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Influenza Virus RNA-Dependent RNA Polymerase and the Host Transcriptional Apparatus

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Abstract

Influenza virus RNA-dependent RNA polymerase (FluPol) transcribes the viral RNA genome in the infected cell nucleus. In the 1970s, researchers showed that viral transcription depends on host RNA polymerase II (RNAP II) activity and subsequently that FluPol snatches capped oligomers from nascent RNAP II transcripts to prime its own transcription. Exactly how this occurs remains elusive. Here, we review recent advances in the mechanistic understanding of FluPol transcription and early events in RNAP II transcription that are relevant to cap-snatching. We describe the known direct interactions between FluPol and the RNAP II C-terminal domain and summarize the transcription-related host factors that have been found to interact with FluPol. We also discuss open questions regarding how FluPol may be targeted to actively transcribing RNAP II and the exact context and timing of cap-snatching, which is presumed to occur after cap completion but before the cap is sequestered by the nuclear cap-binding complex.

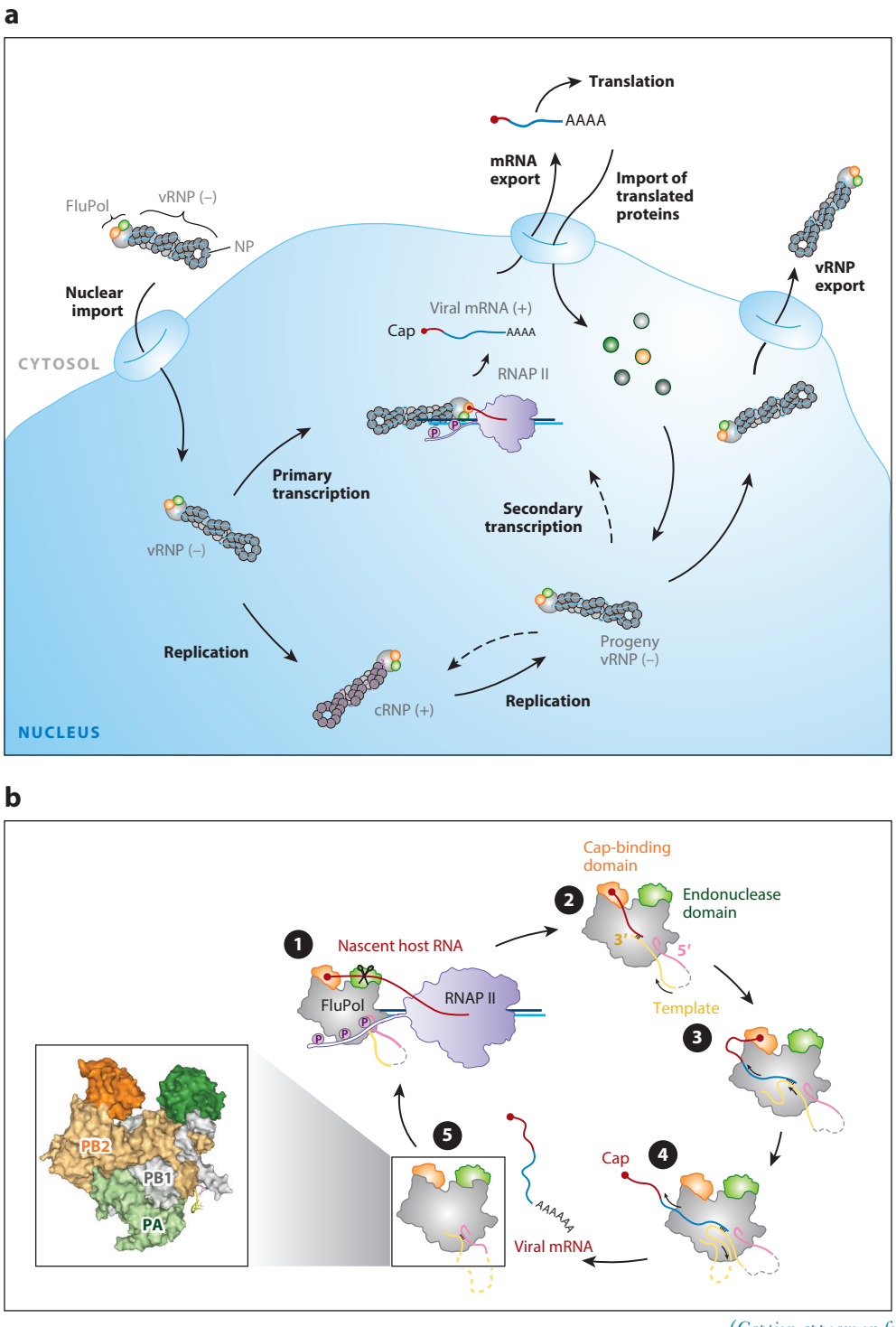
Contents

1. INFLUENZA VIRUS AND CAP-SNATCHING

Influenza is an acute infectious respiratory disease that is mainly caused by influenza viruses of the genera A and B. While human infections with influenza A (IAV) and B viruses cause annually recurring epidemics of seasonal influenza, which affect 10–30% of the global population and kill 290,000–650,000 people each year, influenza C viruses usually cause milder respiratory syndromes. Occasionally, IAVs of animal origin cross the species barrier to humans causing pandemic influenza, which can have devastating consequences in terms of mortality and economic loss and poses a perennial worldwide threat (1). Understanding the mechanism of viral replication is key to improving the prevention and treatment of influenza disease.

Influenza viruses have a segmented, single-stranded RNA genome of negative (−) polarity and, unlike most RNA viruses, replicate in the nucleus of infected cells (**Figure 1*a***) (2). Each of the eight genomic viral RNA (vRNA) segments is encapsidated by multiple copies of the viral nucleoprotein (NP) together with a single copy of the RNA-dependent RNA polymerase (FluPol). This complex is referred to as the viral ribonucleoprotein complex (vRNP) and is the functional unit for transcription and replication (3). After virus internalization, vRNPs are released into the cytosol and subsequently imported into the nucleus, where the first rounds of viral messenger RNA (mRNA) transcription occur (primary transcription) (**Figure 1*a***). FluPol replicates the viral genome by copying vRNAs into intermediate positive-sense complementary RNAs (cRNAs), which in turn serve as templates for the synthesis of new vRNAs. The cRNAs and vRNAs are cotranscriptionally packaged with newly synthesized NP and FluPol to form progeny vRNPs and cRNPs. Progeny vRNPs serve as a template for further (secondary) transcription and replication (see the dashed lines in **Figure 1*a***). At late stages of the infection cycle, viral transcription declines, and vRNPs are exported from the nucleus to the host-cell plasma membrane, where they are incorporated into new virions (2).

