

Influenza virus RNA polymerase: insights into the mechanisms of viral RNA synthesis

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Abstract

The genome of influenza viruses consists of multiple segments of single stranded negative-sense RNA. Each of these segments is bound by the heterotrimeric viral RNA-dependent RNA polymerase and multiple copies of nucleoprotein, forming viral ribonucleoprotein (vRNP) complexes. It is in the context of these vRNPs that the viral RNA polymerase carries out transcription of viral genes and replication of the viral RNA genome. In this Review, we discuss our current knowledge of the structure of the influenza virus RNA polymerase, how it carries out transcription and replication, and how its activities are modulated by viral and host factors. Furthermore, we discuss how advances in our understanding of polymerase function could help identifying new antiviral targets.

Influenza viruses belong to the family of *Orthomyxoviridae* and are the causative agents of influenza, a respiratory disease in humans. Influenza A and B viruses cause substantial morbidity and mortality in humans and a considerable financial burden worldwide, whereas influenza C viruses cause sporadic outbreaks of mild respiratory disease, mainly in children^{1, 2}. Although vaccines against influenza A and B viruses are available, the protection that they offer is limited by antigenic variation in the haemagglutinin (HA) and neuraminidase (NA) envelope glycoproteins of influenza virus strains³. Additionally, influenza A viruses have a reservoir in birds and pigs from which pandemic viruses with novel HA and NA proteins can emerge against which there is little pre-existing immunity in the human population. Available vaccines are unlikely to protect against such strains. Although antiviral drugs can be a first line of defense in the case of an emerging pandemic virus, the number of antiviral drugs that can be used for prophylaxis and therapeutic treatment of severe infections is limited and emerging antiviral resistance is a continuing problem^{4, 5}. Therefore, understanding of the molecular mechanisms of influenza virus replication is critical for the development of new antiviral drugs.

Influenza viruses contain a single-stranded negative-sense RNA genome that consists of eight segments in influenza A and B viruses and seven segments in influenza C viruses⁶. In all three species, the viral RNA (vRNA) genome segments are bound by a heterotrimeric RNA-dependent RNA polymerase, forming a viral ribonucleoprotein (vRNP) complex^{7, 8}. In the vRNP, the 5' and 3' termini of vRNA are bound to a polymerase heterotrimer, while the rest of the vRNA

associates with oligomeric nucleoprotein (NP) (FIG. 1a). Structural models based on cryo-EM imaging of native vRNPs show that the vRNP is an anti-parallel double helix of NP-coated vRNA that contains a polymerase at one end and an NP loop at the other end^{9, 10}. The influenza virus RNA polymerase consists of three subunits: polymerase basic 1 (PB1), PB2 and polymerase acidic (PA) in influenza A and B virus or polymerase 3 (P3) in influenza C virus^{7, 8}. Upon viral infection, the vRNPs are transported into the nucleus of the host cell, where the RNA polymerase carries out transcription of viral genes and replication of the viral RNA genome in the context of the vRNP¹¹ (BOX 1). Transcription is a primer-dependent process that produces mRNAs with a 5' cap structure and a 3' poly(A) tail. The influenza virus polymerase does not have inherent capping activity and it relies on host capped RNAs as cap-donors¹². In a process called 'cap-snatching', the viral polymerase uses its PB2 cap-binding domain to capture the 5' cap of nascent host capped RNAs and its PA/P3 endonuclease domain to cleave the capped RNA about 8-14 nucleotides downstream of the cap structure¹³⁻¹⁵. It then uses these capped RNA fragments as primers to initiate transcription. In contrast to transcription, replication is primer-independent and proceeds via a complementary RNA (cRNA) replicative intermediate. Progeny vRNPs are exported from the nucleus and traffic to the cell membrane to be incorporated into new virions (BOX 1).

Until recently, lack of high-resolution structural information on the influenza virus RNA polymerase greatly hampered our understanding of influenza virus transcription and genome replication at the molecular level. The RNA polymerases of negative-strand RNA viruses have been refractory to structural analyses, primarily due to their large size and low expression levels in recombinant systems and, in the case of influenza virus, multi-subunit nature^{16, 17}. Recent developments in the expression and purification of large proteins and macromolecular complexes, combined with improved methods for the structural analysis of proteins, resulted in high resolution RNA polymerase structures for influenza A, B, and C viruses as well as for other negative-strand RNA viruses, such as La Crosse virus (a bunyavirus) and Vesicular Stomatitis Virus (VSV; a paramyxovirus)¹⁸⁻²². These novel structures provide unprecedented insights into their molecular mechanisms. This Review focuses on these novel structural insights and their potential to advance our understanding of viral pathogenicity and host adaptation as well as to facilitate the development of new prophylactic antivirals or treatments for influenza virus infections.

The RNA synthesis machine of influenza viruses

Viral polymerase structure. The past decade witnessed the emergence of high-resolution structural information on fragments of the three subunits of the influenza virus polymerase^{13-15, 23-27}. Moreover, electron microscopy (EM) of the polymerase heterotrimer in isolation or as part of vRNP revealed that the polymerase has a globular structure^{9, 10, 28, 29}. However, these structures provided limited information on the arrangement of the subunits in the polymerase heterotrimer. This changed dramatically when high-resolution x-ray crystal structures of the complete polymerase heterotrimers of the bat influenza A and human influenza B and C viruses became available (FIG. 1b)^{19, 21, 22}. In addition, a 4.3Å resolution structure of an H5N1 avian influenza virus polymerase complex composed of the full-length PB1, PA and an N-terminal fragment of PB2 has been obtained by cryo-EM³⁰.

The x-ray crystallography structures of the complete polymerase heterotrimer revealed that the PB1 subunit is the centre of the polymerase (FIG. 1b and FIG. 2a,b). PB1 contains the pre-A (also known as F) and A-E motifs which are characteristic of RNA-dependent RNA polymerases (FIG. 2a)³¹. Accordingly, PB1 has the canonical right-hand-like fold, possessing fingers, fingertips, palm, and thumb, but with additional N- and C-terminal extensions that facilitate interactions with the PA/P3 and PB2 subunits, respectively (FIG. 2b; see also BOX2 panel b). PB1 is sandwiched by the N-terminal endonuclease domain and the large C-terminal domain of the PA/P3 subunit (FIG. 1b and FIG. 2c). A long linker connects the two domains and wraps around the external side of the PB1 fingers and palm subdomains. PB2 consists of multiple domains (FIG 1b and FIG. 2d). The N-terminal third of PB2 interacts with PB1, making contacts mostly with the PB1 thumb and C-terminal extension. The arrangement of the domains within the remaining two thirds of PB2 differs dramatically in the influenza A and B virus polymerase structures compared to that of influenza C virus, indicating that these form flexible domains (Supplementary Video 1). These include the mid-domain, the cap-binding domain, the cap-627 linker, the so called 627-domain, named after amino acid residue 627, a host range determinant of influenza viruses³², and the C-terminal nuclear localization signal (NLS) domain (FIG. 2d). The mid and cap-627 linker domains form one rigid unit referred to as the ‘mid-link’³³. Overall the polymerase can be viewed as a complex made up of a polymerase core consisting of PB1, the

C-terminal domain of PA/P3 and the N-terminal region of PB2, and flexible peripheral appendices that are formed by the PA/P3 endonuclease and the PB2 cap-binding, mid-link, 627, and NLS domains (FIG. 2e,f).

