNP possesses two types of surface grooves: a major groove that is well separated, and a minor groove that is maintained by interactions between NP molecules associated with opposing RNA strands. While cohesion between the two anti-parallel strands in a DNA duplex is maintained by base pairing, the interaction between the two opposing arms of an RNP hairpin is solely mediated by the NP. Interactions between adjacent NP molecules on the same RNA strand are facilitated by the extended tail loop. The double-helical RNP is ∼15 nm wide and ∼65 nm long for the second smallest gene segment (∼1000 nts long) of the influenza A virus [54].

It is also important to note that the two models by Moeller et al. and Arranz et al. exhibit significant variations in helical parameters and NP orientations. Using crystal structure docking, Arranz et al. proposes that the NP molecules from opposing strands contact each other through their body domains at a region near the disordered N-terminus of the NP structure. Moeller et al., however, suggests that the RNP helix is stabilized by the NP-RNA strand interacting with the opposing strand near the NP head domains. The most likely cause of the model difference is likely due to the different handedness of the two reconstructions, with Arranz et al. showing a left-handed helix and Moeller et al. showing a right-handed helix (Fig. 2d, f). It is expected that the modest resolution, rotational freedom of the NP molecules, and the source of RNP samples (viral particles vs. cells) may also contribute to some inter-model variations as well. It is worth mentioning that Ye et al. recently reported a NP dimer crystal structure with a dimer interface that does not involve the tail loop [24]. Mutational analysis indicated that the dimer interface is biologically relevant, suggesting a possible role in RNP assembly. Comparing the NP

dimer structure with the RNP reconstructions may help to interpret interactions made between the two opposing NP-RNA strands.

The two cryoEM reconstructions of the RNP also offer a new look at the viral polymerase. Both Arranz et al. and Moeller et al. located the viral polymerase at the open end of the RNP hairpin, simultaneously interacting with both the 5′ and 3′-ends of the vRNA [54,55]. The closed end of the RNP hairpin contains a small loop formed by a curved array of three to eight NP molecules. It was proposed by Moeller et al. that the PA C-terminal domain is structurally flexible and may help to feed the vRNA template into the polymerase active site, based on structural homology between the PA C-terminal domain and the N-terminal domain of the reovirus RNA polymerase [38,55,56]. Arranz et al. observed that the RNP-associated polymerase samples two alternative conformations, but higher resolution structural information is needed to address the biological relevance of this distribution and the possible implications.

5. RNP replication and transcription

The polymerase complex of the influenza A virus is the core machinery for viral RNA replication and transcription [1,57]. Viral RNA replication is primer independent, but transcription initiation requires short, 10–13 nts long, capped RNA fragments snatched from host pre-mRNAs. The termination process for viral RNA replication and transcription is also different. Viral RNA transcription prematurely terminates at a polyU tract ∼25 nts away from the end of the viral template, a which point the polymerase engages in repetitive copying of the polyU sequence for the synthesis of a polyA tail. In contrast, during replication the viral polymerase is able to read through the polyU, resulting in a faithful copy of the entire template.

The new RNP structures provide insights into our understanding of influenza virus transcription and replication [54,55]. As the RNP structure is predominantly maintained by NP and the bound RNA is fully solvent exposed, the viral polymerase moving along the RNA template during viral RNA synthesis should result in little or only local disruption in the double-helical hairpin structure. During infection, viral mRNAs were detected immediately, but cRNAs were detectable only after viral protein synthesis started [58]. Viral RNA replication requires soluble NPs for the elongation of nascent RNA chains, as a polymerase-RNA complex can only synthesize small-sized RNAs if NP is not present [59–61]. Several models, including the template modification model, the polymerase modification model and the stabilization model, have been proposed to explain NP's role in replication and how the transcription and replication activities of the viral polymerase appear to occur during different phases of virus infection [41]. These models, which are not necessarily mutually exclusive, entail NP binding to viral polymerase, viral template RNA and product RNA, respectively.