FluPol is a heterotrimer composed of the subunits PA (polymerase acidic protein), PB (polymerase basic protein) 1, and PB2 (**Figure 1*b***) (4). X-ray crystallography and cryo–electron microscopy (cryo-EM) have revealed that FluPol is a highly dynamic molecule with many flexible linked domains that can adopt multiple conformations corresponding to different functional states (5–9). FluPol performs transcription and replication of the viral genome through very



(Caption appears on following page)

Figure 1 (*Figure appears on preceding page*)

FluPol transcription and replication. (*a*) Incoming vRNPs are imported into the nucleus and used as templates for primary transcription. Viral mRNAs are exported from the nucleus and translated by the cellular translation machinery. Genome replication involves an unprimed mechanism that produces full-length positive-strand cRNA (+) that is then replicated into progeny vRNA (−). Newly synthesized NP and polymerase subunits are reimported into the nucleus and cotranscriptionally package replicated cRNA and vRNA into RNPs. Progeny vRNPs are substrates for secondary transcription and replication. (*b*) The FluPol transcription cycle starts with (❶) cap-snatching from nascent 5′-capped RNAP II transcripts via the binding of the PB2 cap-binding domain to the capped moiety and cleavage 10–15 nt downstream by the PA endonuclease domain, followed by (❷) repositioning of the 3′ end of the primer to the polymerase active site where viral mRNA synthesis is initiated. (❸) Elongation proceeds with the addition of nucleotides to the 3′ end of the capped primer, and (❹) after exiting the active site cavity, the 3′ extremity of the template binds into a secondary site on the polymerase surface. Both template ends thus remain in close proximity throughout transcription, allowing efficient recycling for the next round of transcription by reformation of the promoter after termination. (❺) The released product has a 5′ cap derived from the snatched host RNAP II transcript and a poly(A) tail, which is produced by the FluPol by a stuttering mechanism. Abbreviations: cRNP, complementary ribonucleoprotein; FluPol, influenza virus RNA-dependent RNA polymerase; NP, nucleoprotein; PA, polymerase acidic protein; PB, polymerase basic protein; RNAP, RNA polymerase; vRNP, viral ribonucleoprotein.

different processes. Whereas replication is initiated by a primer-independent mechanism (10, 11), transcription of viral mRNAs is primer dependent (9, 12). Replication generates exact, full-length genome copies, while transcription results in mRNAs with a 5′ terminal N^7-methylguanosine (m^7G) cap and a 3′ poly(A) tail (9, 13) that are competent for translation by the host translation machinery (14).

In contrast to many other RNA viruses, FluPol does not possess any inherent capping activity (15). This initially puzzling observation was explained in the late 1970s by the Krug laboratory. They demonstrated that FluPol uses short, capped oligomers derived from capped host RNAs to prime transcription of viral mRNAs (16, 17). In a process referred to as cap-snatching, the PB2 cap-binding domain binds to the 5′ cap of nascent host RNA polymerase II (RNAP II) transcripts (18), and the PA endonuclease cleaves 10–15 nt downstream of the cap to generate the capped primers that initiate transcription (see step 1 of **Figure 1*b***) (19, 20). Polyadenylation is achieved by a noncanonical mechanism involving stuttering of the viral polymerase at a 5′ proximal oligo(U) polyadenylation signal present on each genomic vRNA (9, 21). Recently, the initiation, elongation, polyadenylation, and recycling states (see steps 2–5 of **Figure 1*b***) of the complete FluPol transcription cycle have been visualized by a combination of X-ray crystallography and cryo-EM (4, 9, 22). The 5′ and 3′ vRNA extremities always remain bound to the polymerase while it moves along the vRNA, thereby allowing efficient recycling from the termination back to the initiation state of viral transcription (see steps 4 and 5 of **Figure 1*b***) (9, 23, 24).

The cap-snatching mechanism is common to all segmented, negative-sense RNA viruses (25). However, for orthomyxoviruses such as influenza, cap-snatching uniquely occurs in the nucleus, whereas members of the large *Bunyavirales* order perform cap-snatching in the cytoplasm. Influenza viral replication has long been known to be dependent on active host RNAP II (26, 27). Moreover, it has been shown that cap-snatching requires an intimate association with the RNAP II transcription machinery (**Figure 1*a*,*b***) (28, 29). The RNA targets of FluPol cap-snatching, as well as the effect of an influenza infection on RNAP II transcription, have been recently reviewed (30). Here, we focus on the recent significant, often structure-based advances in the mechanistic understanding of both FluPol and RNAP II transcription with the aim of trying to understand how the two processes are coupled. We review FluPol-associated host factors and discuss possible steps of RNAP II transcription that could allow cap-snatching by FluPol. Moreover, we discuss the recent discoveries of RNAP II compartmentalization and phase separation in the context of the cap-snatching process.

2. THE CELLULAR CONTEXT OF CAP-SNATCHING

2.1. RNAP II Transcription

Eukaryotic cells encode three multisubunit RNA polymerases, RNAP I–III (31). RNAP II transcribes all protein-coding mRNAs and diverse noncoding RNAs, including long noncoding RNAs (32), micro RNAs (33), small nuclear RNAs (snRNAs) (34), and small nucleolar RNAs (35). RNAP II is composed of 12 subunits, of which the largest subunit, RPB1, has a long unstructured C-terminal domain (CTD) (36). The CTD consists of three regions: a tip, a middle region of repetitive nature, and a linker, which connects the CTD to the RPB1 core. The middle region consists of heptapeptide repeats with the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser ($Y_1S_2P_3T_4S_5P_6S_7$). While the heptad motif is conserved between species, the number of repeats, and hence CTD length, differs markedly between species, as illustrated by the presence of 26 CTD repeats in *Saccharomyces cerevisiae* and 52 repeats in mammals (37). The CTD is subject to diverse posttranslational modifications including phosphorylation, glycosylation, methylation, ubiquitination, and acetylation (36). The modification pattern of the CTD evolves in a regulated fashion during RNAP II transcription, thereby defining the CTD code, which is fundamental for the spatiotemporal control of transcription. The CTD directly binds or indirectly recruits cotranscription factors and thereby serves as a scaffold for diverse RNA processing factors and transcriptional regulators (38).

RNAP II transcription is initiated by the recruitment of general transcription factors (GTFs) and RNAP II to the promoter region, thereby forming the preinitiation complex (PIC) (see step 1 of **Figure 2**) (39). A crucial regulator of transcriptional initiation is the Mediator complex, a large protein complex with variable subunit composition (40) that stabilizes the PIC (41) and functionally couples the PIC to chromatin remodelers and transcriptional regulators (42). The Mediator complex interacts with GTFs (43) as well as the unphosphorylated RNAP II CTD (44, 45), facilitating CTD Ser5 and Ser7 phosphorylation by the transcription factor IIH (TFIIH) subunit

