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Review

A structural understanding of influenza virus genome replication

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Influenza virus contains a single-stranded negative-sense RNA genome. Replication of the genome is carried out by the viral RNA-dependent RNA polymerase in the context of the viral ribonucleoprotein (RNP) complex, through a positive-sense complementary RNA intermediate. Genome replication is tightly controlled through interactions with accessory viral and host factors. Propelled by developments in recombinant protein expression, and technical improvements in X-ray crystallography and cryo-electron microscopy, snapshots of the replication process have been captured. Here, we review how recent structural data shed light on the molecular mechanisms of influenza virus genome replication, in particular, encapsidation of nascent RNA, *de novo* RNP assembly, and regulation of replication initiation through interactions with host and viral cues.

Highlights

Influenza virus RNA genome replication is carried out by a dimeric form of the influenza virus RNA polymerase with one of the polymerases acting as a replicating polymerase, synthesizing product RNA, and the other acting as an encapsidating polymerase, capturing the nascent RNA product and initiating its assembly into a ribonucleoprotein (RNP) complex.

Proteins of the acidic nuclear phosphoprotein 32 (ANP32) family, essential host factors for influenza virus RNA genome replication, bridge the replicating and encapsidating polymerase.

The disordered highly acidic C-terminal tail of ANP32 proteins acts as a platform for binding viral nucleoprotein, increasing nucleoprotein density around the site of RNA genome replication, and thus facilitating its recruitment to nascent viral RNA.

Using a dimeric form of the viral RNA polymerase to replicate viral genomic RNA could be a general feature of negative-sense RNA viruses.

Influenza virus RNA polymerase

Influenza viruses belong to the family Orthomyxoviridae and are divided into four types: A, B, C, and D viruses [1]. Influenza A and B viruses are causal agents of seasonal epidemics in humans, and influenza A virus can lead to pandemics when a zoonotic strain infects humans. Influenza C virus causes rare mild illness in humans, while influenza D virus primarily infects livestock [2]. Despite the differences in host range and virulence, all influenza viral types undergo a similar life cycle (Figure 1A).

Influenza viruses contain a segmented negative-sense single-stranded RNA genome, with each RNA segment packaged into a viral ribonucleoprotein (vRNP) complex. Within a vRNP, a single RNA-dependent RNA polymerase molecule captures the 3′ and 5′ termini of the viral RNA (vRNA), forming a pseudo-circularised piece of RNA [3]. The rest of vRNA associates with nucleoprotein (NP), with an estimated ratio of 24 nucleotides per one NP [4,5]. NP oligomerises, forming a helical scaffold that packages and protects the viral genome [6–10]. During infection, the virus binds to a cell-surface receptor and enters the host cell via endocytosis. Subsequently, vRNPs are released into the cytoplasm and are trafficked to the nucleus (Figure 1A). Once inside the nucleus the viral polymerase transcribes and replicates the viral genome [11,12].

The influenza virus RNA polymerase is a heterotrimer formed from three individual polypeptides, polymerase basic 1 (PB1), polymerase basic 2 (PB2), and polymerase acidic (PA) (or P3 in the case of influenza C and D viruses) proteins [12] (Figure 1B,C). Complete structures in various conformations and in complex with different RNA/cofactors have been solved for influenza A, B, and C viruses, and an almost complete structure has been reported for the influenza D virus polymerase [13–22]. The polymerase contains a globular core and several discrete flexible domains which rearrange as the polymerase performs its various functions. The polymerase core is composed of the PB1 subunit, the C-terminal domain of the PA/P3 subunit (PA/P3-C), and the N-terminal domain of the PB2 subunit (PB2-N). The PB1 subunit has a canonical right-hand shape and contains subdomains called the fingers ($PB1^{Fingers}$), palm ($PB1^{Palm}$) and thumb ($PB1^{Thumb}$).