Promoter binding. The highly conserved sequences present at the termini of each vRNA segment have the potential to form a partially double-stranded ‘panhandle’ structure³⁴. The polymerase needs to associate with the 5′ end of the vRNA template in order to bind and cleave host capped RNA, as well as to initiate transcription at the 3′ end of the vRNA template³⁵⁻³⁹. These observations lead to the proposal that the vRNA promoter is formed by the 5′ and 3′ terminal sequences of the vRNA segments³⁶.

The structures of the influenza A and B virus RNA polymerases were obtained in the presence of short RNAs mimicking the 5′ and 3′ terminal vRNA sequences, which revealed how the vRNA promoter is bound to the polymerase. The crystal structures show that nucleotides 1-10 of the 5′ end of the vRNA form a compact stem-loop (hook) structure that inserts into a deep pocket at the interface of the PB1 fingers and the PA C-terminal domain (FIG. 1b and 2a, b). Multiple amino acid residues from both PB1 and PA create a highly specific and strong interaction^{16, 21, 22, 40}. Nucleotides 1-9 of the 3′ end of the vRNA bind in a nearby binding site on the surface of the polymerase with amino acid residues from all three polymerase subunits contributing to the association. These findings are consistent with UV-cross-linking experiments, which showed that all three subunits intimately interact with the vRNA termini^{36, 41}. The distal part of the vRNA promoter forms a double-stranded structure involving nucleotides 11-16 of the 5′ end and 10-15 of the 3′ end, which in the polymerase crystal structures projects away from the body of the polymerase. The overall arrangement of the vRNA promoter is consistent with vRNA promoter models based on mutagenic studies that predicted a 5′ hairpin-loop and base-pairing in the distal region^{36, 37, 42, 43}. An RNA secondary structure similar to the vRNA promoter has been predicted for the cRNA replicative intermediate and is called the cRNA promoter^{44, 45}. Although the 5′ end of the cRNA differs in sequence from the vRNA 5′ end (being the complement of the vRNA 3′ end), it forms a similar hook structure and is bound by the influenza B virus polymerase at the same site as the vRNA 5′ end³³.

Conformational rearrangements of the viral polymerase. Different conformations of the polymerase can be observed in the crystal structures of the RNA-free (apo form) influenza C

virus polymerase and the vRNA promoter-bound influenza A and B virus polymerases (FIG. 1b). Whereas the polymerase core changes little when vRNA is bound, the peripheral C-terminal two-thirds of PB2 (including the cap-binding, mid-link, 627, and NLS domains) and the N-terminal PA/P3 endonuclease domains undergo major rearrangements (Supplementary Video 1). In the apo structure of the influenza C virus polymerase, the PB2 cap-binding domain is immobilized by several inter-domain interactions that involve the PB1 palm subdomain. The cap-binding pocket is occluded by a part of PB2. The P3 endonuclease is located on the side of the fingers subdomain of PB1 with the PB2 NLS domain packing against the endonuclease. This arrangement of the cap-binding and endonuclease domains is incompatible with cap-snatching activity^{19, 33}. By contrast, in the vRNA promoter-bound influenza A and B virus polymerase structures, the PB2 cap-binding and PA endonuclease domains are oriented in a way that is compatible with cap-snatching (FIG. 1b), consistent with previous observations that cap-snatching is activated by the binding of the polymerase to vRNA^{35, 39}. In this arrangement, the cap-binding domain makes little contact with the other domains, so it can rotate freely *in situ*, while the endonuclease is stabilised by the PB1 C-terminus and PB2 N-terminus interaction motif. In addition, the NLS of PB2 no longer contacts the endonuclease and has instead moved with the PB2 627-domain to a site that is occupied by the PB2 cap-binding domain in the apo form (Supplementary Video 1).

The conformations captured by X-ray crystallography most likely represent the polymerase in two different functional states. The apo form of the influenza C virus polymerase corresponds to a transcriptionally inactive state and may represent the structure of the newly assembled polymerase. However, a structure of the influenza B virus polymerase in complex with 5' cRNA shows a similar, though not identical arrangement of its peripheral domains as the apo structure of the influenza C virus polymerase³³. Therefore this conformation may also be relevant for a polymerase engaged in primer-independent cRNA to vRNA replication, which does not require cap-snatching. By contrast, the structures observed in the presence of vRNA represent the polymerase in a transcription pre-initiation state, with the cap-binding and endonuclease domains aligned such that the polymerase could bind and cleave host capped RNA (FIG. 1b). Structural and biophysical characterisation of the polymerase in solution, with and without RNA, suggests that multiple conformations exist in equilibrium^{19, 33}. Thus, the influenza virus RNA polymerase is inherently flexible. The nature of RNA present influences the equilibrium and a particular

conformation may further be stabilised through interactions with other viral or host factors (see below), which may preferentially promote a particular function of the polymerase^{19, 33}. Therefore it is possible that vRNA-bound polymerase could also exist in a transcriptionally inactive state, for example in vRNPs packaged into virions and vRNPs that are in transit between the host cell nucleus and the plasma membrane.

Polymerase active site. The polymerase active site is located at the edge of a large central cavity that is formed by PB1 and the N-terminus of PB2^{19, 21, 22}. The active site can be accessed via several channels (FIG. 2e). The putative template entry channel that leads from the site of 3' vRNA binding into the active site is lined by conserved amino acid residues from all three subunits and the NTP entry channel is made up by highly conserved basic amino acids from PB1. In contrast to positive strand RNA viruses, influenza viruses lack a helicase, and the template and product RNA strands are separated before they exit the polymerase. Potentially, an element of PB2 called the lid-domain (FIG. 2d) could be responsible for separating the two strands which, when modelled into the available crystal structures, appear to clash with this element²². The putative template exit channel is located on the same side of the polymerase as the template entry channel and it has been identified by comparing the influenza virus RNA polymerase structure to that of the La Crosse bunyavirus, which shows a more obvious channel for template exit¹⁸. The close proximity of the template entry and exit channels on the same side of the polymerase could facilitate the copying of the RNA genome with minimal disruption of the vRNP complex (see below). The product exit channel is likely to be defined by the path that the capped RNA primer takes when its 3' end is inserted into the active site (FIG. 2f). An important feature of the central cavity is the priming loop, a β -hairpin in the case of the influenza virus polymerase, that protrudes from the PB1 thumb subdomain towards the active site (FIG. 2a,f) and may support the sugar-base of the first NTP during *de novo* initiation as observed in other RNA-dependent RNA polymerases^{22, 46-48}. A recent study has shown that the priming loop is important for primer-independent replication initiation on the vRNA template⁴⁹ (see below).