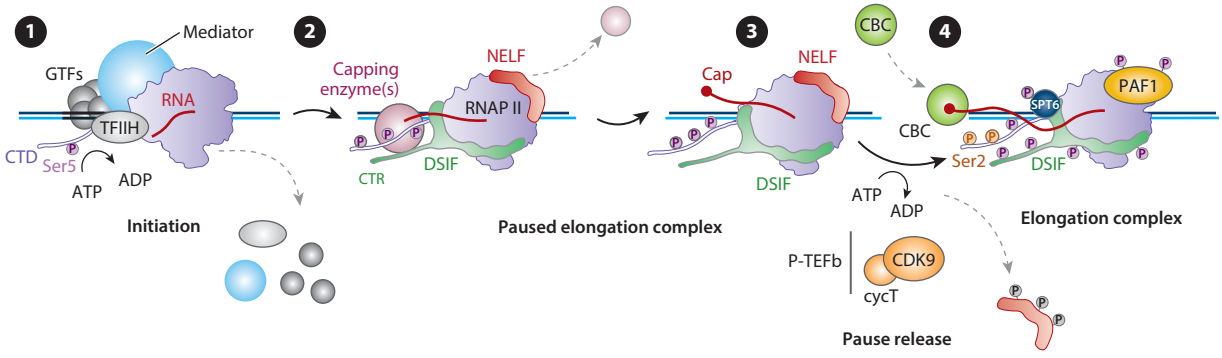


Figure 2

The initiation, pausing, and pause-release steps of early RNAP II transcription. (❶) Initiation starts with the recruitment of GTFs to the promoter region, followed by recruitment of RNAP II and the Mediator complex, which binds to the unphosphorylated RNAP II CTD. TFIIH phosphorylates the CTD on Ser5, thereby triggering promoter escape. (❷) The capping apparatus binds to the Ser5P CTD and the unphosphorylated DSIF CTR, leading to the synthesis of the cap structure on the 5′ end of the nascent RNA. (❸) Promoter-proximal pausing is associated with binding of the pausing factors DSIF and NELF to RNAP II. (❹) Phosphorylation of DSIF, NELF, and RNAP II CTD on Ser2 by CDK9, the kinase component of P-TEFb, leads to RNAP II pause release and recruitment of the elongation factors PAF1 and SPT6, resulting in an active elongating complex. The 5′ cap structure is bound by the nuclear CBC. Abbreviations: CBC, cap-binding complex; CDK, cyclin-dependent kinase; CTD, C-terminal domain; CTR, C-terminal region; DSIF, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole sensitivity-inducing factor; GTF, general transcription factor; NELF, negative elongation factor; P-TEFb, positive-transcription elongation factor b; RNAP, RNA polymerase; Ser5P, phosphorylated Ser5; TFIIH, transcription factor IIH.

cyclin-dependent kinase (CDK) 7, which in turn leads to Mediator release and RNAP II promoter escape (see step 2 of **Figure 2**) (46).

RNAP II pausing 20–100 bp downstream from the transcription start site is a decisive step for the control of transcriptional elongation (47). RNAP II pausing rates are highly regulated and contribute to gene-specific transcriptional outputs (48–50). RNAP II pausing is dependent on DNA sequence elements in the promoter-proximal region (51), as well as on specific negative elongation factors that provoke tilting of the DNA-RNA hybrid within the active-site cavity of the paused RNAP II complex, thus preventing RNA chain elongation (52, 53). Paused RNAP II is stabilized by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) sensitivity-inducing factor (DSIF), a dimeric complex formed by SPT4 and SPT5 (54), and the negative elongation factor (NELF), a heterotetramer formed by subunits NELF-A, B, C/D, and E (see steps 2 and 3 of **Figure 2**) (55, 56). SPT5 comprises multiple subdomains, which extensively interact with the RNAP II surface and the DNA template, as well as the exiting RNA (52, 53, 57). The C-terminal region (CTR) of human SPT5 consists of pentapeptide repeats with the consensus sequence Gly-Ser-Gln/Arg-Thr-Pro, with the Ser and Thr residues undergoing phosphorylation (see step 4 of **Figure 2**) (58). Similar to the RNAP II CTD repeats, the SPT5 CTR plays a role in the recruitment of transcription-associated factors (59). NELF also interacts with RNAP II at multiple sites, restricting its mobility and preventing the binding of TFIIS (60), a factor that aids the realignment of the DNA-RNA hybrid and the restarting of elongation after transient pausing or transcriptional arrest (53, 61).

Capping of nascent RNAP II transcripts occurs immediately after the emergence of the RNA 5′-end triphosphate from the RNA exit tunnel and is tightly coupled to RNAP II pausing (see step 2 of **Figure 2**) (62–64). Capping is crucial for transcript stability, subsequent processing, intranuclear transport, nuclear export, and, in the case of mRNA, translation (65). Shortly after cap completion, the modified 5′ end of the nascent RNA is bound by the nuclear cap-binding complex (CBC) (see step 4 of **Figure 2**). The heterodimeric CBC consists of nuclear cap-binding protein 1/2 (NCBP1/2) (66, 67) and interacts with several RNA processing complexes, including those for splicing (68), U snRNA export (69), RNA degradation (70), and 3′ end processing (71), thereby playing a fundamental role in mediating the function of the 5′ cap structure.

The 5′ cap structure is characterized by an m^7G linked via an inverted 5′–5′ triphosphate bridge to the 5′-terminal nucleoside of the transcript, and its synthesis requires a series of enzymes (**Figure 3**) (72). The formation of the minimal cap 0 structure is catalyzed by three enzymes, namely RNA 5′-triphosphatase (RT), guanylyltransferase (GT), and RNA guanine-N^7 methyltransferase (RNMT) (**Figure 3*a–c***) (65, 73). In mammals, γ-phosphate hydrolysis and guanylyl transfer are catalyzed by the capping enzyme (CE) (74, 75). The guanosine-capped structure is a substrate for a series of further methylations. RNMT transfers a methyl group to the N^7 of the guanosine to form the cap 0 structure (76), which is crucial for CBC binding and efficient translation of mRNA (**Figure 3*c***) (77). The cap 0 structure normally undergoes further methylation of the 2′-OH on the ribose of the first nucleotide, catalyzed in higher eukaryotes by cap-specific mRNA methyltransferase 1 (CMTR1) (78), thereby generating the cap 1 structure (**Figure 3*d***). The cap 1 structure is a hallmark of bona fide cellular RNAs, whereas cap 0 is recognized as nonself by innate immune receptors such as RIG-I (79, 80). The 2′-O ribose of the second nucleotide can be methylated by CMTR2 (81), resulting in the cap 2 structure (**Figure 3*e***), which is present only in approximately half of capped mRNAs (82) and has been suggested to increase RNA stability (83). Moreover, it was recently demonstrated that the majority of mRNAs that start with an A are methylated at the N^6A position by cap-specific adenosine methyltransferase (CAPAM) (**Figure 3*f***) (84).

(*Caption appears on following page*)

Figure 3 (*Figure appears on preceding page*)

Enzymatic reactions of cap synthesis. The addition of each chemical group is highlighted with a different color. (*a*) The γ-phosphate is hydrolyzed by RT, and (*b*) guanylyl transfer is catalyzed by GT. In mammals, RT and GT activity reside in the CE. (*c*) Methylation of the N^7 of the guanosine by RNMT leads to the formation of the cap 0 structure. A series of methylations by (*d*) CMTR1 and (*e*) CMTR2 further modify the hydroxyl groups of the first and second nucleotides, respectively, producing the cap 1 and cap 2 structures. (*f*) Additional methylation on the N^6 of the first adenine by CAPAM occurs in some capped RNAs. Abbreviations: CAPAM, cap-specific adenosine methyltransferase; CE, capping enzyme; CMTR, cap-specific mRNA methyltransferase; GT, guanylyltransferase; RNMT, RNA guanine-N^7 methyltransferase; RT, RNA 5′-triphosphatase.