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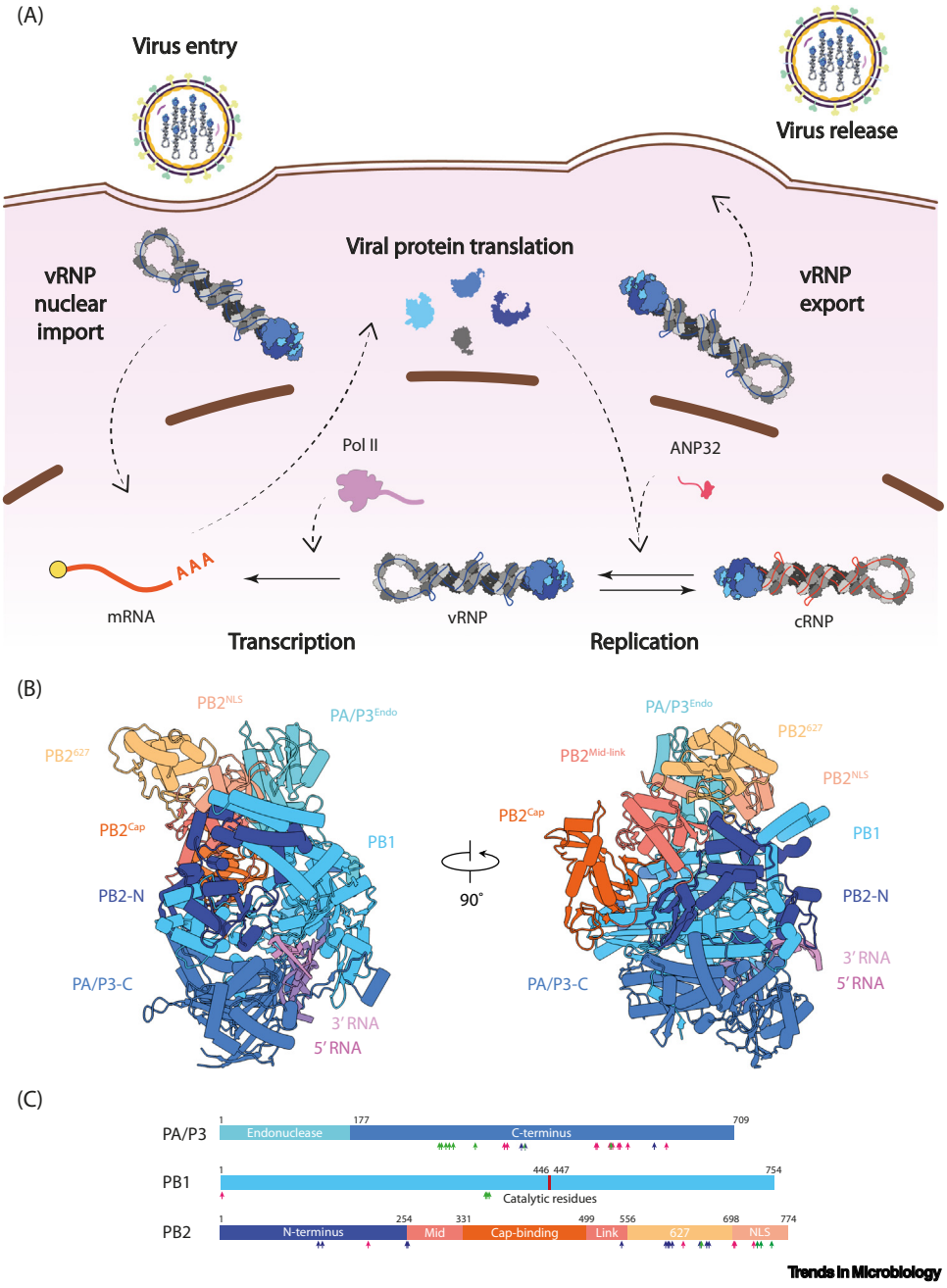

Figure 1. Influenza virus life cycle and structure of the influenza virus RNA polymerase. (A) Life cycle of the influenza virus with processes involving the viral polymerase highlighted. (B) Cartoon diagram showing the influenza C virus RNA polymerase in complex with vRNA promoter in a replicating conformation, derived from an acidic nuclear phosphoprotein 32A (ANP32A)-bound structure (PDB ID 6XZR). Subunits and domains of interest are shown in different colours. (C) Schematic of the polymerase subunits. Major domains are highlighted in the same colours as in (B). The PB1 catalytic aspartates, residues 446 and 447, are highlighted in red. Arrows beneath the schematic indicate key interaction sites with viral polymerase and host ANP32A. Blue, residues on a replicating polymerase that interact with an encapsidating polymerase; green, residues on an encapsidating polymerase that interact with a replicating polymerase; pink, residues on a polymerase that interact with host ANP32A. Abbreviations: cRNP, complementary ribonucleoprotein; vRNP, viral ribonucleoprotein.

Glossary

Acidic nuclear phosphoprotein 32 (ANP32): a protein family involved in the regulation of many cellular processes, including tumour suppression, apoptosis, cell-cycle progression, and transcription, as well as influenza virus RNA genome replication. Species-specific differences in ANP32 proteins are responsible for the poor replication of avian influenza viruses in mammalian hosts.

Co-replicative assembly: assembly of nascent RNA into a ribonucleoprotein complex with polymerase and nucleoprotein.

Encapsidating polymerase: a polymerase that captures the 5′ terminus of nascent RNA produced by the replicating polymerase initiating the assembly of nascent RNA into a ribonucleoprotein complex.

Internal initiation: *de novo* synthesis of ApG dinucleotide opposite the fourth and fifth 3′ nucleotides on a cRNA template during replication initiation.

Priming loop: a structural feature of polymerases that protrudes into the active site and coordinates the positioning of template and initiating nucleotides.

Replicating polymerase: a polymerase that is bound to template RNA in the context of a ribonucleoprotein complex and is directly involved in synthesising an RNA.

Replication platform: a multisubunit protein complex involved in the initiation of viral RNA genome replication and coordinating ribonucleoprotein assembly, consisting of a replicating polymerase, an encapsidating polymerase, and a host ANP32 in the case of influenza virus.

Terminal initiation: *de novo* synthesis of ApG dinucleotide opposite the first and second 3′ nucleotides on a vRNA template during replication initiation.

Transactivating polymerase: a polymerase that interacts with the cRNA-bound replicating polymerase to trigger a conformational rearrangement in the replicating polymerase leading to template realignment in the polymerase active site.

Together, PB1 and PB2-N form an easily identifiable central cavity in the polymerase, containing the catalytic active site, and acting as a hub where nucleotide triphosphates (NTPs) and the template enter, and the template and the product exit. The flexible domains are the N-terminal endonuclease domain of PA/P3 (PA/P3^{Endo}) and the C-terminal two-thirds of PB2 (PB2-C), including the mid domain (PB2^{Mid}), the cap-binding domain (PB2^{Cap}), the 627-linker (PB2^{Link}), the 627 domain (PB2^{627}), and the nuclear localization signal (NLS) domain (PB2^{NLS}). In most structures solved, the PB2^{Mid} and PB2^{Link} form a single rigid domain referred to as mid-link (PB2$^{Mid\text{-}link}$). The position of the flexible domains around the polymerase core have been linked to different functional states of the polymerase. Several unique positions of the flexible domains have been observed, highlighting the flexibility of the polymerase which underpins its multifunctional nature.