Models for transcription and replication

Transcription. After vRNPs reach the nucleus, the resident polymerase, that is the polymerase that is bound to the vRNA termini in the vRNP, synthesizes 5' capped and 3' polyadenylated viral mRNA (FIG. 3a). For this process the viral polymerase requires primers which are 5'

capped RNA fragments derived from nascent host capped RNAs. Accordingly, viral transcription is sensitive to actinomycin-D and α -amanitin, an inhibitor of cellular DNA-dependent RNA polymerase II (Pol II). Host snRNAs, snoRNAs and promoter-associated capped small (cs)RNAs, which are generated by “paused” Pol II during transcription initiation, are the preferred substrates for cap-snatching over mRNAs or pre-mRNAs⁵⁰⁻⁵². Cap-snatching is carried out by the viral polymerase that is in a transcription pre-activation state (as illustrated by the influenza A and B virus polymerase structures) with the PB2 cap-binding and PA endonuclease domains aligned such that the polymerase can bind and cleave host capped RNA. After cleavage of the capped RNA, the 3' end of the capped primer must transfer from the endonuclease active site into the polymerase active site via the product exit channel. This transfer is likely facilitated by the rotation of the cap-binding domain as observed in one of the influenza B virus polymerase structures (FIG. 1b)²². Concomitantly, the 3' end of the vRNA template must move from its binding site near the surface of the polymerase into the polymerase active site via the template entry channel (FIG. 3a). Currently, it is not clear how this is triggered, but an equilibrium might exist between conformations with the 3' end at the surface or in the active site. Interactions between the capped RNA primer and the 3' end of the vRNA template in the active site or binding of an initiating nucleotide might stabilise the vRNA 3' end in the active site. Transcription initiation takes place by the addition of a G or a C residue to the 3' end of the capped primer, directed by the penultimate C residue (C2) or the G residue at position 3 (G3) in the vRNA template. In infected cells there is a preference for transcription initiation with primers with a “CA” 3' end, but the structural basis for this sequence specificity remains unknown^{53, 54}. The four conserved residues at the tip of the priming loop in the polymerase active site are redundant for this process as they can be deleted without affecting transcription initiation⁴⁹.

Currently it is unknown how the polymerase copies the vRNA template in the context of the vRNP. It is likely that only local restructuring of the NP helix takes place when the polymerase ‘pulls’ the template vRNA from the NP helix into its active site. The proximity of the template entrance and exit is compatible with a model in which template vRNA is progressively dissociated from NP, translocated via the entrance channel into the polymerase active site and then re-associated with NP at the exit channel (FIG. 3a). Elongation continues in a template-dependent manner by pulling the vRNA template through the polymerase active site until a 5-7 nucleotide long U-stretch, 16 nucleotides from the 5' end of the vRNA template, reaches the

active site. Stuttering of the polymerase on the U-stretch leads to the repeated incorporation of AMP and the production of a poly(A) tail (FIG. 3a). Evidence for the stuttering mechanism has been provided by replacement of the U-stretch with an A-stretch, which resulted in the generation of mRNAs with a poly(U) tail^{55, 56}. Although the exact mechanism that triggers stuttering is unclear, it has been proposed that steric hindrance caused by the 5' end of the vRNA template remaining associated with the transcribing polymerase plays a role^{36, 38, 43}. Return of the already copied 3' end of the vRNA template to its original position near the surface of the polymerase and its base-pairing with the 5' end potentially helps to 'lock' the 5' end in its binding site, thereby preventing its release during polyadenylation (FIG. 3a).

At the point of transcription initiation the 5' cap is associated with the PB2 cap-binding domain. However, as the polymerase enters elongation the cap is released from the PB2 cap-binding domain⁵⁷. Subsequently, the 5' cap is likely bound by the nuclear cap-binding complex, triggering the assembly of the viral mRNA into a host mRNP-like structure^{58, 59}.

Replication. Replication is a two-step, primer-independent process. In the first step of replication, incoming vRNPs carry out the synthesis of cRNA, a replicative intermediate (FIG. 3b). Biochemical data show that the resident polymerase is capable of catalyzing cRNA synthesis: vRNPs isolated both from purified virions as well as vRNPs isolated from infected cells can catalyze the generation of cRNA in a primer-independent manner *in vitro*^{60, 61}. Similar to transcription initiation, replication initiation requires the translocation of the 3' terminus of vRNA into the active site. Next, a pppApG dinucleotide is formed on residues U1 and C2 of the vRNA 3' end (terminal initiation)⁶². As in other *de novo* initiating RNA polymerases, the formation of the dinucleotide on the terminus of the RNA template is dependent on structural support by a priming loop^{22, 49}. Elongation presumably proceeds in a manner similar to that described for mRNA synthesis with minimal disruption of the vRNP structure. However, in contrast to transcription, during the termination of cRNA synthesis the 5' end of the vRNA template must be released from its binding pocket to be copied (FIG. 3b). Currently it is unclear how this difference between mRNA and cRNA termination is achieved.

The cRNA product assembles into complementary ribonucleoprotein (cRNP) complex, a vRNP-like structure, with polymerase bound at the 5' and 3' termini and NP associating with the body of the cRNA. Negative stain EM of isolated cRNPs revealed a twisted, double helical

arrangement of the cRNA-NP complex very similar to that of the vRNP⁶¹. Assembly of cRNA into cRNP is likely started as soon as its 5' end emerges from the product exit channel and is bound by a second, newly synthesized polymerase. The second polymerase then recruits the first NP through polymerase-NP interactions. However, subsequent NPs are recruited via groove and tail-loop interactions between NP monomers in solution and cRNA-bound NPs⁶³⁻⁶⁶. Multiple mechanisms that include phosphorylation and interaction with host factors, i.e. importins, have been proposed to maintain NP in a monomeric form prior to being incorporated into an oligomer of NPs within the RNP⁶⁷⁻⁷⁰. A similar detailed mechanism, involving binding of polymerase to the 5' end of emerging nascent replication product and subsequent binding of NP, has been proposed for bunyavirus replication¹⁸.

In the second step of replication, cRNA acts as template for vRNA synthesis (FIG. 3c). In contrast to replication on a vRNA template, replication initiation on a cRNA template occurs on residues U4 and C5 (internal initiation)⁶². The pppApG dinucleotide product is then aligned to residues U1 and C2 of the cRNA 3' terminus to allow elongation into a full-length copy of the cRNA. Recent data show that the full-length priming loop is not required for internal initiation on a cRNA template, possibly because the 3' UCG overhang contributes to the stabilization of the template-dinucleotide complex⁴⁹. However, replication from the cRNA template is critically dependent on the presence of a second polymerase additional to the resident polymerase. In particular, cRNPs isolated from infected cell lysates were inactive *in vitro* unless 'extra' polymerase was added⁶¹. Interestingly, this second polymerase does not need to be catalytically active, suggesting that it fulfills a role that does not involve RNA synthesis and that it is *trans*-activating rather than *trans*-acting⁶¹. It is tempting to speculate that the *trans*-activating polymerase might induce a conformational rearrangement in the resident polymerase that favours the transfer of the cRNA 3' terminus to the active site and/or internal initiation on the cRNA 3' terminus. Although it is not known where and how the cRNA 3' terminus binds the polymerase, it can be assumed based on the binding of the 5' cRNA end to the influenza B virus polymerase³³, which is similar to the binding of the vRNA 5' end, that in a resting state the 3' end of cRNA occupies the same site as the 3' end of the vRNA template. In this model of vRNA synthesis, the *trans*-activating polymerase would also fulfill the role of the polymerase that binds to the 5' end of the emerging nascent vRNA and recruits the first NP to start the assembly of vRNP that likely proceeds in a manner similar to that described for cRNP (see above).

