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Structure and Function of Influenza Polymerase

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Influenza polymerase (FluPol) plays a key role in the viral infection cycle by transcribing and replicating the viral genome. FluPol is a multifunctional, heterotrimeric enzyme with cap-binding, endonuclease, RNA-dependent RNA polymerase and polyadenylation activities. It performs its functions in the context of the viral ribonucleoprotein particle (RNP), wherein the template viral RNA is coated by multiple copies of viral nucleoprotein. Moreover, it interacts with a number of host proteins that are essential cofactors and, consequently, adaptive mutations in the polymerase are required for crossing the avian–human species barrier. In this review, we show how mechanistic understanding of how FluPol performs its multiple functions has greatly advanced over the last decade through determination of high-resolution structures by X-ray crystallography and cryo-electron microscopy. These have revealed not only the detailed architecture of FluPol but highlighted the remarkably conformational flexibility that is inherent to its functioning as a dynamic RNA synthesis machine. Structural studies are also underpinning current attempts to develop next-generation anti-influenza drugs that directly target FluPol.

Influenza virus possesses a heterotrimeric RNA-dependent RNA polymerase (FluPol) composed of subunits PA (polymerase acidic protein, P3 in influenza C), PB1 (polymerase basic protein 1), and PB2 (polymerase basic protein 2) of total molecular weight of ~270 kDa (Fig. 1A). Each of the negative-sense, single-stranded (ss) RNA genome segments of influenza viruses is packaged as a distinct ribonucleoprotein (RNP) by multiple copies of the viral nucleoproteins (NPs) together with one polymerase complex. The RNP is the functional context in which FluPol transcribes and replicates the viral RNA (vRNA). In the RNP, the polymerase is bound to the conserved and quasicomplementary 3' and 5' ends of the vRNA, jointly known as the “promoter,” which pseudocircularizes the

ssRNA. Synthesis of viral mRNA (transcription) starts with “cap-snatching” of short, capped oligomers (10–15 nt in length) from nascent host Pol II transcripts, using the PB2 cap-binding and PA endonuclease activities of FluPol (Plotch et al. 1981). These oligomers prime template-directed RNA synthesis at the PB1 active site, following the typical polymerase sequence of initiation, elongation, termination, and recycling. However, in the case of FluPol transcription, termination involves stuttering at the 5' proximal oligo(U) polyadenylation signal, thus generating the viral mRNA poly(A) tail (Poon et al. 1999). FluPol thus uses unique mechanisms to synthesize capped and polyadenylated viral mRNAs fully compatible with the downstream processing (e.g., transport, splicing, and translation) by the

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Figure 1. Overall structure of influenza polymerase. (A) Schematic of the three subunits showing major domains. Colors used throughout this review are PA-N endonuclease (dark green), PA linker (light green), PA-C (medium green), PB1 (cyan), PB2-N (red), PB2 midlink (purple), PB2 cap-binding domain (orange), PB2 627 domain (salmon), PB2 NLS domain (dark red). (B) Front and back views of influenza polymerase in the transcription active conformation in surface (*top*) and ribbon (*bottom*) representation showing the major domains colored as in A. Drawn from PDB: 4WSB.

host gene expression machinery. Synthesis of unmodified genome copies (replication) occurs first by FluPol copying vRNA into complementary, positive-sense cRNA, with the cRNA cosynthetically being packaged with newly synthesized NP and FluPol into cRNPs. Subsequently, cRNPs replicate by a similar but not identical process into vRNPs that can be the template for further (secondary) transcription or exported from the

nucleus and packaged into progeny virions. Replication differs from transcription in being unprimed, and termination occurs at the template 5' extremity with read-through of the polyadenylation signal.

It has always been a goal to understand in atomic resolution detail how FluPol carries out its unique functions, but solving the structure of the complete heterotrimer proved a tough nut

to crack, largely because of the difficulty of producing sufficient amounts of pure, active polymerase. The first, atomic-resolution crystal structures of independently folded domains and subunit interfaces were published in 2007–2009 (Tarendeau et al. 2007, 2008; Guilligay et al. 2008; He et al. 2008; Obayashi et al. 2008; Dias et al. 2009; Sugiyama et al. 2009; Yuan et al. 2009; for review, see Ruigrok et al. 2010) and only in 2014 was the structure of the complete heterotrimer determined (Pflug et al. 2014; Reich et al. 2014). More recently, crystallographic and/or cryo-electron microscopy (cryo-EM) structures of the full-length heterotrimer have been obtained for influenza viruses of types A, B, C, and D in several functional states. Together, these structures give detailed insight into the mechanism of action of this remarkably dynamic, multifunctional RNA synthesis machine as well as underpinning new opportunities for anti-influenza drug development. Here we summarize the current understanding of the architecture of FluPol and outline its structure-based mechanism of action, particularly for transcription.