The vRNP-associated polymerase can perform transcription and replication. In transcription, viral messenger RNA (mRNA) coding for viral proteins is produced. Transcription is primed by capped oligonucleotides obtained from nascent host capped RNAs in a process called cap-snatching, and terminates when the polymerase stutters on a short track of uridines near the 5′ terminus of each vRNA segment, adding a poly(A) tail [21,23]. Having a 5′ N7-methyl guanosine (m7G) cap and a 3′ poly(A) tail, viral mRNAs resemble host mRNAs. Transcription occurs early during infection and is in close physical association with the host transcriptional machinery – host RNA polymerase II [24,25]. Only after transcription, and production of sufficient levels of viral proteins, can replication proceed [26–28].

In replication, the negative-sense vRNA in the vRNP is used by the associated polymerase as a template for the production of positive-sense complementary RNA (cRNA), which is assembled into a cRNP, a structure similar to vRNP. Subsequently the cRNA is used as a template to produce new molecules of vRNA for assembling progeny vRNPs [12]. The nascent RNA is captured by viral polymerase and bound by nucleoprotein to provide both stability and protection for the RNA [26–28]. In contrast to transcription, replication initiation happens in a primer-independent (*de novo*) manner [29]. Influenza polymerase uses two distinct types of *de novo* initiation during vRNA and cRNA synthesis: **terminal initiation** (see Glossary), where synthesis begins at the first 3′ nucleotide of the vRNA template, or **internal initiation** where an initial dinucleotide is generated downstream of the first 3′ nucleotide on the cRNA template. Since initiation occurs downstream on the 3′ cRNA template, realignment prior to elongation is essential for the maintenance of genome integrity [30,31].

Recently, snapshots of RNA-bound influenza virus polymerase, captured by X-ray crystallography and cryo-electron microscopy (cryo-EM), have shed light on the molecular detail of key steps in replication. Here we review new data that provide structural insights into RNA replication and the role of the **acidic nuclear phosphoprotein 32** (**ANP32**) family of host proteins.

Influenza virus RNA polymerase forms a dimer to replicate genomic RNA

During genome replication, viral polymerase and NP are recruited to nascent cRNA and vRNA. This **co-replicative assembly** into cRNP and vRNP complexes is important to prevent the degradation of newly produced RNA and to hide the influenza viral genome from host innate immunity. Host nuclear proteins from the ANP32 family (ANP32A/ANP32B in mammals and ANP32A in birds) participate in this co-replicative assembly [32,33]. ANP32A has been observed in a complex, which represents a **replication platform**, with an asymmetric dimer of the influenza C virus polymerase heterotrimer [20]. In the structure of the replication platform, one polymerase is bound to the terminal promoter regions of a 47-nucleotide vRNA, while the other polymerase contains no RNA (Figure 2A). The product exit site of the RNA-bound polymerase is positioned so that the nascent RNA would be readily bound by the 5′ RNA binding site on the RNA free