The recruitment of the CE to paused RNAP II and its allosteric activation is mediated by a direct interaction with the phosphorylated Ser5 (Ser5P) in the RNAP II CTD (85–88), with additional interactions being made with DSIF, particularly its SPT5 CTR (89, 90). CTD-independent interactions with RNAP II position the CE in proximity to the emerging transcript at the RNA exit tunnel, further enhancing CE activity (74, 91). The methyltransferases CMTR1 (92) and CAPAM (84) also bind to the Ser5P CTD, illustrating the crucial role of the Ser5P modification in the cotranscriptional capping of nascent RNAP II transcripts.

The kinase activity of the positive transcription elongation factor b (P-TEFb) essentially regulates RNAP II pause release (93). P-TEFb consists of CDK9 in complex with cyclin T1/2 (94) (see step 4 of **Figure 2**). Before its activation, P-TEFb is sequestered by the 7SK snRNP (small nuclear ribonucleoprotein) complex in an inactive state (95). P-TEFb activation and recruitment to promoter-proximal regions are tightly regulated, and several different mechanisms of activation have been proposed (93). These include the concerted actions of Brd4 (96) and the protein phosphatases PP2B and PP1α (97, 98), as well as the recruitment of P-TEFb to promoter-proximal regions by TRIM28 (99). Upon activation and recruitment to paused RNAP II, CDK9 phosphorylates the CTR of SPT5 (58), NELF (100), the positive elongation factor PAF1 complex (PAF), and Ser2 of RNAP II CTD (101), which triggers the formation of an activated RNAP II elongation complex (102). DSIF phosphorylation is critical for transcriptional elongation and converts DSIF into a positive elongation factor (58). PAF1 binding competes with NELF, leading to the exclusion of NELF from the elongating RNAP II complex (53, 102). CDK9 phosphorylation of the RNAP II–CTD linker region enables the binding of the elongation factor SPT6 (102). Overall, the activity of P-TEFb leads to RNAP II release from the paused state and transition into productive elongation (103).

2.2. FluPol Sensitivity to RNAP II Inhibitors

Early investigations into the effect of RNAP II inhibitors, such as α-amanitin and actinomycin D, on influenza virus multiplication first established that FluPol transcription requires active RNAP II transcription. The inhibitor α-amanitin traps an RNAP II translocation intermediate (104), thereby inhibiting nucleotide incorporation and blocking both RNAP II initiation and elongation. Actinomycin D is a DNA intercalating agent that generally interferes with DNA-templated RNA synthesis (105). Actinomycin D and α-amanitin efficiently inhibit multiplication of influenza virus, but not cytoplasmically replicating RNA viruses (27, 106), when added early in infection (27, 107–109). Inhibition by α-amanitin is specifically related to RNAP II activity, as the virus is insensitive to the drug in cells that express an α-amanitin-resistant RNAP II (26, 110, 111). Treatment with α-amanitin or actinomycin D prevents the accumulation of all three types of viral RNAs (vRNAs, cRNAs, and mRNAs). However, there is ample evidence that only viral transcription is directly dependent on RNAP II activity. For instance, if FluPol and viral NP are expressed prior to α-amanitin or actinomycin D treatment and infection, vRNAs and cRNAs still accumulate, whereas mRNA transcription is strongly impaired (26, 112), thereby demonstrating

the drug's specific effect on viral transcription. The effect of α-amanitin or actinomycin D on viral replication is indirect, as replication is strictly dependent on viral protein expression and, hence, on viral transcription (26, 110, 113).

Influenza virus growth was also reduced in the presence of CDK9 kinase inhibitors, such as DRB (114) and flavopiridol (115). However, these compounds also inhibit other kinases to a lesser extent [e.g., CDK7 for DRB and CDK1, CDK2, CDK4, and CDK8 for flavopiridol (116)], complicating the interpretation of the observed effects. Both inhibitors prevent RNAP II hyperphosphorylation and elongation (117–119). DRB was reported not to inhibit transcription of viral mRNAs, and its effect on influenza virus multiplication is at least partly explained by its inhibition of viral mRNA export (26, 120). This, together with the fact that FluPol preferentially associates with the Ser5P CTD of RNAP II (see Section 2.4) (28), suggests that RNAP II activity prior to hyperphosphorylation by P-TEFb is sufficient for FluPol cap-snatching.

2.3. FluPol Interactions with the Host Transcription Machinery

Several observations suggest that multiple interactions between FluPol and the host transcriptional machinery are required to allow efficient cap-snatching. FluPol directly interacts with the RNAP II CTD (28), and this interaction was shown to be essential for viral transcription (see Section 2.4) (29). Moreover, the intranuclear dynamics of vRNPs suggest that the association of FluPol with RNAP II is established by multiple interactions (121). For instance, indirect interactions with RNAP II through other transcription-associated factors could be involved. In recent years, several proteomic studies and genome-wide loss-of-function screens using CRISPR-Cas9 knockouts or siRNA-mediated knockdown have documented IAV–host protein interactions. There is little overlap between the hit lists of the different screens (122, 123), which is likely due to differences in the experimental setting and selection criteria for the hits. A limitation of loss-of-function screens is the toxicity that might result from the depletion of essential host proteins. Nevertheless, these high-throughput approaches provide extensive data on the physical and functional connections between influenza proteins and host transcription-related factors (122–125). Hits that were found in at least two independent screens and that are potentially relevant with respect to the RNAP II context of cap-snatching are listed in **Table 1**. Few have been validated, and their precise roles during influenza infection remain poorly characterized.

Interestingly, few of the identified host factors correspond to the basal transcription initiation machinery or the Mediator complex (**Table 1**), suggesting that host factors associated with these steps of RNAP II transcription are not involved in the recruitment of FluPol. In contrast, several factors involved in the control of RNAP II pausing and elongation have been identified. Independent proteomic studies report an interaction between FluPol and the DSIF subunits SPT4 and SPT5 (124–126), and one validated this interaction by coimmunoprecipitation experiments (124). Other factors known to regulate or cooperate with SPT5 (102, 127, 128) have been found to interact with FluPol, namely, the arginine methyltransferase PRMT5 (124) and the transcription elongation factors SPT6 (125), PAF1, and Tat-SF1 (124). PARP1, which ADP-ribosylates NELF and promotes transcriptional elongation (129, 130), and CDK9, a component of the P-TEFb kinase responsible for pause release into productive elongation (131), were also identified. Moreover, TRIM28, a negative regulator of transcriptional elongation (132) and CDK9 activity (133), was identified as a FluPol interaction partner (126). However, functional and mechanistic data regarding the potential role of the described factors in influenza infection are scarce and sometimes contradictory. A positive effect of TRIM28 on influenza replication was reported by independent investigations (134–137), and this was attributed to a negative regulatory effect of TRIM28 on the innate immune response (136, 138). However, TRIM28 is also reported to inhibit FluPol activity (139), suggesting it might have a multifunctional role during IAV infection.

Two independent RNA interference screens have pointed to a role for SPT6 in the viral life cycle (140, 141). PARP1 (142, 143) and P-TEFb (131, 139), when overexpressed or depleted, were found to affect FluPol activity. Moreover, Tat-SF1 was shown to positively regulate polymerase activity (139) and stimulate viral replication by possibly playing a role in vRNP assembly, even though this was suggested to happen through interaction with NP rather than the polymerase (144).