FluPol is a highly flexible enzyme, comprising a catalytic core to which are attached flexibly linked peripheral domains (Thierry et al. 2016). It therefore has multiple different interchangeable conformations, even though in a crystal, a particular conformation can be selectively trapped. Indeed the first promoter-bound crystal structures trapped what is now referred to as the “transcriptionally active” state, which will now be described. Unless otherwise stated, residue type and number will refer to the avian/human influenza A strains.

OVERALL STRUCTURE OF INFLUENZA POLYMERASE

Transcriptionally active FluPol has a U-shaped structure, with approximate height, width, and depth of $115 \times 100 \times 75$ Å, respectively (Fig. 1B). The two protruding arms are capped by the PA-N endonuclease (Dias et al. 2009; Yuan et al. 2009) and PB2 cap-binding (Guilligay et al. 2008) domains, which face each other across a solvent channel. The endonuclease is held in place by one of its helices contributing to the

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helical bundle formed by the interface between the carboxyl terminus of PB1 and the amino terminus of PB2 (Sugiyama et al. 2009). The bottom of the U is formed by the large PA-C domain (He et al. 2008; Obayashi et al. 2008). The carboxy-terminal two-thirds of PB2 (PB2-C) form one side of the U and comprise the midlink, 627, and NLS (nuclear localization signal) domains (Tarendeau et al. 2007, 2008). The body of the trimer is formed by the catalytic core of PB1, which has the canonical right-handed polymerase architecture comprising fingers, palm, and thumb. On the thumb side, it is decorated by the amino-terminal third of PB2 (PB2-N), on the opposite side, by three consecutive short helices of the PA linker that wraps around PB1 and connects the PA-N endonuclease with PA-C. Apart from the two hydrophobic intersubunit interfaces between PA-C and PB1-Nter (He et al. 2008; Obayashi et al. 2008) and PB1-Cter and PB2-Nter (Sugiyama et al. 2009), which stably link the subunits in head-to-tail fashion, there are numerous other hydrophilic intersubunit interactions, which can be reversibly broken or formed, and flexible interdomain linkers that permit remarkable conformational flexibility (Thierry et al. 2016).

FUNCTIONAL DOMAINS

Cap-Binding Domain

The cap-binding domain (residues 320–485 of PB2) (Fig. 2A,B) has a unique fold, distinct from cellular cap-binding proteins such as the translation initiation factor eIF4E or the nuclear cap-binding complex (CBC) (Guilligay et al. 2008). It does, however, preserve the aromatic sandwich formed by residues His357 and Phe404, as the means by which specificity for the cap-specific methylated guanosine (m^7G) is enhanced compared to the unmethylated base (Fig. 2B, left). The isolated FluA cap-binding domain binds relatively weakly to the cap analog m^7Gppp (Guilligay et al. 2008), and even more weakly for FluB (Xie et al. 2016), but in the heterotrimer context, additional interactions to the capped RNA physiological substrate are provided by the adjacent PB2 midlink domain (Fig. 2A; Pflug

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Figure 2. (Legend on facing page)

et al. 2018). Even so, it is not yet clear how influenza polymerase is able to preferentially pirate fully capped nascent Pol II transcripts in competition with the much higher affinity nuclear CBC that normally sequesters the cap early in transcription. A key insight emerging from the original crystal structures of the complete heterotrimer was that the cap-binding domain was flexibly linked to the polymerase core allowing its rotation, presumably stochastically, over a large angle ($\sim 70^\circ$) (Fig. 2D; Reich et al. 2014; Pflug et al. 2018). One extreme position is consistent with the cap-bound nascent Pol II transcript reaching across to the endonuclease to be cleaved during cap-snatching. The other extreme position allows the cleaved primer to be directed into the RNA synthesis active site to initiate transcription (Pflug et al. 2018; Kouba et al. 2019). As for the endonuclease, the cap-binding domain is a promising target for anti-influenza drugs (Pautus et al. 2013; Mifsud et al. 2019). The most advanced compound to date is VX-787/JNJ63623872/pimodivir, which is very potent against FluA polymerase because of picomolar affinity for the cap-binding site (Fig. 2B, right; Clark et al. 2014). In contrast, it has no antiviral activity against FluB because it binds too weakly to the FluB cap-binding domain as a result of amino acid differences in the cap-binding site (Pflug et al. 2018). Interestingly, part of the potency of pimodivir derives from its binding to not only the cap-binding domain but also to the adjacent midlink domain, thus stabilizing a particular configuration of these domains that is transcriptionally inactive

(see the section Conformational Flexibility and Dimer Formation; Ma et al. 2017; Pflug et al. 2018). However, resistant mutations to pimodivir (e.g., PB2 S324K/N/R, F325L, S337P, M431I, N510T) have been selected in vitro (Byrn et al. 2015; Ma et al. 2017; Pflug et al. 2018) or in treated patients (Trevejo et al. 2018; Finberg et al. 2019). Combination therapy with other anti-influenza drugs may be a solution to this problem (Mifsud et al. 2019).