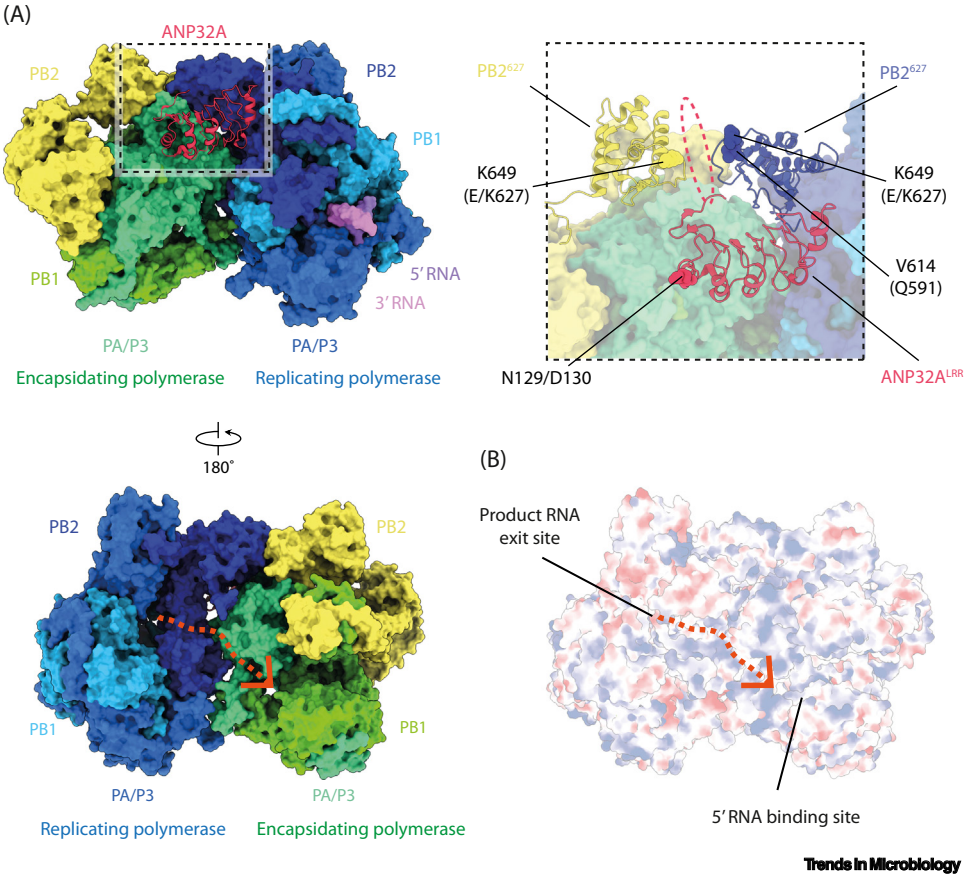

Figure 2. Replication platform for the influenza virus RNA genome. (A) Surface representation of the influenza C virus polymerase in complex with chicken acidic nuclear phosphoprotein 32A (ANP32A) (chANP32A) (PDB ID 6XZR). Subunits of the replicating and encapsidating polymerases are shown in shades of blue and green/yellow, respectively. Viral RNA (vRNA) promoter is shown in pink and purple for the 3′ and 5′ termini, respectively. chANP32A is shown as a cartoon in red. In the lower panel the reverse of the dimer is shown with the putative path of nascent RNA indicated with an arrow. The PB2^{Cap} domain of the replicating polymerase has been made transparent to show the full path. Inset panel shows the positions of the PB2^{627} domains of the replicating and encapsidating polymerases as cartoon with residues of particular interest highlighted (influenza C virus polymerase residue numbering with corresponding influenza A virus residue numbering in brackets). The dashed ellipse shows the likely path of the disordered chANP32A^{LCAR}. (B) Electrostatic potential map of the replication platform with the potential path of nascent RNA, sites of nascent RNA exit from the replicating polymerase and location of the binding site for the 5′ terminus of the nascent RNA in the encapsidating polymerase shown (blue, electropositive; red, electronegative; white, neutral). Note that the PB2^{Cap} domain of the replicating polymerase has been made transparent to show the path of RNA.

polymerase. A track lined with basic amino acid residues links the two sites, forming a path for guiding the negatively charged product RNA (Figure 2B). This asymmetric arrangement suggests a potential role for each polymerase in the dimer: the RNA-bound polymerase acts as **replicating polymerase** and the RNA-free polymerase is an **encapsidating polymerase**. Formation of this dimer is mediated by the P3-C (PA-C), PB2^{627}, PB2^{NLS} of the encapsidating polymerase, and PB2-N, PB2$^{Mid\text{-}link}$ of the replicating polymerase. The conformation of the replicating polymerase is closely related to the structures of RNA-free influenza A and influenza C virus polymerases and the 5′ cRNA promoter-bound influenza B virus polymerase [15,18,34]. In contrast, the conformation of the encapsidating polymerase is different compared to those previously reported. While P3^{Endo} and several domains of PB2-N are flexible, PB2-C

(including PB2^{Cap}, PB2$^{Mid\text{-}link}$, PB2^{627}, and PB2^{NLS}) are observed to pack against P3-C, in a unique arrangement. This conformation orients the 5′ promoter binding site of the encapsidating polymerase to accept newly synthesized RNA from the replicating polymerase.

Although the structure of this asymmetric dimer has only been observed for the influenza C virus polymerase bound to vRNA, functional studies have demonstrated the importance of the dimer interface for the influenza A virus polymerase [20]. It is proposed that a similar replication platform is formed by the influenza A and B virus polymerases, as well as by cRNA-bound polymerases, given the fundamental similarities between cRNA and vRNA and the requirement of their assembly into cRNPs and vRNPs, respectively.

Host ANP32 family proteins are involved in influenza virus genome replication

Host proteins of the ANP32 family are shown to specifically support influenza viral genome replication [35–39]. Carrique and colleagues showed that the dimeric polymerase assembly is maintained by a single copy of ANP32A, which bridges the dimer and forms extensive contacts with both polymerases via its N-terminal leucine-rich repeat (ANP32A^{LRR}) domain [20]. Host ANP32 is shown to support replication in a species-specific manner, and has been linked to the avian to mammalian adaptive mutation at PB2 amino acid residue 627 (residue 649 in influenza C virus). While influenza A viruses encoding Glu627 replicate in avian host, a Glu627Lys mutation in the PB2^{627} domain is responsible for the adaption of avian influenza virus to replication in mammalian cells [40]. Compared to avian hosts, mammals have an ANP32A that is missing a 33-amino acid long sequence between ANP32A^{LRR} and the disordered C-terminal low complexity acidic region (ANP32A^{LCAR}) [38,41,42]. In complex with the influenza virus polymerase dimer in the replication platform, both avian and human ANP32A are observed to bridge the replicating and encapsidating polymerases via the N-terminal ANP32A^{LRR} domain in essentially identical positions (Figure 2A). In particular, N129 and D130 of ANP32A were observed to interact directly with K391 and K608 of the encapsidating polymerase P3 subunit, respectively. These key interactions explain why amino acid polymorphisms N129I and D130N in avian ANP32B render it incapable in supporting influenza virus replication [43].

The C-terminal ANP32A^{LCAR} is not resolved in the available structures but continuous electron density suggests that it inserts between the two PB2^{627} domains of the polymerase dimer (Figure 2A). A region of ANP32A^{LCAR} (residues 176–183), which in human ANP32A contains only acidic amino acid residues, is suggested to interact with PB2^{627} of the replicating polymerase. Specifically, this acidic LCAR region is positioned next to PB2 residue 649 (equivalent of residue 627 in influenza A virus) and residue 614 (equivalent of residue 591 in influenza A virus). These molecular details provide a structural explanation to why nonacidic residues (such as lysine) are preferred in PB2^{627} domain at position 627 or 591 in mammalian-adapted influenza viruses [20,44,45]. A strong interaction between ANP32A^{LCAR} and PB2^{627} is speculated to contribute to the stabilization of the replicating-encapsidating polymerase dimer, in agreement with NMR data implicating the PB2^{627} domain in multivalent interactions with ANP32A^{LCAR} [46].