An alternative model for the second step of replication, cRNA to vRNA synthesis, involves a catalytically active, *trans*-acting polymerase. In this model, a new polymerase, possibly associated with small viral RNA (see below), is recruited to the resident polymerase and gains access to the 3' terminus of the cRNA template. The 3' terminus enters the active site of the *trans*-acting polymerase that performs replication initiation internally on the cRNA template as described above for the *trans*-activating polymerase model. However, in this model, a third polymerase is proposed to bind to the 5' end of the nascent vRNA to initiate the assembly of a vRNP. Genetic complementation experiments as well as the observation of branched RNPs in cryo-EM support this alternative model^{10, 71}. Further studies are required to confirm whether vRNA synthesis is performed by the resident or a *trans*-acting polymerase.

Regulation of transcription and replication

Regulation by viral factors. Several viral proteins have been implicated in the regulation of viral transcription and replication (FIG. 4). In particular, NP has long been considered a factor that switches the polymerase from a transcriptase to a replicase^{72, 73}. However, more recent studies challenge this view by proposing that the initiation of mRNA or cRNA synthesis is stochastic, with no switch regulating the initiation of RNA synthesis⁷⁴. In this model, newly synthesized viral polymerase and NP are required to stabilize nascent cRNA, which would otherwise be degraded by host cell nucleases. Furthermore, short genome segments (up to 76 nucleotides), with large internal deletions but preserved 5' and 3' terminal sequences, were found to be efficiently transcribed and replicated in the absence of NP in cell-based transcription/replication assays^{66, 75}. NP had no effect on the ratio of transcription and replication products under these conditions. However, NP has been observed to stimulate polymerase activity and it is required for the processivity of the polymerase on long RNA templates⁷⁶⁻⁷⁸. Two other viral proteins, non-structural protein 1 (NS1) and nuclear export protein (NEP; formerly known as non-structural protein 2 (NS2)) have also been implicated in the regulation of transcription and replication (FIG. 4). NS1, recently identified as a component of the influenza virion⁷⁹, was found to interact with NP and several studies suggest a role for NS1 in viral RNA synthesis⁸⁰⁻⁸³ and the splicing of viral mRNAs^{84, 85}. NEP is primarily considered to play a major role in mediating the nuclear export of progeny vRNPs⁸⁶. However, there is also evidence for a regulatory role of NEP in viral RNA synthesis, with NEP expression leading to an increase in replication^{87, 88}. This function has

been linked to the requirement of NEP for the generation of 22-27 nt long short viral RNAs (svRNAs) that correspond to the 5' terminus of vRNAs and are highly expressed in infected cells^{89, 90}. svRNAs have been implicated as viral factors involved in the replication of cRNA into vRNA⁹¹. svRNAs might associate with the *trans*-acting polymerase through binding to the vRNA 5' end binding pocket.

Regulation by host factors. A wide range of methods, including proteomics and genome-wide RNAi-based screens, have been applied to search for host factors involved in viral replication^{5, 92-100}. Targeted interaction screens using proteomics to identify host interacting partners of the viral polymerase, NP, and RNPs have also been performed and revealed hundreds of candidate proteins that could affect viral RNA synthesis^{96, 99, 101-109}. However, the role of very few of these host factors has been investigated in detail.

Viral transcription and replication are carried out in the nucleus of the infected cell. vRNPs are targeted to the nuclear matrix or to chromatin components, and viral transcription and replication are proposed to take place in DNase-insensitive nuclear fractions that include chromatin and the nuclear matrix^{81, 110-113}. Specific interactions between the influenza virus polymerase and chromatin remodelers CHD1 and CHD6, nuclear matrix protein NXP2/MORC3, and Pol II function modulator CLE/C14orf166 may play a role in this targeting¹¹⁴⁻¹¹⁷. Viral mRNA synthesis is proposed to take place in close association with host Pol II. It has been demonstrated that the viral polymerase interacts directly with the serine-5 phosphorylated form of the C-terminal domain (CTD) of the large subunit of Pol II^{118, 119}. This form of the CTD is known to recruit cellular capping enzymes to cap nascent Pol II transcripts near the transcription start site¹²⁰. The interaction of the viral polymerase with the same form of the CTD presumably facilitates access of the viral polymerase to nascent host capped RNAs (FIG. 4). However, this interaction also brings viral mRNA synthesis close to sites of high concentration of splicing factors and host factors involved in the assembly of host mRNPs and it may therefore facilitate not only cap-snatching but also RNA processing and viral mRNP assembly⁵⁹. Viral M1, NS1 and PB2 mRNAs can be spliced¹²¹, a function carried out by the cellular splicing machinery¹²². Cellular factors related to splicing, including RED, SMU1, SF2/ASF, SFPQ/PSF, hnRNP K and NS1-BP have been identified as important for influenza virus replication¹²³⁻¹²⁶. Splicing factor SFPQ/PSF that interacts with vRNPs has been shown to be important for viral mRNA synthesis by increasing the efficiency of viral mRNA polyadenylation¹²⁴.

The cellular transcriptional machinery may provide the viral polymerase with a platform inside the host cell nucleus around which not only viral mRNA synthesis and processing but also viral RNA genome replication is organized. The minichromosome maintenance (MCM) complex, a cellular helicase involved in DNA replication but also participating in Pol II elongation via its interaction with the Pol II CTD¹²⁷, was found to stimulate viral RNA replication. MCM interacts with the PA subunit of the viral RNA polymerase and it appears to stimulate the transition from *de novo* initiation to elongation on the vRNA template¹²⁸. Members of the ANP32 family of proteins, implicated in multiple cellular pathways, including transcriptional regulation by chromatin remodelling, mRNA export and cell death, were found to promote the second step of replication, i.e. vRNA synthesis from a cRNA template. In particular, human ANP32A (also known as pp32) and ANP32B (also known as APRIL) were shown to interact with the viral RNA polymerase^{102, 104, 129}, up-regulate vRNA synthesis from cRNA *in vitro*¹²⁹, and be required for viral RNA synthesis in infected cells⁹⁹. Importantly, ANP32A was found to underlie PB2 position 627-mediated polymerase host restriction. More specifically, a species-specific difference in ANP32A accounts for the suboptimal function of avian influenza virus polymerase with PB2 containing glutamic acid at position 627 (627E) in mammalian cells¹³⁰. In comparison with its human counterpart, avian ANP32A possesses 33 additional amino acids between its leucine-rich repeats and the C-terminal low-complexity acidic region. This insertion enables ANP32A to stimulate the activity of avian influenza virus polymerase to levels similar to mammalian-adapted polymerase in mammalian cells. Thus, ANP32A represents a host-specific factor of the influenza virus polymerase. The requirement of ANP32 proteins for cRNA to vRNA replication is consistent with previous findings that linked PB2 627-mediated host restriction to a block in avian influenza virus cRNP activity in mammalian cells⁸⁸. Although the molecular mechanisms behind the action of the ANP32 proteins remain obscure, it is tempting to speculate that they might facilitate the recruitment of the *trans*-activating polymerase to the cRNP-resident polymerase and promote its initiation of vRNA synthesis.