Screening hits relevant to capping include the CBC subunits NCBP1 (125, 137), NCBP2 (122, 137), and NCBP3 (137, 145), as well as the methyltransferase CMTR1 (122) (**Figure 3*d***). Downstream functional analyses confirmed a positive effect of CMTR1 on viral replication (122). However, it remains unclear whether direct interactions between CMTR1 and FluPol are important for viral replication or simply its cap-modifying activity.

Table 1 Host factors involved in cellular mRNA biogenesis that have been identified by high-throughput screening as interacting with influenza virus

Gene	Loss-of-function screen reference(s)	Interaction screen reference(s)	Functional study reference(s)
Basal RNAP II transcription			
CCNT1/CDK9	NR	NR	131
CMTR1	122	NR	122
GTF2I	NR	125 146	NR
HTATSF1 (Tat-SF1)	139 142	124	144
MED6	140 145	NR	NR
NCBP1	NR	125 137	199
NCBP2	122	137	NR
NCPB3	137 145	NR	NR
PARP1	142	129	143 205
POLR2A (RPB1)	NR	124 125 130	28
POLR2B (RPB2)	NR	125 126 130 146	NR
SUPT5H (SPT5)	NR	124 125 126	NR
SUPT6H (SPT6)	140 141	125	NR
TRIM28	134 135 137	126 129 137	136 138

(*Continued*)

Table 1 (*Continued*)

Gene	Loss-of-function screen reference(s)	Interaction screen reference(s)	Functional study reference(s)
Chromatin-associated factors			
CHD1	NR	NR	149
CHD6	NR	130	148 206
DDB1	142	125 126 129 146	147
RRP1B	207	NR	208
RNA processing factors			
DDX3X	137 209	137 210	210 211 212
DDX5	142 209	125 130 137 210	210
DDX17	142 209	129 137	NR
DDX39B (BAT1)	209	124 129 137	213
EFTUD2	134 137	137	NR
FUS	134 137	125 130 137	NR
HNRNPM	142	129 210	NR
NS1-BP	NR	NR	214
NUDT21 (CPSF5)	122	137	NR
PRPF8	134 137 140	124 125 137	215
RED-SMU1	NR	NR	216
SART3	122	125 137	NR
SF3A1	135 140	NR	NR
SF3B1	134 137 140	137	NR
SF3B2	134 137	125 137	NR

(*Continued*)

Table 1 (*Continued*)

Gene	Loss-of-function screen reference(s)	Interaction screen reference(s)	Functional study reference(s)
SF3B3	134 137	137	NR
SFPQ	122 142 217	137 210	210 218
SNRNP70	135 140	125	NR
SNRPB	134 137	137	NR
SNRPD3	134 137	137	NR
SRSF10	NR	125 126	219

Genes are tabulated for which an interplay with influenza virus was documented in at least two independent high-throughput screens and/or in at least one dedicated functional study.
Abbreviations: CDK, cyclin-dependent kinase; CMTR, cap-specific mRNA methyltransferase; GTF, general transcription factor; NCBP, nuclear cap-binding protein; NR, not reported; RNAP II, RNA polymerase II.

Other nuclear proteins interacting with FluPol and/or potentially regulating FluPol activity include chromatin-associated proteins and mRNA processing factors (**Table 1**). Only a few have been investigated in detail. Although the multifunctional DDB1 protein was identified as a hit in five independent proteomic studies or genetic screens (125, 126, 129, 130, 146) and was shown to mediate PB2 ubiquitination (147), its precise role in the viral life cycle has not been uncovered. Two chromatin-remodeling proteins, CHD6 and CHD1, were shown to interact with FluPol in infected cells and to act as a negative and positive regulator of FluPol activity, respectively (148, 149). A physical association between FluPol and the nuclear RNA exosome complex was also proposed to contribute to chromatin targeting of the viral polymerase to promoters, thereby promoting cap-snatching (150). Many transcription factors are multifunctional and are involved in various steps of cellular RNA biosynthesis. However, to our knowledge, there is no evidence that any of the RNA processing factors listed in **Table 1** are directly involved in the influenza cap-snatching process.

2.4. FluPol Binding to the RNAP II C-Terminal Domain

Biochemical and structural evidence demonstrate a physical association between FluPol and the RNAP II CTD. Coimmunoprecipitations showed that FluPol specifically binds to CTD repeats when transiently expressed in the absence of other viral proteins and vRNA (28), as well as in the context of vRNPs in infected cells (151, 152). Moreover, CTD binding enhances the in vitro transcriptional activity of FluPol, suggesting that CTD binding stabilizes FluPol in a transcriptionally active conformation (30, 153).

Biophysical and structural investigations using synthetic peptides corresponding to a few heptad repeats of Ser5P, Ser2P, or unphosphorylated RNAP II CTD show that the FluPol–CTD interaction is direct and specific for Ser5P (29, 152, 153). The structure of the bat $FluPol_A$ Ser5P CTD complex shows that highly conserved basic residues at two distinct sites directly interact with the phosphate groups of two Ser5Ps in the CTD (**Figure 4**) (29). Moreover, $FluPol_A$ mutants carrying single alanine mutations of any of these basic residues, which partially disrupt the