The C-terminal LCAR of ANP32 plays a role in NP recruitment to nascent RNA

Assembly of RNP is proposed to occur co-replicatively so that no naked RNA is generated that could trigger innate immunity. Following the capture of the 5′ end of the nascent replication product by the encapsidating polymerase, the RNA product is likely to be packaged into RNP by associating with NP as elongation proceeds. Unlike nonsegmented negative-sense RNA viruses which possess viral phosphoprotein (P protein) for NP recruitment [12,47–49], segmented negative-sense RNA viruses do not encode a P protein equivalent. In the structure of the replicating-encapsidating polymerase dimer in complex with ANP32A, the highly acidic

ANP32A^{LCAR} is disordered, indicating the flexibility of this region in the complex [20]. Recently, it was shown that the ANP32^{LCAR} can interact with RNA-free NP via the NP G1 and G2 RNA binding grooves and is important for full-length vRNA replication which requires NP for RNP assembly [37]. Together, these data suggest an NP recruitment mechanism in influenza viruses: at the start of RNA elongation in replication, an ANP32 molecule bridging the replicating-encapsidating polymerase dimer plays a role equivalent to the P protein in nonsegmented negative-sense RNA viruses (Figure 3, Key figure). Specifically, the long and flexible ANP32^{LCAR} captures RNA-free NP, increasing its local concentration, promoting NP association with nascent RNA.

Key figure

Acidic nuclear phosphoprotein 32A (ANP32A) recruits nucleoprotein (NP) to nascent viral RNA

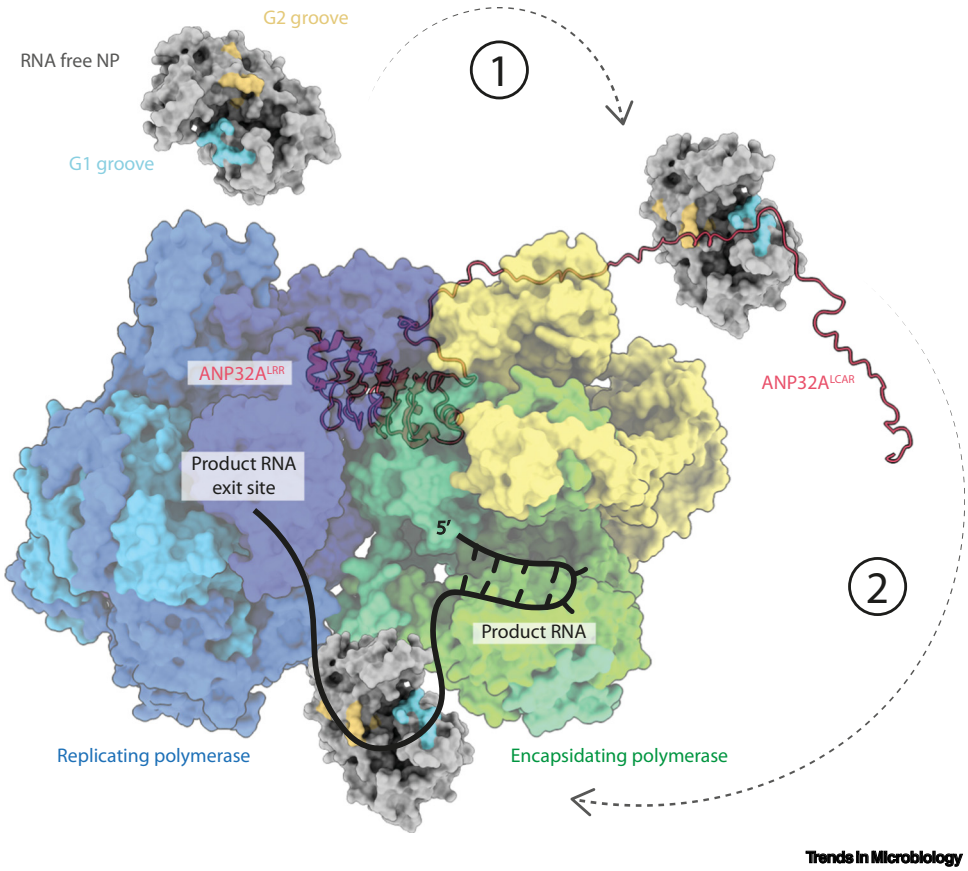

Figure 3. Putative model of NP recruitment to the emerging nascent viral RNA. In step 1, the ANP32A^{LCAR} binds to the RNA free NP increasing the local concentration of NP. Later in step 2, NP is displaced from ANP32^{LCAR} by the higher affinity interaction with the viral RNA. The influenza C virus polymerase dimer bound to human ANP32A (huANP32A) was modelled by combining the replicating-encapsidating polymerase dimer model (PDB ID 6XZR) with the full length alphafold2 model for huANP32A (https://alphafold.ebi.ac.uk/entry/P39687). A monomer of influenza virus NP (PDB ID 7NT8) is coloured grey with the G1 (R74/R75/R174/R175) and G2 (R150/R152/R156/R162) amino acid residues, involved in RNA binding, coloured cyan and yellow, respectively. Product RNA is shown as a black line.