Several other host factors, including the RNA helicase DDX39B (also known as Bat1/UAP56), HTAT-SF1 (also known as Tat-SF1), and the mRNA export and translation stimulator FMR1 have been described to stimulate viral RNA replication by promoting either NP interactions with the viral RNA polymerase or NP recruitment to nascent viral RNA during RNP assembly^{77, 96, 131}. Host importin- α isoforms have been reported to fulfill a role in influenza virus RNA replication,

beyond their role as nuclear import factors, by interacting with the PB2 polymerase subunit in a host-dependent manner¹³².

Interactions between the viral and host transcriptional machineries on one hand provide the viral polymerase with a platform to co-ordinate cap-snatching, transcription, replication, RNA processing, as well as mRNA and vRNA nuclear export^{59, 86, 110}, but on the other these interactions might also provide the virus with an opportunity to interfere with host gene expression, inhibiting the expression of antiviral host genes. The viral NS1 protein is well documented to interfere with the 3' end formation of host mRNA via its interaction with the cellular polyadenylation machinery^{133, 134}. Additionally, interaction of the viral RNA polymerase with Pol II and cleavage of nascent capped host RNA is presumably significant for the inhibition of host gene expression, including antiviral host genes^{118, 135}. Moreover, degradation of the large subunit of Pol II has also been observed in influenza virus infected cells and been associated with increased pathogenicity of influenza virus in mice^{136, 137}.

Outlook and conclusions

The recently published high-resolution structures of the influenza virus RNA polymerase provide unprecedented insights into the mechanisms of this molecular machine. The PB1 subunit together with the C-terminal domain of PA/P3 and the N-terminal one third of PB2 form the core of the polymerase. The architecture of this core is similar to that of other viral RNA-dependent RNA polymerases. The core encompasses the polymerase active site, which is connected to the exterior by several channels, including channels for template and NTP entry as well as template and product exit. Near the active site, the priming loop is a feature that is essential for primer-independent terminal initiation on the vRNA template, but that is not required for primer-dependent transcription and, surprisingly, also not for primer-independent internal initiation on the cRNA template. The core of the polymerase is decorated by several flexible domains that include the PB2 cap-binding and PA/P3 endonuclease domains. These domains can be arranged in two different conformations that either enable cap-snatching or are incompatible with it, suggesting that the polymerase exists in multiple functional states. These functional states are controlled by the association of the polymerase with viral RNA and possibly other viral and host factors.

Although unprecedented progress has been made in understanding the structure and function of the influenza virus RNA polymerase, many long-standing questions remain unanswered. The available crystal structures have provided insights into subunit and domain organisation. However, this is only a starting point for further investigations into how this complex machine works. For example, neither of the available structures shows the polymerase in an active conformation with template RNA in the active site and we cannot rule out that the polymerase may adopt different conformations during initiation and elongation. Likewise, the conformation of the terminating polymerase might be different. Further structural analysis by crystallography and new powerful cryo-EM technologies, will be needed to visualize other functional states of the influenza virus polymerase; i.e. in complex with RNA templates, RNA primers, and incoming nucleotides during initiation, elongation and termination.

The *trans*-acting and *trans*-activating replication models discussed above indicate that multiple polymerases are required and although oligomerisation of polymerase heterotrimers and sub-complexes has been observed^{30, 138}, there is virtually no information available on how these polymerases might interact. Single-molecule techniques could greatly facilitate our understanding of the mechanics and kinetics of the polymerase during transcription and replication using fluorescently labelled polymerase and RNA templates⁴⁰.

Another avenue of interest concerns the location, host interactions and dynamics of RNPs in the nucleus. Importantly, it remains unclear whether RNPs associate with specific nuclear sites for transcription and genome replication. Do these two processes occur at the same site or different sites? It will be important to determine how host factors, such as ANP32A contribute to polymerase function and host adaptation. It will be equally important to study what cellular factors participate in the assembly of vRNPs and viral mRNPs and their nuclear export. Ultimately, understanding these molecular mechanisms will not only improve our basic scientific knowledge of the workings of an important human and animal pathogen, it could also contribute to our understanding of virulence and adaptation mechanisms of influenza viruses. Furthermore, understanding how the influenza virus RNA synthesis machine works at the molecular level and its interaction with viral and host factors could lead to the identification of targets that might be exploited for the development of drugs that can prevent or treat influenza virus infections (BOX 2).

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Competing interests statement

The authors declare no competing interests.

Display units

Box 1 Influenza A virus life cycle. Viral infection initiates with the binding of a virion to cell surface receptors containing sialic acid, followed by the endocytosis of the virion. After fusion of the viral and endosomal membranes, the vRNPs are released into the cytoplasm and then transported into the nucleus. In the nucleus the viral RNA polymerase transcribes the vRNA segments into mRNAs, which are 5' capped and 3' polyadenylated. Viral mRNA is exported to the cytoplasm for translation by cellular mechanisms. The viral RNA polymerase also performs replication of vRNA by copying it into complementary RNA (cRNA), which serves as a template for the production of more vRNA. Newly synthesised viral polymerase and nucleoprotein are imported into the nucleus and bind to cRNA and vRNA to assemble vRNPs and cRNPs, respectively. Following nuclear export, progeny vRNPs are transported across the cytoplasm on recycling endosomes in a Rab11-dependent manner to the cell membrane, where assembly of progeny virions takes place. Mature virions incorporate a substantial amount of host proteins⁷⁹ and are released by budding. Auxiliary viral proteins, PB1-F2, PB1-N40, PB2-S1, PA-X, PA-N155, PA-N182, M42, and NS3 that have been detected in infected cells, but are not known to be incorporated into progeny virions, are not shown¹³⁹.