Endonuclease

The cap-snatching endonuclease (residues 1–196 of PA) is a member of the PD-(D/E)xK two-metal nuclease superfamily (Dias et al. 2009; Yuan et al. 2009). Conserved residues His41, Glu80, Asp108, and Glu119 chelate the two essential divalent cations (probably magnesium in vivo, but manganese works better in vitro), and Lys134 also plays an important catalytic role (Fig. 2C; Crepin et al. 2010; Kowalinski et al. 2012; Datta et al. 2013). The isolated endonuclease domain has weak affinity for RNA, which probably explains why there is still no structure available of an RNA substrate complex. However, in the context of the complete heterotrimer, the *cis*-acting cap-binding domain considerably enhances the affinity for the capped nascent Pol II transcript, which is the physiological substrate (Pflug et al. 2018). Because the endonuclease is essential to provide the capped primers for initiating transcription, endonuclease inhibitors that chelate the two-metal ions are potential anti-influenza drugs. Since the 1990s, many

Figure 2. Cap-snatching. (A) Capped RNA primer (slate blue) binding to the PB2 cap-binding and midlink domains and descending into the PB1 active site to initiate transcription. Drawn from PDB: 6QCX. (B) Close-up of (*left*) cap-analog m^7 GTP (slate blue) and (*right*) VX787/pimodivir (pink) binding in the influenza A cap-binding domain showing conserved interacting residues (orange sticks). Drawn from PDB: 4CB4 and 6EUU, respectively. (C) Surface representation of influenza A endonuclease with bound baloxavir acid (BXA) in the active site, showing two divalent cations (purple spheres) and conserved interacting residues (yellow sticks). The substitution I38T confers partial resistance to the drug. Drawn from PDB: 6FS6. (D) Schematic of how cap-snatching is performed. FluPol (gray surface with cap-binding domain in orange and endonuclease in green) binds to the serine 5 phosphorylated (pS5) carboxy-terminal domain (CTD) of host polymerase II (Pol II, light blue surface). This gives FluPol access to emerging nascent capped transcripts (red) that bind to the cap-binding domain and are cleaved 10–15 nt downstream by the endonuclease. The cap-binding domain then rotates to direct the capped primer into the PB1 RNA synthesis active site to initiate transcription.

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inhibitors have been described (Tomassini et al. 1994; DuBois et al. 2012; Kowalinski et al. 2012; Sagong et al. 2014; Stevaert and Naesens 2016; Mifsud et al. 2019) culminating in the discovery of baloxavir acid (BXA), which potently inhibits FluA and FluB endonuclease. Under the trade name Xofluza, a pro-drug of BXA is approved for clinical use in Japan and the United States (Noshi et al. 2018). However, an I38T mutation in the endonuclease active site, which can emerge during treatment, significantly reduces susceptibility to the drug (Omoto et al. 2018; Mifsud et al. 2019), and such resistant viruses can propagate between humans (Imai et al. 2020). Combination therapy with, for instance, Tamiflu is being tested as a potential means to get around this problem.

627-NLS Double Domain

The 627-NLS double domain (PB2/538-757) forms the carboxy-terminal region of PB2 and is separable into two independently folded subdomains, the “627 domain” and the “NLS domain,” separated by a linker (PB2/679-684) (Fig. 3A; Tarendeau et al. 2008; Kuzuhara et al. 2009). The 627 domain (PB2/538-678) is so-called because it contains the host-specific residue 627 (see the subsection Host-Specific Mutations). The NLS domain (PB2/685-759) contains a flexible carboxy-terminal extension beyond PB2/736 that features the PB2 bipartite nuclear localization sequence with minor (737-RKR-739) and major (752-KRIR-755) basic motifs that are recognized and bound by the

Figure 3. Host-protein interacting domains. (A) Ribbon diagram showing the closed state of the PB2 627-NLS double domain. (B) Binding of the PB2 NLS domain, which carries a bipartite nuclear localization signal (NLS) in its carboxy-terminal extremity, to nuclear import factor importin- α . Drawn from PDB: 2JDQ. (C) Serine 5 phosphorylated Pol II CTD binding to influenza A, B, and C polymerases. In each case, two distinct sites are observed.