A transactivating polymerase is required for cRNA to vRNA synthesis

Primer-independent replication initiates differently in vRNP and cRNP, that is, terminal and internal initiation is used, respectively, for cRNA and vRNA synthesis. The difference in initiation mechanisms is determined by the difference between the RNA promoters, the 3′ and 5′ termini of vRNA and cRNA [50]. In the context of the polymerase, the ten nucleotides at the 5′ terminus of both vRNA and cRNA fold into a hook structure which is bound tightly in a site formed by the PB1^{Fingers} and PA-C [13]. The subsequent five or six nucleotides of the 5′ terminus of both vRNA and cRNA pair with complimentary bases at the 3′ terminus, forming a 5–8 base-pair duplex. In vRNA, base pairing starts from U10 at the 3′ terminus, leaving nine nucleotides of the 3′ terminus to enter the template entry channel, positioning U1 and C2 in the polymerase active site (Figure 4A). In cRNA, base pairing starts from C12 at the 3′ terminus, leaving 11 nucleotides to be inserted into the template entry channel, positioning U4 and C5 in the active site (Figure 4B).

Terminal initiation results in the synthesis of a pppApG dinucleotide opposite nucleotide positions U1 C2 of the 3′ vRNA terminus. This process requires support from the **priming loop**, a flexible

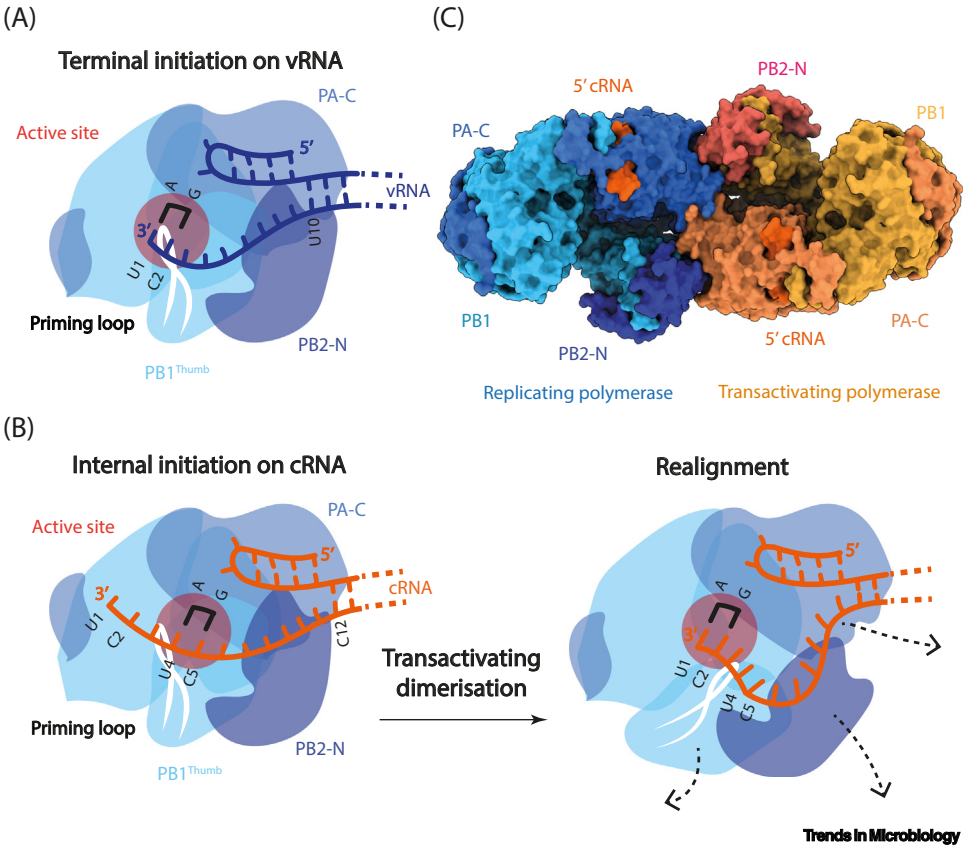

Figure 4. Modes of replication initiation on complementary RNA (cRNA) and viral RNA (vRNA) templates and polymerase dimerization required for template realignment. (A) Terminal initiation on a vRNA template showing the positioning of the vRNA and priming loop. (B) The two steps of internal initiation on cRNA template. In the first step a dinucleotide is formed opposite bases 4 and 5 of the 3′ terminus of the cRNA template. In the second step a second polymerase, termed the transactivating polymerase, interacts with the replicating polymerase, opening the polymerase core and triggering a movement of the priming loop that result in the realignment of the 3′ cRNA terminus such that bases 1 and 2 are positioned opposite the previously formed dinucleotide. (C) Dimer of the influenza A virus RNA polymerase (PDB ID 6QX8). Subunits of the replicating and transactivating polymerases are shown in shades of blue and orange, respectively. The 5′ cRNA terminus is shown in red-orange.

region of the PB1 subunit that protrudes into the active site of the polymerase and controls the position of template and initial dinucleotides [30,51]. Currently a structural model showing the polymerase at the point of replication initiation is not available. Extrapolating from models of transcription initiation, it is thought that the priming loop buttresses the 3′ end of the template in the active site through P651 at its tip and provides a surface upon which free nucleotides are stabilized to facilitate formation of the dinucleotide [21,51]. These interactions facilitate correct positioning of the 3′ terminus opposite the catalytic residues in the influenza A virus PB1 subunit D445/D446 (or D446/D447 in the case of influenza C virus, see Figure 1C), favouring terminal dinucleotide formation in vRNA to cRNA replication [18,51] (Figure 4A).