Box 2: Therapeutic applications. Most attempts to develop antivirals targeting the influenza virus RNA polymerase have so far focused on the PB1 active site, the PB2 cap-binding and PA endonuclease domains¹⁴⁰. These attempts lead to the discovery of compounds that act as RNA chain terminators, mutagens or as inhibitors of the cap-snatching activity of the polymerase¹⁴¹⁻

¹⁵⁰. Structural studies of some of these compounds in complex with either the PB2 cap-binding or PA endonuclease domain have produced valuable data that are currently being used to improve the pharmacokinetic parameters of these compounds. The novel structural data on the polymerase heterotrimer will be a source for new drug discoveries. The structures have revealed the conformation of the active site, the presence of a priming loop, the sites of viral RNA binding, and channels for template, NTP and capped primer entry as well as template and product exit. All these features of the influenza virus RNA polymerase are putative targets for small molecule inhibitors (see figure, part **a**; cartoon model of the influenza A virus polymerase structure (PDB: 4WSB)). Disruption of heterotrimer assembly is an alternative strategy for the development of antivirals targeting the influenza virus polymerase (see figure, part **b**; cartoon model of the influenza A virus polymerase structure (PDB: 4WSB) with the interaction between the PB1 N-terminal helix and three helices of the C-terminal PA domain highlighted; additional interfaces revealed by the structures of the heterotrimeric polymerase complex are also highlighted). Studies have so far focused on the interaction of the PA C-terminal domain with the PB1 N-terminus, and the interaction between the PB1 C-terminus and PB2 N-terminus as revealed by previous structural analyses of isolated domains²³⁻²⁵. These interactions have been targeted by antiviral peptides, peptidomimetics or small molecules¹⁵¹⁻¹⁵⁸. The structures of the complete polymerase heterotrimer reveal numerous interaction interfaces, such as the interface between PB2 helix 6 and PB1 helices 17 and 19 of the PB1 thumb subdomain, that could be targeted using similar strategies (see figure, part **b**). In addition, interfaces may be targeted that are only present in one of the various conformational states that have been observed in the crystal structures. Examples are the interaction between the penultimate helix of PB1 and helix 4 of the PA endonuclease, which is only evident in the vRNA promoter-bound polymerase, and the various inter-domain interactions in the C-terminal two thirds of PB2. ‘Locking’ the polymerase in a particular conformation using a small molecule might be yet another viable strategy for the development of antivirals. Structural studies of the influenza virus polymerase in complex with other viral, e.g. NP, or host factors are likely to reveal additional interaction interfaces that could be targeted. For example, the site of the interaction with the CTD of Pol II is currently unknown but it could represent an attractive target for an antiviral. Similarly, ANP32A has emerged as an interactor of the influenza virus polymerase and a critical factor for the replication activity linked to the PB2 627-domain^{129, 130}. Uncovering the mode of interaction of ANP32A with the viral

polymerase could reveal yet another site in the polymerase that could be targeted. The availability of techniques to express and purify large quantities of the heterotrimeric polymerase will further facilitate these studies in the context of the polymerase complex and RNPs.

Figure 1 Model of the vRNP complex and structures of the influenza A, B and C virus RNA polymerases. **a** Model of the vRNP complex. In the vRNP, the 5' and 3' termini of vRNA are bound by a heterotrimeric polymerase complex and the rest of the vRNA is coated by nucleoprotein. The complex is twisted into an anti-parallel double helix, the structure of which is maintained by contacts between NP monomers. The RNA forms a loop at the end opposite the polymerase-bound end^{9, 10}. **b** Front and side views of the influenza A (PDB: 4WSB), B (PDB: 4WSA) and C (PDB: 5D98) virus RNA polymerase structures shown in surface (top and middle row) or cartoon model (bottom row) representation. PB1, PB2 and PA/P3 subunits are coloured in blue, pink and light green, respectively. The PB2 cap-binding (PB2-cap), PB2 627-domain (PB2-627), PA/P3 endonuclease (PA-endo) and PA/P3 C-terminal (PA-C) domains are indicated. The 5' and 3' termini of vRNA in the influenza A and B virus polymerase structures are shown in dark grey and yellow, respectively. In the influenza A virus polymerase the cap-binding and endonuclease domains face each other, configured for cap-snatching (left, bottom row). In the influenza B virus polymerase structure the cap-binding domain is rotated to face the product exit channel consistent with insertion of the capped primer into the active site via the product exit channel (middle, bottom row). In the influenza C virus polymerase structure the cap-binding and endonuclease domains face opposite directions, incompatible with cap-snatching (right, bottom row).

Figure 2 Polymerase architecture and channels. **a-d** Cartoon models of the influenza A virus polymerase (PDB: 4WSB), in different orientations, showing PB1 polymerase motifs A to F and the priming loop (**a**), the right handed arrangement of the PB1 fingers, palm and thumb subdomains and the fingertips (**b**), the PA (**c**) and PB2 (**d**) domain structures. The 5' and 3' termini of vRNA are coloured as in FIG. 1. **e, f** Models of the RNA polymerase in the 'inactive' (**e**) and 'transcription pre-initiation' states (**f**) showing the conformational re-arrangement of the flexible peripheral cap-binding, 627 and endonuclease (Endo) domains. The temple entry and exit, NTP entry and product exit channels, the priming loop near the active site as well as the binding of the 5' and 3' vRNA termini and the positions of nucleoproteins (NP) in the 'transcription pre-initiation' model are shown. The U-stretch near the 5' end of the vRNA that

acts as a poly(A) signal is indicated.

Figure 3 Models of viral transcription (mRNA synthesis) and replication (cRNA and vRNA synthesis).

a Model for mRNA synthesis. The polymerase is depicted as in FIG. 2f in the ‘transcription pre-initiation’ state. Host capped RNA is bound by the PB2 cap-binding domain and cleaved by the PA/P3 endonuclease domain. The cap-binding domain rotates to allow the insertion of the 3’ end of the capped RNA primer into the active site via the product exit channel²². The 3’ end of the vRNA template also inserts into the active site and NTPs enter via the NTP entry channel. Transcription is initiated by the addition of a GTP to the 3’ end of the capped primer that is templated by the second residue in the vRNA template. During elongation, product and template are separated and they exit through their respective exit channels. As the template is pulled into the active site, NP detaches from the entering vRNA, translocates via the surface of the polymerase and joins the vRNA template as it emerges through the template exit channel. The 5’ cap is released from the PB2 cap-binding domain as the polymerase enters elongation⁵⁷. Termination is achieved through polyadenylation that occurs as the result of repeated copying of the U-stretch of the vRNA template⁵⁶. The 5’ end of the vRNA remains bound to its binding site and is stabilized by base-pairing with the 3’ end of the vRNA that re-binds at its binding site near the surface of the polymerase during elongation. **b** Model for cRNA synthesis. The polymerase is shown in the same conformation as for mRNA synthesis. The 3’ of the vRNA inserts into the active site and NTPs enter via the NTP entry channel. *De novo* initiation occurs at the first residue of the vRNA template (terminal initiation) and the initiating nucleotide is stabilized by the priming loop^{22, 49, 62}. During elongation, product and template are separated and they exit through their respective exit channels. NP translocates via the surface of the polymerase to join the vRNA template as it emerges through the template exit channel. The 5’ end of the cRNA product is bound by a second polymerase as it emerges from the product exit channel and the rest of the RNA associates with NP to assemble cRNPs. For termination, the 5’ end of the vRNA template is released from its binding site and is pulled through the polymerase active site while the 3’ end re-binds at the surface of the polymerase. **c** Model for vRNA synthesis. The polymerase is depicted as in FIG. 2e in the ‘inactive’ state consistent with the conformation of the polymerase bound to the 5’ end of cRNA³³. For replication initiation to take place the polymerase needs to interact with a *trans*-activating polymerase⁶¹. The 3’ of the cRNA inserts into the active site and NTPs enter via the NTP entry channel. *De novo* initiation occurs at

the fourth and fifth residue of the cRNA template (internal initiation) without the participation of the priming loop^{22, 49, 62}. The resulting pppApG dinucleotide re-locates to the 3' end of the cRNA template (not shown) and is elongated by the polymerase. Elongation and termination proceed as described above for cRNA synthesis. The 5' end of the vRNA product is bound by the *trans*-activating polymerase and the rest of the vRNA associates with NP to assemble vRNPs. Note that an alternative model, involving a *trans*-acting polymerase, has also been proposed for cRNA to vRNA replication (not shown)^{10, 71}.