nuclear import adapter importin- α (Fig. 3B; Tarendeau et al. 2007; Pumroy et al. 2015). In crystal structures of the isolated double domain and in the “transcriptionally active” conformation of the trimer, the two folded subdomains are packed against each other (Fig. 3A), whereas in the so-called “apo”-conformation of the polymerase (in which the promoter is not bound, see the section Conformational Flexibility and Dimer Formation) the two domains are separated and the linker is extended (Hengrunga et al. 2015; Thierry et al. 2016). In solution the equilibrium between the open and closed configurations of the 627-NLS double domain has been characterized by NMR, and it has been shown that the open configuration is required for importin- α binding (Delaforge et al. 2015). The 627-domain is not required for transcription in vitro (Nilsson et al. 2017), but in vivo it contributes to binding the Pol II CTD during cap-snatching (see the subsection The PA-C Domain and Host Pol II CTD Binding). The domain is also required for replication, perhaps for recruiting a second polymerase to package the nascent replicate (Nilsson et al. 2017).

Host-Specific Mutations

Both the 627 and NLS domains of PB2 are hotspots for host-specific residues that mutate during interspecies transmission (Peacock et al. 2019). First and foremost is residue 627 itself, which is almost invariably a glutamate in avian strains but needs to be a lysine for efficient polymerase activity in mammalian cells (Subbarao et al. 1993; Hatta et al. 2001). However, the combination of the avian Glu627 signature with additional substitutions G590S and Q591R in the same domain, as found in the 2009 pandemic A strain, also confers high activity in mammalian cells (Mehle and Doudna 2009). Consistently, these avian-to-human substitutions render the surface of the 627 domain more extensively positively charged in human compared to avian variants (Tarendeau et al. 2008; Mehle and Doudna 2009). The specific role of the different signatures at position 627 is still not understood mechanistically. However, a recent breakthrough has correlated charac-

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teristic differences between the avian and human versions of the host factor ANP32, which is important for vRNA to cRNA replication (Sugiyama et al. 2015), with host differences in the 627 domain signature (Long et al. 2016; Mistry et al. 2019). PB2 residues 701 and 702 of the NLS domain are also strongly implicated in host adaptation (Chin et al. 2017). Residue 701 is normally an aspartate in all strains but the D701N substitution is selected upon adaptation of highly pathogenic avian strains to mice (Li et al. 2005; Gabriel and Fodor 2014) and also in cases of avian to human or guinea pig transmission (de Jong et al. 2006; Steel et al. 2009). Although it has been suggested that the D701N substitution could affect the interaction with importin- α (Tarendeau et al. 2007), the exact role of these two residues remains to be elucidated.

The PA-C Domain and Host Pol II CTD Binding

The large PA carboxy-terminal domain (PA/258-713) has an important structural role in tightly binding the extreme amino terminus of PB1 and more generally buttressing the entire trimer. However, it also has specific functional roles in promoter binding (see the section Different Modes of Promoter Binding), in interacting with the carboxy-terminal domain (CTD) of host Pol II to enable cap-snatching, and in contributing to the recently discovered secondary 3' end binding site (see the section Different Modes of Promoter Binding). Cap-snatching is thought to occur while Pol II is paused to allow transcript capping, soon after transcription initiation (Fig. 2D). At this stage, the 52 heptad repeats of the CTD are preferentially phosphorylated at the serine 5 position. Although it was shown some time ago that FluPol interacts, probably directly, with the serine 5 phosphorylated form of the CTD (Engelhardt and Fodor 2006), the structural basis for binding was only revealed recently for influenza A and B (Lukarska et al. 2017) and C (Serna Martin et al. 2018) polymerases (Fig. 3C). In each case, there is a different bipartite binding site, exclusively or partially on PA-C. For influenza A, there are two phosphoserine binding sites on PA-C with

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the phosphates bound respectively by conserved Lys635 and Arg638 (site 1A) and Lys289 and Arg449 (site 2A). Mutation of any one of these residues severely attenuates viral replication (Lukarska et al. 2017), suggesting that disruption of CTD binding by FluPol could be a novel antiviral strategy. However, the highly deleterious R638A mutation can be largely compensated by a second site C453R mutation that restores phosphoserine binding (Fodor et al. 2003; Lukarska et al. 2017). Interestingly, site 2A involves residues from the PA 550-loop, some of which are also implicated in interspecies adaptation (Lukarska et al. 2017). In influenza B, site 1 is conserved (and denoted site 1B) but site 2A is replaced by a distinct site 2B that crosses over from the PA subunit to the PB2 627 domain (Lukarska et al. 2017). For influenza C, both sites 1C and 2C are distinct from previously described sites. They are primarily on PA-C, although site 1C also involves PB1 residues (Serna Martin et al. 2018). These results show that FluPol binds multiple, not necessarily adjacent Pol II CTD repeats, with each individual interaction being only in the micromolar range but, through avidity and cooperativity, resulting in an overall high-affinity interaction. Finally, there is evidence that CTD binding across to the 627 domain stabilizes the transcriptionally active form of the polymerase (Serna Martin et al. 2018).