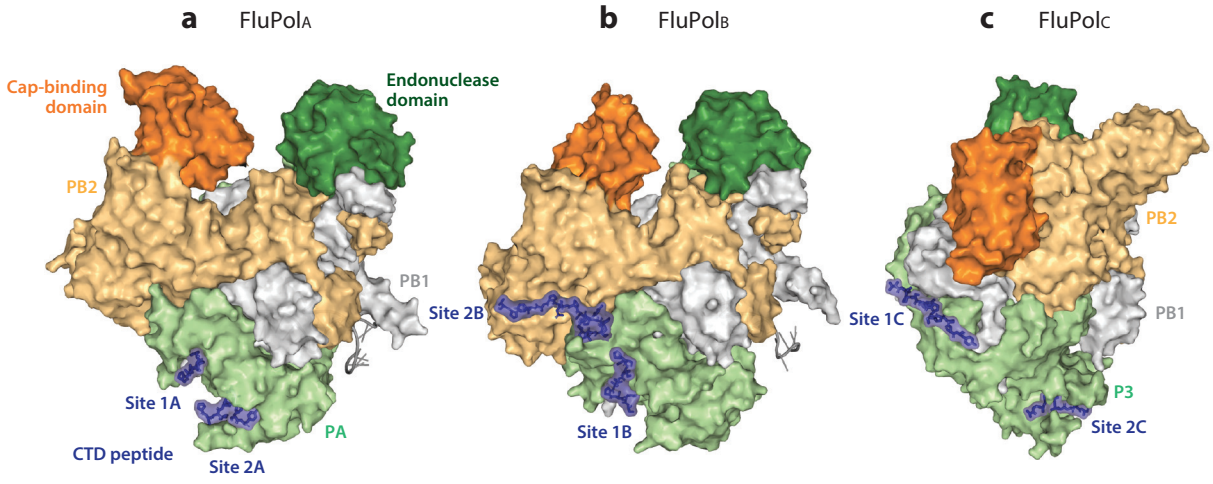


Figure 4

Modes of FluPol binding to the Ser5P CTD. Cocrystal structures of influenza A, B, and C polymerases bound to CTD-mimicking peptides. (*a*) Polymerase from Influenza A/little yellow-shouldered bat/2010/H17N10 strain ($FluPol_A$), PDB ID: 5M3H; (*b*) Influenza B/Memphis/13/03 ($FluPol_B$), PDB ID: 5M3J; (*c*) Influenza C/Johannesburg/1/66 ($FluPol_C$), PDB ID: 6F5O. Polymerases are color coded with PA (P3 for $FluPol_C$), in green, PB1 in gray, and PB2 in orange. The PA endonuclease and PB2 cap-binding domains are highlighted in darker shades of green and orange, respectively. The bound CTD peptides are shown in blue. Abbreviations: CTD, C-terminal domain; FluPol, influenza virus RNA-dependent RNA polymerase; PDB ID, Protein Data Bank identifier; PA, polymerase acidic protein; PB, polymerase basic protein.

CTD interaction, display strongly impaired transcriptional activity in the cellular context but not in vitro when a capped RNA primer is provided, suggesting that the FluPol–CTD interaction provides access to nascent host-cell RNAs for cap-snatching. Recombinant viruses carrying these mutations were highly attenuated and genetically unstable but could acquire second-site mutations that partially restored infectivity (29).

The CTD-binding patterns of polymerases from different influenza subtypes have common and distinct features (**Figure 4**). Similar to $FluPol_A$, cocrystal structures of $FluPol_B$ (29) and $FluPol_C$ (153) show bipartite CTD-binding sites. In $FluPol_A$, both binding sites (sites 1A and 2A) are on the C-terminal region of PA (PA-C) (**Figure 4*a***). In $FluPol_B$, site 1 is conserved (site 1B), while site 2B is distinct from site 2A and crosses over from PA-C to the PB2 627–NLS domain (**Figure 4*b***) (29). The $FluPol_C$ CTD-binding sites 1C (at the interface between P3-C and PB1) and 2C (on P3-C) are distinct from any of the sites observed in $FluPol_A$ and $FluPol_B$ (**Figure 4*c***) (153). A parallel can be drawn between the CTD-binding strategies evolved by divergent influenza polymerases and the recruitment of CEs from different species to RNAP II. Whereas the CEs from yeast, fungal, and mammalian species directly interact with Ser5P RNAP II CTD repeats, the binding interfaces and the conformations of the bound CTD peptides differ between species (85, 86, 154). A similar process of divergent evolution of CTD binding might have occurred for FluPol, as the influenza genera differ in host range (155). Despite the general conservation of the CTD heptad repeats (37), subtle differences in degenerate residues of the RNAP II CTD might have an effect on FluPol binding and therefore might affect the cap-snatching efficiency. Additionally, the context of the host factors associated with the FluPol-RNAP II complex may reflect host specificities, as seen for other host factors that are essential for influenza replication (155).

The binding of CTD-mimicking peptides to FluPol indicates that the affinity of each individual interaction at site 1 or 2 is in the micromolar range. However, binding of a CTD repeat to one site on the polymerase increases the likelihood of a nearby CTD repeat binding at the second

site (29). Avidity and cooperativity mechanisms are therefore likely to result in an overall high-affinity interaction between FluPol and the full-length CTD in the cellular context, although this association is likely to be highly dynamic. The CTD domain is located adjacent to the RNAP II mRNA exit tunnel (102), thereby allowing the coordinate binding of proteins involved in post-transcriptional processing (37). Therefore, it is possible that binding of FluPol to a distal CTD repeat stimulates subsequent binding to a proximal repeat, looping out a long CTD stretch in between and thereby bringing FluPol closer to the RNAP II mRNA exit tunnel.

3. THE LOCALIZATION AND TIMING OF CAP-SNATCHING

3.1. Intranuclear Sites of RNAP II and FluPol Transcription

A prerequisite for efficient FluPol transcription is access to a constant supply of RNAP II–derived nascent 5′-capped RNAs. Given that viral mRNAs can constitute up to 50% of the total mRNA in influenza-virus-infected cells (156), it is plausible that a highly efficient mechanism targets vRNPs, especially incoming parental vRNPs, to specific subnuclear localizations that are enriched in actively transcribing RNAP II. However, so far there is no clear evidence for such a mechanism.

Analyses of nuclear fractions with different nuclease sensitivities provided the first evidence that actively transcribed genes correspond to regions of open chromatin, where DNA is not tightly packaged into nucleosomes and is therefore more accessible to transcription factors (157). Based on microscopy and profiling of high-salt fractions from fixed cells, it was proposed that active RNAP II transcription occurs at discrete sites in the nucleus named transcription factories, which contain clusters of RNAP II and transcription factors tethered to the insoluble nuclear matrix (158). Using a similar approach, influenza vRNPs were found to be associated with chromatin and components of the nuclear matrix (159, 160), and vRNA synthesis was suggested to occur in the same insoluble subnuclear compartment (161–163).

Later studies led to more dynamic models for the regulation of chromatin topology and RNAP II clustering that better account for rapid transcriptional gene activation in response to external stimuli. Chromatin remodeling was shown to be mediated by histone modifications such as acetylation (164) or methylation (165) and to play a central role in the regulation of gene expression (166). The chromatin remodelers CHD1 and MORC3, which recognize transcriptionally active chromatin regions, were both found to bind FluPol and to enhance viral mRNA transcription (149, 167). It is possible that CHD1 and MORC3 target vRNPs to sites of open chromatin and active RNAP II transcription.

Recently, live-cell superresolution microscopy revealed transient dynamic foci of RNAP II that are referred to as RNAP II condensates (168, 169). A growing body of evidence suggests that these foci are formed by liquid–liquid phase separation, which is established by multivalent interactions between proteins with low-complexity disordered regions (LCDRs) (31, 170, 171). Transcription factors frequently possess LCDRs (172), which can attract the Mediator complex and RNAP II, thereby concentrating transcription initiation factors at enhancer and promoter regions (173, 174). The CTD of RNAP II is itself an LCDR that can undergo phase separation (175–177) and is suggested to drive the establishment of Mediator-containing promoter condensates where transcription initiation occurs (31, 176, 177). CTD phosphorylation enhances RNAP II incorporation into phase-separated droplets formed by P-TEFb (178) and major components of the splicing apparatus (176). A condensate-based model of transcription was therefore proposed (171) in which CTD phosphorylation drives RNAP II relocalization from promoter condensates to gene-body condensates (31).

So far only a few studies have documented the behavior of FluPol in the nucleus in live cells. Fluorescence recovery after photobleaching studies have shown that the nuclear mobility

of transiently expressed vRNPs is increased upon RNAP II inhibition with α-amanitin (121). Single-particle analyses of incoming vRNPs have demonstrated two distinct nuclear diffusion patterns corresponding to a simple and a restricted diffusion (179). It is tempting to speculate that the FluPol binding preference for Ser5P CTD repeats drives the incorporation of vRNPs into gene-body condensates, thereby restricting their diffusion and providing access to nascent capped RNAs. Superresolution microscopy studies of FluPol and its localization relative to key phase-separating factors of the transcriptional machinery are needed to explore this hypothesis.