Due to the different positioning of the 3′ cRNA promoter, initiation occurs internally on the cRNA template which results in the synthesis of a pppApG dinucleotide opposite nucleotide positions 4U 5C of the 3′ cRNA terminus [30]. Since initiation occurs internally on the cRNA template, realignment prior to elongation is required to produce a complete copy of cRNA (Figure 4B). The priming loop and a helix-loop-helix motif of the PB1 subunit have been shown to contribute to the realignment process. Mutations at the tip of both structural features affect the realignment during replication on a cRNA template with mutant polymerases producing failed-realignment products [31]. A mechanism for template realignment has been proposed which utilizes a second copy of the viral polymerase that forms a dimer with the replicating polymerase on the cRNA template (Figure 4C) [18]. This second polymerase, referred to as a **transactivating polymerase**, plays a regulatory role and the formed dimer is distinct from the asymmetric dimer discussed above (Figure 4C).

Comparison of structures of monomeric and dimeric forms of the influenza A virus polymerase in complex with cRNA promoter revealed that dimerization induces a movement of a helical bundle formed by $PB1^{Thumb}$ and $PB2^{N1}$ within the polymerase core. This causes the priming loop to move away by 7 Å from the active site and opening up the RNA binding site in the polymerase, potentially destabilizing the 3′ template in the active site to allow relocation of the 3′ cRNA terminus. Together, these conformational changes facilitate the backtracking of the cRNA template and the alignment of pppApG to the terminus of the 3′ RNA template (Figure 4B). The functional importance of this regulatory dimer has been verified by disrupting the dimer interface with mutations or reducing dimerization with a nanobody, both of which specifically affect the cRNA to vRNA step of replication and suppress viral growth [18].

Molecular cues for replication

Profiles of mRNA, cRNA, and vRNA accumulation during infection reveal that mRNA is produced early in infection, while cRNA and vRNA are not detectable until a sufficient level of viral proteins is reached. In the later stages of infection, vRNA levels keep increasing while cRNA levels plateau, and mRNA levels decrease [52–54]. How does a resident polymerase in an RNP adopt distinct transcriptase and replicase conformations to synthesize different species of RNAs at different times? In addition, how are viral and host factors involved in regulating the relative timing of transcription and replication at various stages during infection?

Previous studies suggested that low levels of cRNA synthesis can occur in the absence of viral protein synthesis [27,30,55]. This led to the proposal of a ‘stabilisation model’, which postulates that transcription and replication initiations are stochastic in vRNPs delivered by the infecting virus and that no molecular switch is involved. Product cRNA is only detectable when being stabilised and assembled into functional cRNPs after free polymerase and NP become available. While this model is plausible, several studies have shown that binding to host Pol II (a source of nascent host mRNA caps) promotes cap-snatching [56,57]. In addition, expression of free polymerase not only

enables cRNA to accumulate but also allows vRNA synthesis in the context of cRNPs [26,28]. It is therefore reasonable to hypothesise that binding of the polymerase to molecular cues determines whether the polymerase carries out transcription or replication at different stages of an infection cycle (Figure 5A). Specifically, at the onset of infection when transcription promoting cues, such as host Pol II, are widely available, a polymerase is more likely to adopt a transcriptase conformation. As infection progresses, replication promoting cues, such as newly produced viral polymerase, accumulate. Free polymerase may bind to both host Pol II and RNP-associated polymerase, decreasing their availability for each other, hence shift RNA synthesis to replication. Influenza virus-induced degradation of the large subunit of Pol II might further reduce the availability of Pol II [52,58]. A recent study has proposed a binding site competition-based mechanism where a dual binding site on polymerase, identified by nanobodies, is responsible for the mutually exclusive binding to either host Pol II C-terminal domain or an additional polymerase [22].

Concluding remarks

Recent structural studies have revealed snapshots of influenza virus polymerase complexes implicated in the replication of the viral RNA genome. Most importantly, a complex of an influenza polymerase dimer bound to host ANP32A has been observed that likely represents a platform for the replication of the viral RNA genome and the assembly of the nascent replication product into an RNP with polymerase and NP. In parallel, complementary functional studies have verified the biological importance of observed interactions between the two polymerase complexes and ANP32A. Combining the structural and functional data, the molecular mechanisms underlying replication have begun to be revealed (Figure 5B). The requirement for polymerase dimerization during RNA genome replication ensures that nascent cRNA and vRNA are bound by an encapsidating polymerase as soon as their 5′ end emerges from the active site of the replicating polymerase, thus minimizing the possibility of releasing RNA free of viral protein that could be a trigger for innate immune activation. Furthermore, the requirement for dimerization in replication also ensures temporal segregation of transcription and replication during the course of infection such that no genomic RNA is produced during early stages of the infection when no newly synthesized viral polymerase and NP would be present to encapsidate it.

As both cRNA and vRNA are assembled into an RNP complex it is likely that a dimer of a replicating and an encapsidating polymerase bound by an ANP32 protein is required for both steps of replication [36]. In addition, the replicating polymerase on the cRNA template also dimerises with a transactivating polymerase (Figure 5B). Maintaining a balance between different forms of dimeric interactions of the influenza virus polymerase is important for the efficiency of influenza replication, especially when different polymerase subunits are combined during reassortment, or upon transmission to a new host encoding different ANP32 homologues and/or replicating viral RNA at a different temperature [59].

Our current knowledge of influenza virus replication is far from complete – currently available structures only provide a glimpse into how influenza virus genome replication might be proceeding. It is necessary to capture the replicating polymerase at the point of initiation, on both vRNA and cRNA templates, with the active site of the polymerase and the priming loop fully ordered, as well as during the elongation and termination stages of RNA replication. It is of particular interest to understand how the 5′ end of the template is released from its binding site to enable the generation of a complete copy of the RNA template, as opposed to transcription, during which the 5′ end remains associated with the polymerase to ensure premature termination and polyadenylation of viral mRNA.