DIFFERENT MODES OF PROMOTER BINDING

The vRNA promoter is composed of the conserved, quasicomplementary pyrimidine-rich 3' (nucleotides 1–12) and purine-rich 5' (nucleotides 1–13) termini (Fig. 4A). The isolated promoter RNA can form an irregular base-paired duplex, but this is not how it binds to the polymerase. Indeed, it is unlikely that the full “panhandle” duplex has any role in influenza replication, although it may bind as such to the innate immune RNA sensor RIG-I (Lee et al. 2016). When bound to the polymerase, nucleotides 1–10 of the 5' end form an intramolecular stem-loop, the so-called “hook,” with four base-pairs, two of them noncanonical

A-A base-pairs (A1:A10 and A4:A7) (Fig. 4A; Pflug et al. 2014). This compact RNA structure, which forms with both vRNA and cRNA (Thierry et al. 2016), binds with high affinity in a specific pocket in the interface between the PA and PB1 subunits. The 5' hook is secured by a latch formed by the so-called PA-arch and PB1 β -hairpin, thus becoming an integral part of the polymerase structure (Fig. 4B; Pflug et al. 2014). This places the loop of the hook (nucleotides 5–6) close to the polymerase active site. Indeed, 5'-hook binding is critical for correctly structuring critical peptides (e.g., the fingertips) on the RNA synthesis active site and thus acts as an allosteric activator.

The proximal vRNA 3' end nucleotides 1–9 (or 1–11 for cRNA), where RNA synthesis initiates, are single-stranded and flexible, allowing different modes of binding to FluPol depending on the polymerase functional state. In the pre-initiation (resting) state (i.e., in the absence of ongoing RNA synthesis), the vRNA 3' end is particularly mobile (Robb et al. 2019) and has been observed to take up three positions. The first corresponds to the 3' end entering through the template entrance tunnel into the PB1 RNA synthesis active site (Fig. 4B; Reich et al. 2017). This has been designated as mode A promoter binding (Peng et al. 2019). Alternatively, for FluB polymerase, the 3' end has been observed on the external surface of the polymerase, interacting with the PB1 β -ribbon (Reich et al. 2014). More recently, it has been found that the 3' end nucleotides 1–5 can bind sequence specifically in a “secondary 3'-end binding site” between PA-C and the PB1 thumb, which has been called mode B promoter binding (Fig. 4C; Fan et al. 2019; Peng et al. 2019; Wandzik et al. 2020). In the case of cRNA, the longer single-stranded 3' end is considerably less flexible (Robb et al. 2019) and preferentially bound in the secondary 3'-end binding site (i.e., mode B) (Fan et al. 2019; Peng et al. 2019). Importantly, the secondary 3'-end binding is mutually exclusive with the homodimer interface (Fan et al. 2019). This suggests that dimerization with a second “activating” polymerase *in trans* might promote transfer of the cRNA 3' end to the active site, thus initiating cRNA to vRNA replication, although the

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Figure 4. Promoter binding. (A) Schematic of the vRNA promoter structure that comprises the conserved 5' (pink) and 3' (yellow) extremities of each genomic vRNA segment. Nucleotides 1–10 of the 5' end form a stem-loop structure called the “hook,” which binds tightly to the polymerase and acts as an allosteric regulator of polymerase function. Nucleotides 1–9 of the 3' end are single-stranded and flexible. The distal duplex region is formed by interstrand base-pairs. The cRNA promoter forms a similar structure, but the single-stranded region of the 3' end is 2 nt longer. (B) Mode A promoter binding showing the 5' hook bound in a pocket between the PB1 and PA-C domains and the 3' end of the template entering the PB1 RNA synthesis site. (C) Mode B promoter binding showing the vRNA 3' end binding in the “secondary 3'-end binding site.”

detailed molecular mechanism of such regulation of replication remains unknown.

The distal parts of the promoter 5' and 3' ends typically make four canonical base-pairs with each other (e.g., 5' nucleotides 11–14 base-pair with 3' nucleotides 10–13 for vRNA, and 5' nucleotides 11–14 base-pair with 3' nucleotides 12–15 for cRNA) (Fig. 4A). This short duplex projects away from the polymerase. Stabilization of the interstrand base-pairing between the promoter ends is one way the polymerase maintains the pseudocircularity of the viral RNA. However, even in the absence of this base-pairing (which must be broken once RNA synthesis starts and the template begins to translocate), the existence of the high affinity 5' hook and secondary 3'-end binding site provide a second means by which the polymerase can

remain bound to both genome segment ends. Indeed, at least for transcription, there is now strong evidence that during the complete cycle of viral mRNA synthesis, the polymerase never lets go of either end of the vRNA (see the section Model for Complete Transcription Cycle).