Figure 4 Regulation of transcription and replication. Viral RNA synthesis is performed in association with the chromatin/nuclear matrix close to sites of host Pol II transcription. This association is mediated by nuclear matrix and chromatin factors. For transcription, vRNPs bind to the serine-5 phosphorylated CTD of the large subunit of Pol II to access nascent capped host RNA for cap-snatching^{118, 119}. Polyadenylation of viral mRNA is stimulated by the host splicing factor SFPQ/PSF; viral NS1 and host RED, SMU1, SF2/ASF, NS1-BP, hnRNP K participate in mRNA processing^{84, 85, 123-126}. The host cap-binding complex (CBC) binds to the 5' cap once it is released by PB2⁵⁸. vRNPs in close proximity to Pol II but not bound to the CTD of Pol II carry out cRNA synthesis. cRNA serves as template for vRNA synthesis. Free viral polymerase and NP are required for the assembly of cRNA and vRNA into cRNP and vRNP, respectively. DDX39B, HTAT-SF1 and FMR1 stimulate viral RNA replication by promoting NP interactions with the viral RNA polymerase or NP recruitment to nascent viral RNA during cRNP and vRNP assembly^{77, 96, 131}. Viral NS1 regulates viral RNA replication by interacting with NP⁸⁰⁻⁸³ and viral NEP promotes viral RNA replication, possibly via the stimulation of svRNA synthesis⁸⁷⁻⁸⁹. svRNAs are implicated as viral co-factors in genome replication⁹¹. The cellular DNA helicase MCM as well as proteins of the ANP32 family are implicated in genome replication^{128, 129}. ANP32A may possibly contribute by recruiting a *trans*-activating polymerase to the resident cRNP-bound polymerase in a host- and PB2 627-domain-specific manner to facilitate vRNA synthesis although the exact mechanism of its involvement remains unknown^{88, 129, 130}. Importin- α , which acts as import factor for PB2, is proposed to have a role in transcription and replication independent of its role as a nuclear import receptor^{26, 132}. Importin-5 (also known as RanBP5) acts as a co-factor for the nuclear import of the PB1-PA dimer^{16, 107, 109}.

Supplementary Video 1 **Conformational flexibility of the influenza virus RNA polymerase.** Morph between the structure of the RNA-free influenza C virus polymerase (PDB: 5D98) and

that of vRNA promoter-bound influenza A virus polymerase (PDB: 4WSB). PB1 and PA are coloured orange and blue, respectively. PB2 is coloured according to its domain structure with the N-terminal one third shown in green, the cap-binding domain in dark yellow, the mid-link in light yellow, the 627-domain in red and the C-terminal NLS in brown. Promoter RNA is shown in red. Modified from Hengrung *et al*¹⁹.

Glossary terms

Haemagglutinin Type I integral membrane glycoprotein that binds to cell-surface receptors and facilitates fusion between the viral envelope and endosomal membrane. It is the main target antigen of the humoral immune response of the host.

Neuraminidase Type II integral membrane glycoprotein, which facilitates viral release from cells by removing sialic acid from sialyloligosaccharides on the cell and viral surface.

Cap-snatching A process in which a cellular capped RNA is cleaved a few nucleotides downstream of the 5' cap by an endonuclease activity encompassed within a viral RNA-dependent RNA polymerase.

Host range determinant Characteristic of a pathogen that allows it to replicate in a particular host.

Panhandle structure A double-stranded RNA structure formed by the conserved 5' and 3' RNA termini of negative-sense viral RNA genomes.

Apo structure A structure of a protein with no ligand bound.

Helicase An enzyme that catalyzes the unwinding and separation of double-stranded DNA or RNA using energy from ATP hydrolysis.

De novo initiation Initiation step of nucleic acid synthesis by means of a primer-independent mechanism.

Carboxyl-terminal domain (CTD) of Pol II An unstructured, evolutionarily conserved domain at the C-terminus of the largest Pol II subunit that comprises tandem copies of the consensus heptapeptide YSPTSPS. Phosphorylation of these repeats is crucial for the regulation of Pol II function.

Pol II pausing A control step in gene transcription by which RNA Pol II pauses at certain sites and requires specific stimuli and elongation factors to overcome the pausing block to enter productive elongation.

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10. Moeller et al 2012 This paper describes the three-dimensional structure of native RNPs derived from cells expressing influenza virus RNP complex components.

19. Hengrung et al 2015 This paper presents the crystal structure of the RNA-free influenza C virus polymerase that reveals a radically different configuration of the PB2 C-terminal domains compared to the influenza A and B virus polymerase structures obtained in the presence of vRNA. SAXS studies show that the polymerase can take up a variety of conformations.

21. Pflug et al 2014 This paper presents the crystal structure of bat influenza A virus polymerase which shows the architecture of the heterotrimeric complex and the structural basis for binding the vRNA promoter which comprises the conserved 5' and 3' termini of the vRNA.

22. Reich et al 2015 This paper presents the crystal structure of the influenza B virus polymerase and shows the rotation of the PB2 cap-binding domain guiding the 3' end of the capped RNA primer into the polymerase active site.

33. Thierry et al 2016 This paper presents structures of the influenza B virus polymerase showing a similar arrangement of the PB2 C-terminal domains as observed for the influenza C virus polymerase. Solution studies show the polymerase can take up a variety of conformations.

49. te Velthuis et al 2016 This paper shows that the priming loop of the influenza A virus polymerase is required for terminal *de novo* replication initiation on the vRNA template but it is not required for internal *de novo* replication initiation on the cRNA template and for capped RNA primer-dependent transcription initiation.

61. York et al 2013 This paper reports the isolation, structural and functional characterization of the influenza A virus cRNP replicative intermediate. It proposes that the cRNP associated polymerase requires a *trans*-activating polymerase to be able to carry out vRNA synthesis.