Structural insights into polymerase dimerization and interactions with host factors during RNA replication have also revealed new drug targets on the surface of influenza virus polymerase

Outstanding questions

What is the sequence of events leading to the assembly of the replicating-encapsidating polymerase dimer bound by an ANP32 protein? How is a balance maintained between different types of polymerase dimers? Does a polymerase trimer state exist during cRNA to vRNA replication?

Does NP interact with the replicating and/or encapsidating polymerase during recruitment to nascent RNA?

What is the arrangement of the active site, RNA, and priming loop during initiation on vRNA and cRNA templates?

How is the binding of the 5′ end of template RNA to the replicating polymerase controlled to enable its release during replication so that the polymerase could produce a complete copy of the template as opposed to transcription during which the 5′ end of the template remains bound to the transcribing polymerase resulting in premature termination and polyadenylation?

Is polymerase dimerization for genome replication a conserved feature of negative-sense RNA viruses?

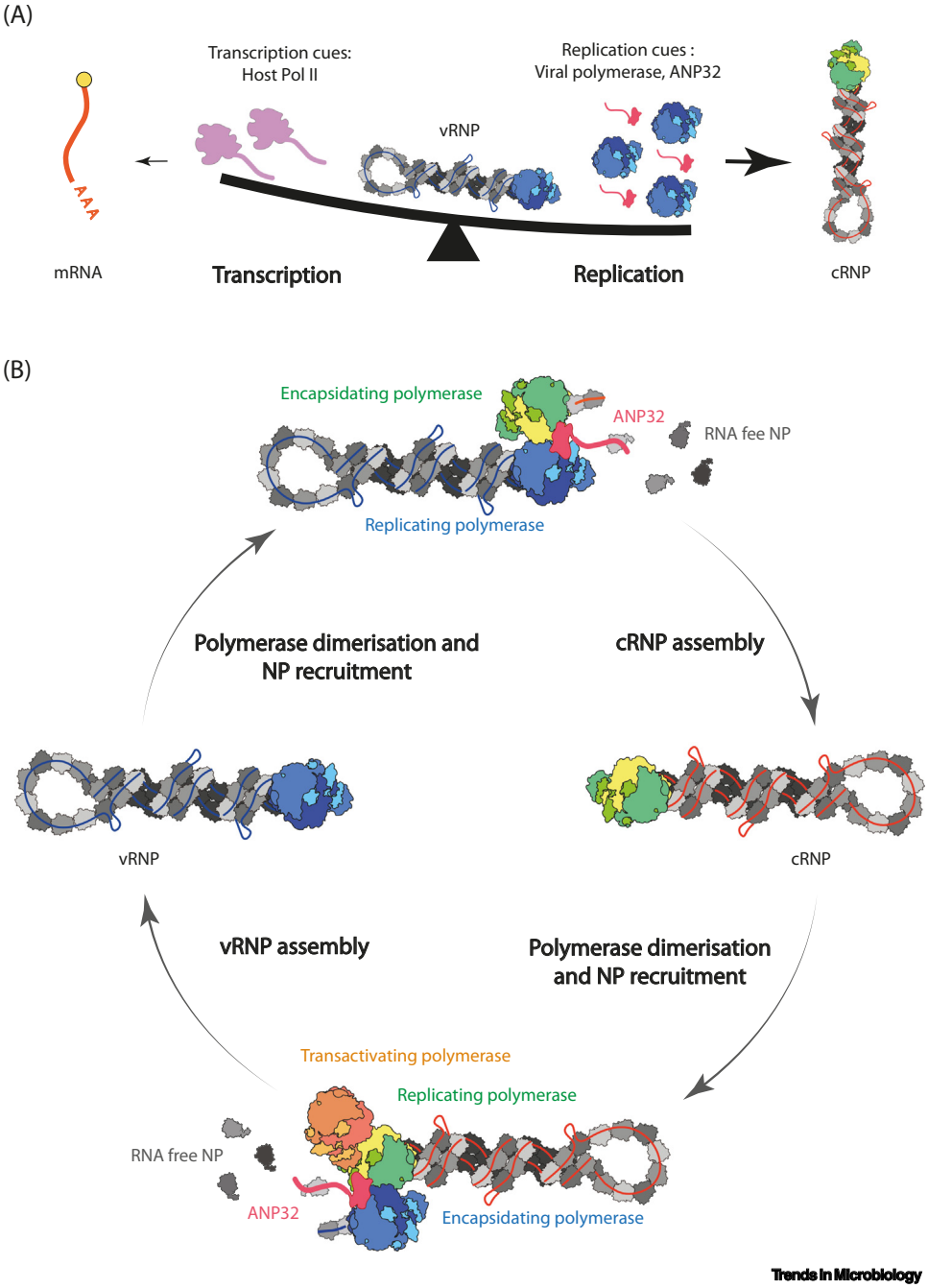

Figure 5. Regulation of the influenza virus RNA polymerase through interactions with molecular cues and proposed model for influenza virus RNA genome replication. (A) Interactions with host RNA polymerase II drive incoming viral ribonucleoproteins (vRNPs) towards transcription to produce viral mRNA while interactions with host acidic nuclear phosphoprotein 32 (ANP32) proteins and newly synthesized viral polymerase drive vRNPs towards replication producing cRNP. (B) Replication cycle by the viral polymerase showing the roles of the replicating, encapsidating, and transactivating polymerases. Abbreviations: cRNA, complementary RNA; cRNP, complementary ribonucleoprotein.

that should be exploited for the development of novel antiviral drugs to inhibit influenza virus replication [22] (see Outstanding questions).

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Declaration of interests

No interests are declared.

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