CONFORMATIONAL FLEXIBILITY AND DIMER FORMATION

As noted above, FluPol comprises a relatively stable core comprising PA-C, PB1, and PB2-N with flexibly linked peripheral PA-N and PB2-C domains that can be repacked in very distinct ways or simply left dangling, depending on the functional state of the polymerase (Fig. 5). As described above, during cap-snatching, the PB2 cap-binding domain rotates in situ by $\sim 70^\circ$

Figure 5. Polymerase conformational flexibility. (A) Schematic showing the FluPol core, comprising PA-C, PB1, and PB2-N, to which are flexibly attached the peripheral PA endonuclease and PB2 midlink, cap, 627-, and NLS domains. (B) Surface representation of domain arrangement in the transcriptionally active state with bound full vRNA promoter. Drawn from PDB: 4WSB. (C) Domain arrangement in the transcriptionally inactive state of FluB polymerase with bound cRNA 5' end only. Drawn from PDB: 5EPI. (D) Domain arrangement in the transcriptionally active state of FluA polymerase with no bound RNA (apo). Similar to C, but endonuclease and 627-domains orientated differently. Drawn from PDB: 5D9A. (E) Symmetric dimeric form of influenza A polymerase as observed in the crystal. The conformation of each monomer is as in D. Drawn from PDB: 6QNW.

(Fig. 2D). Even more dramatically, crystal structures of FluC (Hengrung et al. 2015), FluB (Thierry et al. 2016), and FluA (Fan et al. 2019) have trapped the so-called “apo” state (i.e., in the absence of any promoter RNA or with only the cRNA 5′ hook bound in the case of FluB), in which the PA endonuclease and PB2-C midlink, cap-binding, 627-, and NLS domains have all translated and/or rotated by significant amounts (Fig. 5C,D). For instance, in the “transcriptionally active” polymerase conformation, the 627-NLS double domain is in the closed state and packs against PB1, distant from the endonuclease (Fig. 5B). However, in the “apo”-conformation, the double domain is relocated and opens, the NLS domain moiety binding to the endonuclease, which itself rotates in situ by $\sim 45^\circ$. The carboxy-terminal NLS motif containing an extension to the NLS domain refolds into an α -helix that also binds to the endonuclease (Hengrung et al. 2015; Thierry et al. 2016; Fan et al. 2019). The functional role of the “apo” state has yet to be elucidated, although it is presumed to be transcriptionally inactive. There is also strong evidence that the cap-binding inhibitor pimodivir stabilizes the “apo” state (Ma et al. 2017; Pflug et al. 2018). In solution, the polymerase is in dynamic equilibrium between several different configurations, as is apparent from recent cryo-EM single-particle reconstructions that typically resolve several different 3D structural classes on the same grid (Kouba et al. 2019; Peng et al. 2019).

Structural studies of human A/H3N2, avian A/H5N1, and avian A/H7N9 polymerases show that in the absence of full promoter binding they form homodimers (Fig. 5E; Chang et al. 2015; Fan et al. 2019; Wandzik et al. 2020). However, similar dimers have not yet been reported for bat FluA or influenza B, C, and D polymerases. The extensive FluA dimer interface is twofold symmetric and involves core peptides from all three subunits. These include PA loops 292–297 and 348–356, each interacting with their counterparts in the second monomer, PB1 helix 567–581 and PB2 loop 71–76. In the H3N2 and H5N1 crystal structures, the polymerases forming the dimer are both in the “apo” conformation, whereas in the dimeric H3N2 cryoEM structure only the core is visible, the peripheral

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domains being disordered. Full promoter binding leads to monomerization of the polymerase (Chang et al. 2015; Fan et al. 2019), and the observed dimerization is also incompatible with the promoter 3′-end binding in the secondary site (Fan et al. 2019). Interestingly, in a recent study, mutations PB1/K577G, PB2/G74R, or PA/E349K, all of which weaken the observed dimer interface, were selected that could compensate for a replication defect arising from a mismatched PB2 segment from another strain (Chen et al. 2019). Indeed, numerous reports implicate a second “*trans*-acting” polymerase in the process of replication, particularly cRNA to vRNA synthesis (York et al. 2013; Chen et al. 2019; Fan et al. 2019). However, the exact nature of the interaction between the two polymerases (e.g., whether symmetric or asymmetric) and the mechanistic role of the *trans*-acting polymerase (e.g., releasing the cRNA 3′ end from the secondary site, template realignment during initiation of vRNA synthesis, and/or nucleating encapsidation of the nascent replicate into an RNP) remains elusive.