3.2. FluPol Access to Nascent Capped RNAP II Transcripts

The preferential binding of FluPol to the Ser5P CTD suggests that FluPol is recruited to the promoter-proximal region of RNAP II–transcribed genes, as the RNAP II Ser5P CTD is enriched around the transcription start site (TSS) (26, 30). This model is supported by FluPol chromatin immunoprecipitation followed by DNA sequencing (ChIP-seq) analyses showing that FluPol exclusively binds to RNAP II–associated DNA and preferentially to the TSS when compared to intragenic regions (26). The current mechanistic understanding of the regulation of RNAP II transcription is based on a variety of techniques (180). Mapping of global RNAP II genome occupancy by ChIP-seq (181) and sequencing of nascent RNA associated with RNAP II (182) have proven to be valuable tools. ChIP-seq analyses using antibodies to specific CTD modifications of RNAP II indicate that Ser5 is phosphorylated at the TSS, and this is reversed during transcriptional elongation (181, 183, 184). In contrast to ChIP-seq, mammalian native elongating transcript sequencing (mNET-seq) identifies the 3′-end sequence of nascent RNA in the active site of RNAP II, thereby allowing single-nucleotide-resolution mapping of the position of RNAP II (185, 186). Compared to ChIP-seq, mNET-seq does not indicate strong Ser5P CTD enrichment at the TSS but reveals high levels of Ser5P CTD in exons (182, 186). Indeed, accumulating evidence suggests that Ser5P CTD is not restricted to the TSS but is present during transcriptional elongation and is preferentially associated with splicing factors (38, 187–189). While the discrepancy between ChIP-seq and mNET-seq could be due to methodological differences and needs to be clarified, it raises the question of whether FluPol cap-snatching occurs exclusively at promoter-proximal regions. Currently, knowledge about the distribution of FluPol along RNAP II genes is restricted to the housekeeping genes for β-actin and dihydrofolate reductase (26). FluPol's distribution along genes that are preferentially used as substrates for cap-snatching such as snRNAs and other noncoding RNAs (19, 190, 191) is unclear. Moreover, specific inhibition of RNAP II's transition from the initiation to elongation state, as previously suggested (26), was not observed by mNET-seq in influenza-infected cells (151). The RNAP II occupancy instead progressively declines downstream of the TSS when compared to noninfected cells (151). Therefore, further investigations are needed to gain deeper knowledge about the timing of FluPol cap-snatching in relation to the RNAP II transcription cycle. Genome-wide ChIP-seq analyses of FluPol's position on DNA might improve our understanding of the window of opportunity for FluPol cap-snatching. Moreover, a comprehensive understanding of the timing of cap-snatching could help to identify essential host factors associated with the cap-snatching complex, as each step of RNAP II transcription necessitates a specific set of transcription factors (**Figure 2**).

3.3. FluPol Cap Preference and Competition with the Host Cap-Binding Complex

In early studies on the influenza cap-snatching mechanism, the viral polymerase showed a preference for the cap 1 structure (**Figure 3*d***) (192, 193). Moreover, influenza mRNAs were found to preferentially start with adenine (19, 190), of which a significant amount was m^6A

modified (**Figure 3*f***) (13). Since CAPAM acts on CMTR1-methylated cap 1 (**Figure 3*d***) (84), this suggests that cap-snatching occurs after CMTR1 and CAPAM have modified the nascent RNAP II transcript, although these modifications occurring after the cap is snatched and released from the PB2 cap-binding domain early in viral transcription cannot be ruled out. Indeed recent structures of capped-RNA-bound FluPol with either A or G as the first nucleotide cannot explain the preference for methylated-cap substrates, although direct comparative measurements of affinity have not been made (5, 9, 22, 194). It is possible that the observed in vivo preference for cap 1 is not governed by specific recognition of the methylated ribose or base of the first nucleotide but by FluPol being actively recruited to transcribing RNAP II after CMTR1 and CAPAM have modified the nascent transcript.

This model poses several questions related to the exact timing and regulation of the sequential capping reactions and FluPol cap-snatching. What signals cap completion, and how does FluPol successfully compete with the host CBC for access to the completed cap? This is particularly puzzling as the CBC has a very high affinity to the cap (195), certainly much higher than that of the FluPol cap-binding domain alone (18), although tethering FluPol in the vicinity of the nascent capped RNA through the association with the RNAP II CTD should increase the apparent affinity. In the absence of FluPol, the normal sequence of events connecting cap completion to pause release is thought to be as follows. Nascent transcript capping coincides with promoter-proximal pausing when RNAP II is associated with NELF and DSIF, and the CEs are recruited via interactions with the Ser5P CTD and unphosphorylated SPT5 CTR (see step 2 of **Figure 2**). Subsequent phosphorylation of NELF and DSIF by P-TEFb and recruitment of PAF are required for pause release and the transition to processive RNAP II elongation. But how is the action of P-TEFb coordinated with cap completion and CBC binding? It has recently been shown by NELF depletion that NELF regulates a first step in pause release, and its loss allows RNAP II to advance to the +1 nucleosome dyad position in a P-TEFb-independent manner (196). Importantly, NELF depletion correlates with significantly reduced CBC levels at promoter regions. That NELF has an important role in recruiting the CBC to nascent, capped transcripts is consistent with NELF directly interacting with CBC via the C-terminus of the NELF-E subunit (71, 197). This interaction enhances the affinity of the CBC for the cap 8-fold (197). Thus, as capping progresses to the m^7G methylation step (cap 0) (**Figure 3*a–c***), the affinity of the CBC for the modified 5′ end of the transcript increases by 100–200-fold (195) and is enhanced by the interaction with NELF in *cis* (197). There is further evidence that a direct interaction between the CBC and P-TEFb contributes to the latter's recruitment to paused RNAP II (198). Consistent with this, knockdown of the CBC reduces P-TEFb and Ser2P CTD occupancy at promoters, as well as in coding regions (198). These interactions provide a causal connection between cap completion, CBC binding, and P-TEFb-mediated pause release. However, given that the affinity of the CBC for RNA with additional methylation at the first transcribed nucleotide is not much different from that for cap 0 (195), it is not clear how it is ensured that these additional modifications occur before CBC association.

The next question is, How does FluPol interfere with this process to allow robust cap-snatching, given that its affinity for 5′-capped RNA is substantially lower than that of CBC? A plausible answer is that FluPol somehow manages to block CBC recruitment and/or sterically blocks CBC access to nascent 5′-capped RNAP II transcripts, but how this is achieved is currently unknown. Moreover, this block is only temporary as NCBP1 does associate with viral mRNAs (199). It is possible that FluPol forces the dissociation of NELF, specifically prevents CBC recruitment by NELF-E, or sequesters the CBC such that it cannot bind 5′-capped nascent RNA. Consistent with the last option, the subunits of the CBC were identified as interaction partners of the viral polymerase in proteomics-based interaction screenings (**Table 1**). However, it is unclear whether this interaction is direct or indirect. One study shows that P-TEFb can interact

with FluPol to enhance its interaction with Ser5P RNAP II CTD, thus promoting viral transcription (131). In this scenario, it is possible that FluPol inhibits P-TEFb kinase activity as well as its interaction with the CBC.