Recently, it has been demonstrated that the polymerase molecules catalyze transcription and replication are of different origins [62]. By performing an in vivo complementation assay using mutated polymerase defective in either replication or transcription, Jorba et al. proposed a model in which the transcription takes place in cis – via the same polymerase that is part of the RNP; whereas the replication occurs in trans – with exogenous polymerases synthesizing the RNA and mediating the assembly of progeny RNP [62]. According to the model, viral replication starts with the binding of a second polymerase to the polymerase on the template, and this binding would allow the second polymerase to access the 3′-end of the template RNA to initiate RNA synthesis. As the new RNA is synthesized, a third polymerase, or a second exogenous polymerase, binds to and protects the 5′-end of the new RNA, and possibly recruits NPs for the new RNP assembly. However, contradictory evidence by Vreede et al. showed that cRNA can

accumulate in the beginning of infection if NP and a catalytically-inactive polymerase are provided to protect the RNA from degradation, suggesting that replication can also occur in cis [63]. Therefore, more evidence is needed to elucidate the mechanism for influenza viruses to replicate their genome.

6. RNP assembly in vivo

Newly synthesized NPs are imported back into the host cell nucleus to promote viral RNA replication and RNP assembly [60,64]. As NP binds RNA non-specifically and has a strong tendency to self-polymerize, it is important to keep NP in a soluble, encapsidation-competent state prior to RNP assembly. Unlike non-segmented, negative-sense RNA viruses, the influenza A virus does not encode viral proteins that are known to interact with NP and prevent its self-oligomerization. It has been proposed that phosphorylation may play an important role in regulating the self-polymerization and RNA binding activities of the influenza A virus NP [65–67]. Additionally, a number of host factors have been found to be important for influenza virus replication and RNP assembly [68–72]. For example, RAF-2p48/UAP56 and Tat-SF1 assist the formation of the vRNA-NP complex, possibly by functioning as chaperones to suppress the non-specific aggregation of NP [69,72]. Interestingly, Ye et al. recently reported that the self-oligomerization activity of NP is weak in the absence of RNA, but the interaction is kinetically stable once NP oligomerizes [24]. Therefore, it is possible that NPs remains monomeric until they encounter v/c RNAs in the cell nucleus.

RNP assembly in host cells requires only four viral proteins: NP, PA, PB1 and PB2. As shown by Moeller et al., in vivo amplification of vRNAs with only these four proteins co-expressed in transfected cells produced rod-shaped RNPs with a regular helical symmetry [55]. Therefore, although M1 and NEP are needed for RNP export from the nucleus (see below), they do not play major roles in either the assembly or structural maintenance of the RNP. This feature is again different from some of the non-segmented, negative-strand RNA viruses (i.e. rhabdoviruses), in which the matrix protein plays a major role in organizing the helical nucleocapsids by simultaneously interacting with N proteins from two adjacent helical turns [73].

It is likely that NP initiates viral RNA replication via interaction with the polymerase (possibly PB1 or PB2), during which process the RNA-binding ability of NP is not required [51,52,74]. Therefore, the NP-polymerase interaction should facilitate the initial recruitment of NPs to newly synthesized vRNAs. Further vRNA encapsidation is likely stimulated by cooperative NP-RNA interactions [66]. Tarus et al. reported that in vitro NP oligomerization is a slow process that depends on the RNA length, with the oligomerization rate increasing drastically as the RNA length increases [75]. On average, each NP is associated with ~22–28 nts of RNA in the RNPs [47,48]. The viral polymerase is significant in maintaining the supercoiled RNP structure, since single-stranded RNA was observed when the polymerase was removed from the RNP [9].

It is unclear when the polymerase-NP-RNA complex collapses into the supercoiled, double helical RNP structure during replication. The fact that mini-RNPs consisting of nine NP molecules only form circularly shaped rings suggests that the condensation of NP-RNA polymers into double-helical structures does not occur until nascent RNPs reach certain sizes. Moeller et al. reported the observation of "branched" RNPs and suggested that the branches are made of partially replicated, nascent RNPs budding from the full-length RNP templates [55]. Using immuno-labeling, they showed that a second copy of the polymerase is located at the branching site in some RNPs. The observation of a second polymerase molecule is consistent with the notion that template RNPs are replicated in trans by free polymerase complexes and not by the polymerase

molecule bound to the parental RNP [62]. Although "budding" RNPs sounds like an attractive interpretation for these branched RNP structures, whether they are truly replication intermediates or perhaps misfolded RNPs still awaits further verification. One potential concern is that the length of the budding RNPs does not seem to correlate with the location of the RNP branches [55].