MECHANISM OF RNA SYNTHESIS

Active Site and Nucleotide Addition Cycle

The PB1 subunit contains the conserved catalytic core, comprising right-handed fingers, palm, and thumb domains (Fig. 6). Six characteristic motifs (A–F) with key conserved residues, together with two divalent magnesium cations (denoted A and B), control the template-directed nucleotide addition reaction (Fig. 7). The motifs are contained in the palm domain and the fingertips, a loop that extends from the fingers toward the thumb domain. Recent structures of the actively transcribing polymerase reveal in detail how these motifs, and other parts of the influenza polymerase core, interact with the primer, template, and incoming nucleotide during the nucleotide addition cycle (Kouba et al. 2019). Nucleotides are selected for addition to the growing product by their Watson–Crick complementarity to the template base at the so-called +1 position. Nucleotide addition comprises sequential steps of initial NTP binding

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Figure 6. PB1 RNA synthesis active site. (A) Ribbon diagram showing the conserved core polymerase architecture of the PB1 subunit comprising a right-handed arrangement of fingers (cyan), palm (red), and thumb (green). The fingertips loop (FT, blue) and priming loop (PL, magenta) occupy the central cavity where RNA synthesis occurs (see text). Additional PB1 specific features include the β -ribbon (orange), and the carboxy-terminal helices (wheat) that form a strong interface with PB2-N. (B) Mode A vRNA promoter binding to PB1 showing the template 3' end, where RNA synthesis initiates, in the active site in close proximity to the tip of the priming loop (PL, magenta). Motif C (rose), a β -hairpin in the palm domain that carries two key metal binding aspartates and the two catalytic magnesium ions (gray spheres) are highlighted (see also Fig. 7). (C) Model of how the priming loop might promote vRNA to cRNA replication initiation by stabilizing the first phosphor-transfer reaction that requires ATP and GTP to assemble opposite U1 and C2 of the template, respectively.

(Fig. 7B), active site closure, and catalysis with release of pyrophosphate (Fig. 7C). Subsequently, concerted product/template duplex translocation leaves the +1 position vacant for the next incoming NTP (Fig. 7D).

The template enters the active site by undergoing a characteristic kink at the +1 to +2 junction, with the +1 base held in place by a stacking interaction with Ile241 of motif F. A complementary NTP, entering through the NTP entrance tunnel, induces the motif B methionine-rich loop (409–GMMMGMF-) to flip conformation, allowing Met409 to stack on the incoming base (Fig. 7B). Prior to NTP binding, the active site is in an “open” configuration with metal A coordinated motif C Asp445 and motif E Glu490 in a side location, distinct from its position during catalysis. Upon NTP binding, which brings the metal B, the three conserved aspartates, Asp444 and Asp445 from motif C and Asp305 from motif A, coordinate both the metals in the “closed” catalytic conformation. In this conformation, key active site residues (e.g., Arg239 [motif F], Lys308 [motif A], Ser444

[motif C], Lys481 [motif D], and the two magnesium ions) position the receiving nucleotide ribose and the substrate NTP triphosphate in the precise geometry required for catalysis. Upon nucleotide incorporation, the translocation of the template–product is probably helped by the mobile methionine-rich loop, which serves as the Brownian ratchet/power stroke mechanism, together with pyrophosphate release. Translocation leaves the incoming nucleotide +1 position vacant for another round of the addition cycle. Direct inhibition of the RNA synthesis activity or incorporation of mutagenic nucleotides is an obvious antiviral strategy. However, although some active nucleoside analogs have been identified, such as T705 (favipiravir) (Furuta et al. 2017) and N4-hydroxycytidine (Toots et al. 2019), they are not in clinical use.

Priming Loop

A particular feature of viral RNA polymerases that perform unprimed RNA synthesis, as Flu-Pol does during replication, is the so-called

Figure 7. Nucleotide addition cycle. (A) Details of the PB1 catalytic site showing key residues associated with the conserved polymerase motifs A–F and the two catalytic magnesium ions A and B (gray spheres). The primer/product RNA is in blue and the template strand in yellow. An incoming NTP, here a nonhydrolyzable UTP analog (UpNHpp), is shown in the precatalytic state. (B) First precatalytic step in the nucleotide addition cycle with incoming NTP stabilized by stacking on motif B Met409 (same structure as in A). (C) Postcatalytic, pretranslocation step in which the nucleotide has been added onto the product releasing pyrophosphate (PPi). (D) Translocation step, in which the template–product duplex has moved forward one position, bringing the next unpaired template base opposite the empty NTP binding site. Translocation may be assisted by flipping of the motif B loop into an alternative configuration.