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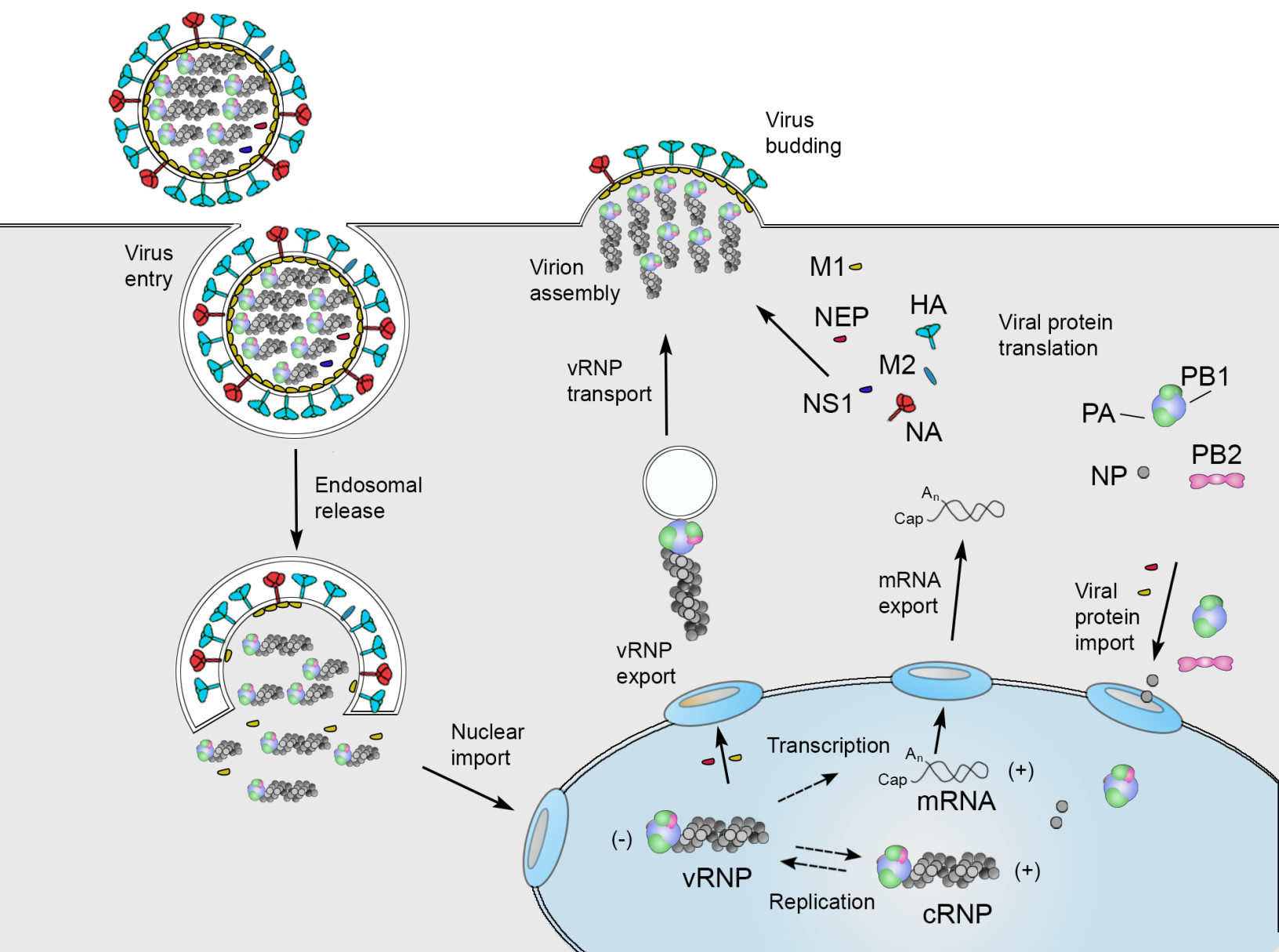
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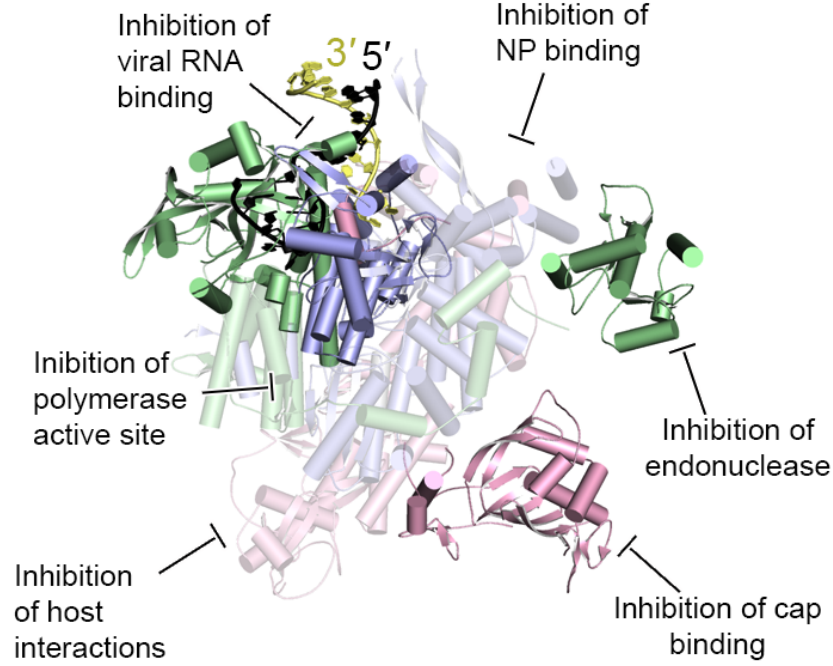
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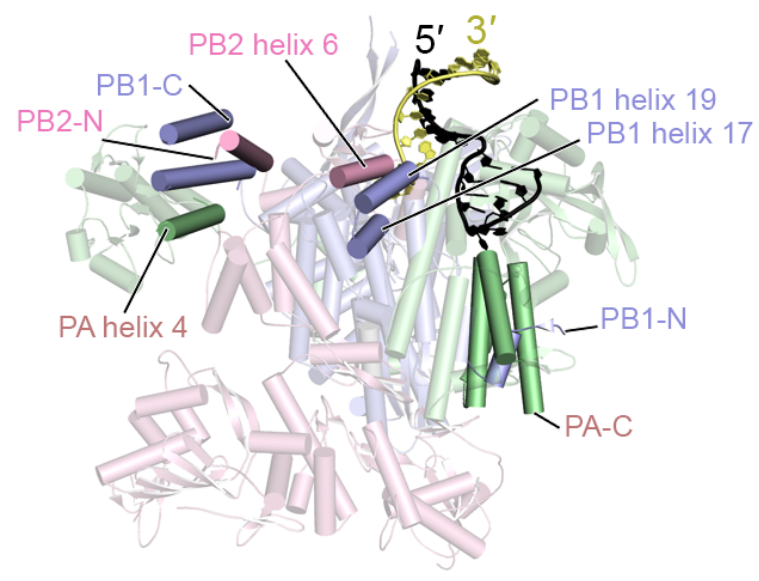
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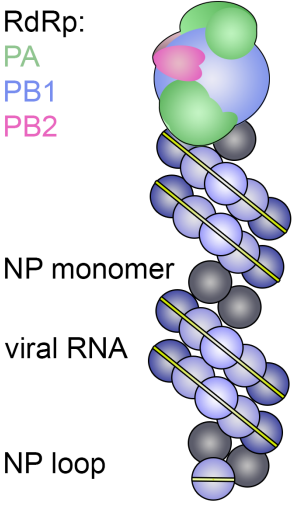
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