7. RNP assembly in vitro

In the late 1980s, it was reported that in vitro transcribed vRNAs, when mixed with NP and polymerase purified from infectious particles, could be replicated in vitro to produce full-length RNA copies [76,77]. Palese and colleagues showed that when these in vitro encapsidated vRNAs were introduced into permissive cells coinfected with a helper virus, they could be amplified, expressed, and packaged into progeny virions, thus leading to the development of the first-generation influenza A virus reverse genetics [13,78]. For influenza viruses and other negative-stranded RNA viruses, free vRNAs or cRNAs are not infectious. The observation that those reconstituted RNPs were replicated in vitro and amplified in vivo indicates that some RNP-like structures might have formed. Additionally, attempts to assemble RNP in vitro by mixing RNA and recombinant NP have also been reported [66,79]. However, these artificial RNPs may be a poor model to study native RNP as they do not seem to have the typical rod-shaped morphology [66,79]. It would be interesting to find out whether the addition of both recombinant polymerase and NP to in vitro transcribed vRNA would produce RNPs that closely resemble those from infectious particles.

8. Nuclear import and export of RNPs

At the onset of infection, RNPs released from the infecting influenza A virus are actively transported from cytosol into nucleus. It is not clear whether all eight RNPs are imported to the nucleus as a large bundle, or if they are separately imported as individual RNPs. All RNP component proteins contain at least one nuclear localization signal (NLS) necessary for nuclear import. Two regions of the PA protein, residues 124–139, and 186–247, were found to contain NLSs [80]. For PB1, a NLS was first found between residues 187–211 [81], but later findings showed that the co-expression of PA was important for the efficient nuclear import of PB1 [82]. PB2 has a linear NLS with a sequence of ^{736}KRKR^{739} that was shown to interact with importin-α in a co-crystal structure [33,83]. Two NLSs have been identified in the NP sequence. One of these was a classical bipartite NLS that was found between residue 198 and 216 with a sequence of ^{198}RX$_{13}$RKTR^{216} [84]. A non-conventional NLS (nNLS) with a consensus sequence of ^{3}SQGTKRSYXXM^{13} was also identified at the N-terminus of NP [85–87]. Although all component proteins of the RNP carry their own NLSs, NP is the major contributor for RNP import [85,86,88]. By dot blotting and immunogold labeling of vRNPs, Wu et al. showed that the nNLS of the NP was much more accessible than the classical bipartite NLS, and that the labeled gold particles showed a regular periodicity which suggested a regular helical conformation of the RNP [89]. In addition, Cros et al. reported that mutations in the nNLS completely abolished NP import, and that short peptides mimicking the nNLS competitively inhibited the nuclear import of the RNP [90]. These findings are consistent with the crystal structure of NP [21,22], as the nNLS is solvent exposed, structurally disordered, and can be easily fitted into the substrate binding pocket of importin-α.