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priming loop (PB1 residues 631–660), which partially fills the active site cavity (Fig. 6A–C). Its role is to provide a stable platform, formed by the conserved tip residues 649–HGP, to promote the assembly of the vRNA to cRNA replication initiation state in which ATP as the priming nucleotide in the +2 position and GTP in the +1 position need to line up opposite terminal template nucleotides U1 and C2, respectively, to form pppApG in the first catalytic step (Fig. 6C; Te Velthuis et al. 2016). However, once this role is fulfilled, the priming loop blocks translocation of the product-template duplex and has to be retracted. The priming loop has been shown to be less important for cRNA to vRNA replication (Te Velthuis et al. 2016). This is because initiation of cRNA to vRNA replication occurs internally opposite nucleotides 4-UC of the cRNA template followed by backtracking of the template and realignment of the formed pppApG opposite 1-UC (Deng et al. 2006; Fan et al. 2019). In the case of transcription, the resting position of the priming loop is incompatible with hybridization of the capped primer with the template during transcription initiation. The priming loop therefore has to progressively retract in the early steps of transcription, as has been recently visualized (Kouba et al. 2019) (see the next section).

MODEL FOR COMPLETE TRANSCRIPTION CYCLE

Recently, a combination of X-ray crystallography and cryo-electron microscopy has enabled snapshots to be determined of key steps in transcription encompassing initiation, elongation, termination/poly(A) tail generation, and recycling, leading to the following model of the complete transcription cycle (Kouba et al. 2019; Wandzik et al. 2020). In this model, the 5' and 3' extremities of the template remain bound to the polymerase throughout (Fig. 8).

In the pre-initiation state with mode A promoter binding, the template 3' end can flip in and out of the active site cavity (Fig. 8A). Transcription initiates when the 3' end of the capped primer hybridizes with the 3' end of the template and this requires partial withdrawal of the

priming loop (Fig. 8B). Typically a primer ending in AG-3' will base-pair with template 3'-UC with the next template base G3 at the +1 position awaiting a CTP as the first nucleotide to be incorporated into the product. After successful initiation, through subsequent nucleotide addition and template translocation, the template-product RNA duplex grows in the active site cavity up to 9 bp, necessitating further retraction of the priming loop (Kouba et al. 2019). At this point, the duplex abuts against the so-called PB2-lid domain, forcing strand separation (Fig. 8C). A specific α -helix of the lid domain faces the growing product-template duplex and conserved Tyr205 changes rotamer to stack on the template base of the last base-pair (Kouba et al. 2019). The separated template strand navigates into a narrow template exit tunnel, which is only open after the full retraction of the priming loop, while the product strand bulges out in the solvent channel between the endonuclease and cap-binding domain (Kouba et al. 2019). In the initiation to elongation transition, concomitant changes occur to the promoter. After a few rounds of nucleotide addition and template translocation, the base-pairs in the promoter duplex must disrupt, resulting in remodeling of the template entry pathway and clamping down of the PB1 β -ribbon that separates the incoming template from the tightly bound 5' end (Kouba et al. 2019). As processive elongation proceeds, the outgoing template extremity eventually reaches the secondary 3'-end binding site, where it tightly binds, thus preventing it from becoming lost and degraded (Fig. 8D; Wandzik et al. 2020). Subsequently the outgoing template will bulge out to form a growing loop while at the same time the ingoing template loop shrinks (Fig. 8E). Once the template is translocated so that there is only a taut connect between the 5' hook and the active site, stuttering occurs on the 5' proximal oligo(U) motif, involving template U17 flipping in and out of the +1 active site position, to generate the poly(A) tail (Fig. 8F). After product disassociation by a mechanism still to be elucidated, further conformational changes involving opening of the polymerase release the template from the active site cavity. This allows reformation of the promoter in the

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Figure 8. Model for the complete transcription cycle (see text).

mode B configuration with the 3' template end still bound in the secondary site (Fig. 8G). Subsequently, upon reclosure of the polymerase, promoter mode A configuration can be reestablished and initiation of the next transcription cycle begin (Fig. 8A).

CONCLUDING REMARKS

Recent high-resolution structural analysis of multiple functional states of FluPol has led to remarkable advances in our understanding of how the viral transcription-replication machine

functions. This is particularly true of cap-dependent transcription, although our understanding of vRNA to cRNA and cRNA to vRNA replication is less well-advanced as it likely involves more than one polymerase. However, most structural studies done so far use recombinant polymerase and do not include NP. Although many polymerase functions can be recapitulated in vitro without NP, notably cap-dependent transcription, the next and considerable challenge is to extend detailed structural studies, first, to the RNP context, to understand the dynamic interplay between polymerase and

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NP, and second, an even greater challenge, to observe the RNP at work in the nuclear context. With regard to the first challenge, a very recent publication uses cryo-electron microscopy to propose a “processive helical track” mechanism of how, within the RNP, antiparallel NP-RNA strands slide past each other during transcription (Coloma et al. 2020). This model is perfectly consistent with the polymerase-centric mechanism of transcription shown in Figure 8 (Wandzik et al. 2020).

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