Because influenza A virus assembly occurs at the host cell membrane, newly synthesized RNPs need to be exported out of the nucleus, thus traveling in the opposite direction compared to their parental RNPs. No nuclear export signal (NES) has been found in
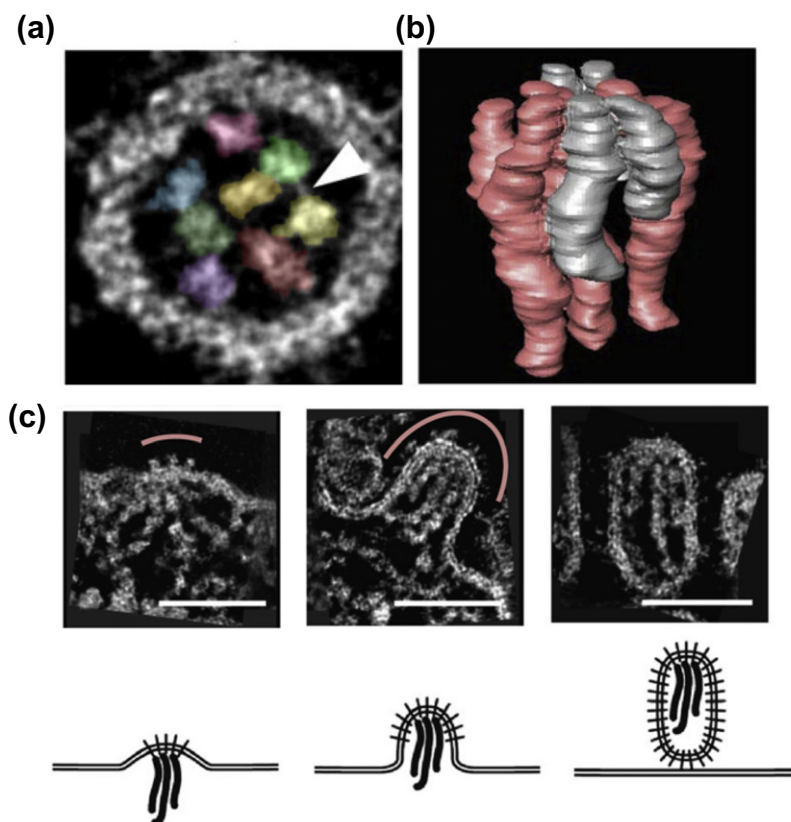
Fig. 3. Specific packaging of the influenza A virus RNP. (a) Linkages among the eight RNPs in a virion [110]. A 0.5 nm-think tomogram is shown with the eight RNPs highlighted in different colors. Short string-like structures can be seen between the RNPs (arrowheads). (b) 3-D model of a multi-segment RNP complex [110]. The four long RNPs are shown in red, while the four shorter ones are shown in grey. (c) Budding (left, middle) and mature (right) virions [110]. Red curves in the left and middle columns indicate the membrane region where viral spike proteins are present. Shown below are schematic diagrams of the RNP packaging process, Scale bar, 100 nm. (a–c) are taken from Noda et al. with permission [110].

the component proteins of the RNP. For RNP export, two other influenza proteins, M1 and NEP, are required [91–93]. The C-terminal domain of M1 is responsible for interaction with RNP [94], and M1-binding to the RNP likely helps to mask the NLSs on the RNP. Meanwhile, M1 can directly interact with the viral protein NEP which possesses a NES signal [91]. The RNP-M1-NEP complex is recognized by the chromosome region maintenance 1 (CRM1) protein, which mediates the nuclear export of NES-containing protein/complexes from the nucleus [95]. The nuclear export of RNP also requires viral activation of the cellular Raf/MEK/ERK (mitogen-activated protein kinase (MAPK)) signaling cascade that is activated late in the infection cycle, as block of the cascade resulted in retardation of RNP export and reduced titers of progeny virus [96]. It has been shown that membrane accumulation of the influenza A virus hemagglutinin triggers activation of the MAPK cascade and induces RNP export. This may represent an auto-regulative mechanism that coordinates the timing of RNP export with virus budding [97].

9. Specific RNP packaging

Early evidence of selective packaging came from the defective-interfering influenza RNAs (DI RNAs) [98,99]. DI RNAs were shown to interfere with the incorporation of some specific gene segments while sparing others, suggesting that each segment contains a unique packaging signal. Later, further evidence was provided by reverse genetics, which revealed that all eight gene segments possess such packaging signals for efficient virion incorporation [100–108]. EM studies have also provided strong evidence for selective packaging (Fig. 3). The observation of the distinctive "7 + 1" pattern of the eight RNPs suggests that specific inter-RNP interactions maintain such a conformation [109] (Fig. 3a). Recent evidence by electron tomography showed that the RNPs of the "7 + 1" bundle are actually different, with four longer RNPs and four shorter RNPs of significantly different lengths, consistent with the length distribution of the eight influenza RNA segments [110,111] (Fig. 3b). Electron tomography studies also revealed that the eight RNPs are aligned at the budding tip and interconnect with each other to form a supra-molecular assembly (Fig. 3c). With fluorescence in situ hybridization (FISH) analysis at the single-virus particle level, Chou et al. confirmed that the eight unique RNPs are incorporated into progeny virions by a selective packaging mechanism [112]. Co-localization tests demonstrated that most of the virus particles have incorporated at least one of the eight RNPs. The exact copy number of each RNP was determined by comparing the photo-bleaching profiles of probes against the HA RNA segment (i.e. the RNA segment encoding the haemagglutinin protein) of the wild-type and a recombinant virus carrying two copies of the HA segments. Their results demonstrated that most virus particles contain only one copy of each of the eight RNP complexes.

The sequence-specific signals for influenza genomic packaging have been discovered on each of the eight genomic RNAs [108]. The "signal regions" cover the untranslated regions (UTRs) of both termini as well as the adjacent coding sequences of the open reading frame (ORF). Many approaches have been employed to map the regions containing the packaging signals. The earliest results were obtained from experiments on DI RNAs [4,98,113–117], which are shorter RNAs derived from the wild-type RNAs with certain

region(s) deleted, while still maintaining the abilities to be replicated and packaged. Examination of these DI RNAs showed that the UTRs as well as the terminal coding sequences are well preserved in the DI RNAs, suggesting a significant role in genomics packaging. Later, reverse genetics experiments confirmed that all eight influenza vRNAs have bipartite packaging signals located at the 5′ and 3′ termini [100,102–105,118,119]. It was demonstrated that the UTRs together with the terminal coding regions can significantly enhance the packaging efficiency than the UTRs alone, suggesting that the coding sequences also contribute to genomic packaging [100]. In addition, codons at the terminal coding regions were found to have synonymous variation rates significantly lower than expected, indicating that the RNA primary sequence is important and thus selectively preserved [120]. Indeed, synonymous nucleotide mutations within the packaging signal regions produce recombinant viruses with reduced replication efficiencies [102,106,107,119–122].

The localization of the packaging signals near the 5′ and 3′ termini of the vRNAs suggest that these RNA sequences should be mapped to the double-helical RNPs near the end where the viral polymerase is located. Using electron tomography 3D-reconstructions, Fournier et al. show that the eight vRNPs contact each other at the budding tip of the influenza A virus particles [111]. This contact region is thick enough to accommodate all described packaging signals. They also demonstrated that in vitro all vRNAs are involved in a single interaction network, with each vRNA segment interacting with at least one other vRNA partner. Fournier et al. thus suggests that the RNPs are likely held together by direct base-pairings between packaging signals. This model raises an intriguing question as to how the packaging signals are presented on the surface of the RNPs. Is vRNA completely denatured by tight wrapping around the NP scaffold, or perhaps some NP-free RNAs exist in the RNP to allow vRNA-vRNA interactions? If there is no NP-free RNA in the RNP, is vRNA able to temporarily dissociate from the NP scaffold during selective RNP packaging? Although packaging signals are identified in all eight vRNAs, much still needs to be learned about the molecular details of their interaction.

10. Summary

Although much has been learned about the structure and function of the influenza A virus RNP in recent years, many long-standing questions remain unanswered. For instance, does the polymerase move along the helical structure of RNP during viral RNA transcription or remain fixed at the terminal end of the RNP with the RNA template threading into the active site during RNA synthesis (polymerase moving vs. RNA moving)? How are nascent RNPs synthesized from the parental RNPs and when do newly synthesized RNPs adopt the double-helical morphology during viral RNA replication? How does RNP interact with M1 and NEP to mediate the nuclear export of RNP? Does NP plays any direct roles in specific RNP recognition? Is RNP-associated RNA completely denatured or retain secondary structures within the RNP. It is anticipated that the influenza virus RNP will remain an active area of research for years to come, and hopefully our improved understanding of RNP structure and function will translate to better methods for influenza prevention and treatment in the near future.

Acknowledgements

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