4. CONCLUDING REMARKS

Recent high-resolution structures of actively transcribing FluPol at different stages of the transcription cycle have led to significant advances in the understanding of this unique process (9, 22). Similarly, a series of cryo-EM structures, corresponding to complexes of the early RNAP II transcription process, reveals details of the transition from the RNAP II promoter-proximal paused state to the elongation state (53, 102, 103, 200). While these advances form the basis for a detailed description of the coupled RNAP II–FluPol cap-snatching complex, central questions remain to be answered. To generate a more comprehensive model of FluPol cap-snatching, it is key to (*a*) identify the host factors present in the active RNAP II–FluPol cap-snatching complex, (*b*) precisely define the time window during RNAP II transcription when cap-snatching occurs, and (*c*) determine the intranuclear localization of cap-snatching. Aided by this information, it may be possible to determine the structure of an active cap-snatching complex either using in situ cryo-tomography or by reconstitution in vitro.

It is well known that the interaction of FluPol with the Ser5P RNAP II CTD is essential for cap-snatching (28, 29). However, it remains to be determined whether this interaction is specific enough to precisely dock FluPol onto the emerging nascent capped RNA or whether, in analogy with the CE (74), other direct or indirect protein-protein interactions are involved (**Figure 5**). The identified protein partners of FluPol, including SPT5, the preferential association of FluPol with the Ser5P CTD, and the need for cap completion prior to cap-snatching suggest that the cap-snatching complex is assembled on RNAP II in its paused elongation state, but precisely which factors are present and their phosphorylation statuses remain to be determined. Moreover, recent genomic mapping of RNAP II has demonstrated that the Ser5P CTD not only is found in the promoter-proximal region of RNAP II–transcribed genes (37) but also is abundant throughout the gene body, especially at splice sites (182, 186). This further suggests that additional interactions, other than Ser5P CTD binding, target FluPol to the paused RNAP II elongation complex. Another intriguing open question concerns how FluPol is able to robustly compete with the high-affinity nuclear CBC for access to the completed 5′ cap. It is possible that FluPol specifically inhibits recruitment of CBC to the nascent capped RNA before cap-snatching by an unknown mechanism, but paradoxically, CBC is eventually recruited to viral mRNAs (199). Binding of another viral protein, e.g., NS1, to the CBC (125) or indirect interference with host factors related to RNAP II pausing and pause release, such as DSIF, NELF, TRIM28, or P-TEFb, could be involved.

Another level of complexity has recently been added by the emergence of the condensate-based model of transcription, which proposes CTD phosphorylation-dependent RNAP II relocalization from promoter condensates to gene-body condensates (31, 171). It is unclear whether FluPol, either alone or in association with host factors, can undergo phase-separation and localize to these condensates (**Figure 5**). However, some FluPol interaction partners, like FUS, are known to promote phase separation (201), and others, like the ANP32 protein family (which are more implicated in viral replication than transcription), contain large LCDRs (202). Studies on the subnuclear localization and genomic associations of FluPol are needed to further define the model of FluPol cap-snatching in the context of subnuclear compartments.

Cap-snatching represents an attractive target for antiviral intervention, as illustrated by the recent development of inhibitors that target the PB2 cap-binding domain (203) and the PA

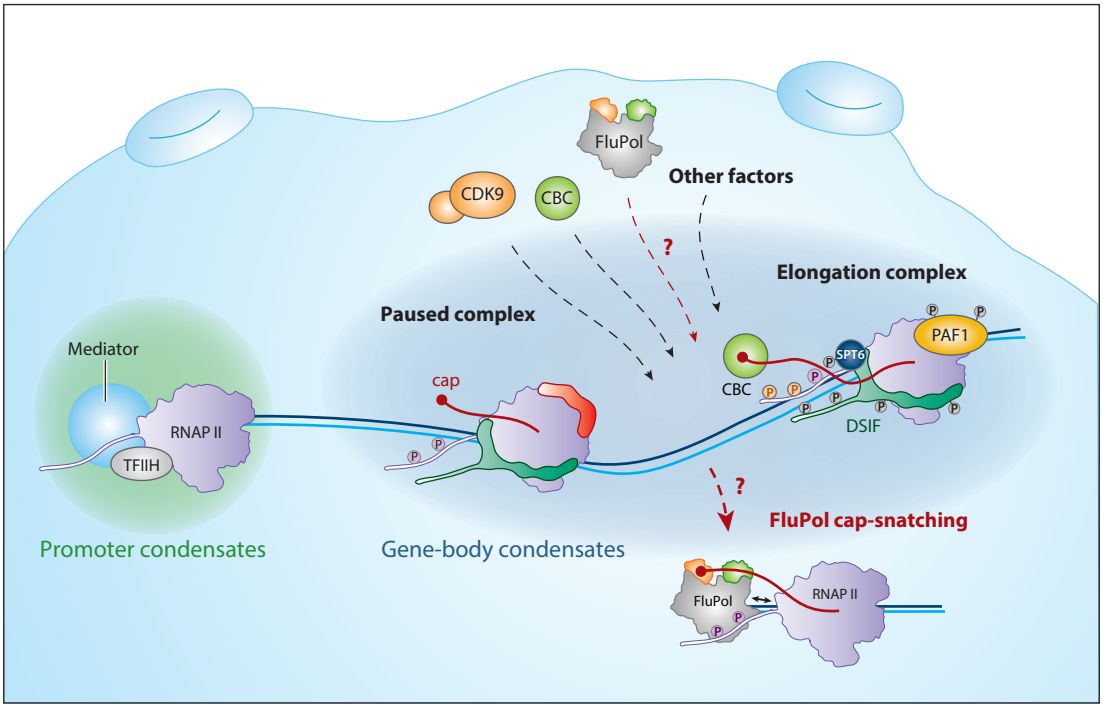


Figure 5

Open questions on FluPol cap-snatching timing and context. The precise nuclear localization of FluPol transcription in the context of promoter and gene-body condensates is unclear. Other unknowns include the precise timing of cap-snatching with respect to cap completion, CBC binding to the nascent capped RNA, and phosphorylation of the transcription machinery by P-TEFb. Potential interactions with other viral factors or cellular factors involved in RNAP II transcription could be involved in coordinating cap-snatching in the context of cellular RNAP II transcription. Abbreviations: CBC, cap-binding complex; CDK, cyclin-dependent kinase; DSIF, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole sensitivity-inducing factor; FluPol, influenza virus RNA-dependent RNA polymerase; PAF1, polymerase-associated factor 1; P-TEFb, positive-transcription elongation factor b; RNAP, RNA polymerase; TFIIH, transcription factor IIH.

endonuclease domain (204). The recently described CTD-binding sites on FluPol possibly represent novel targets of antiviral intervention, even though inhibiting protein-protein interactions is challenging. However, as discussed in this review, it is likely that the FluPol–CTD interaction does not represent the sole interface with the RNAP II transcription machinery. Therefore, gaining deeper knowledge about the cap-snatching process in order to identify novel targets for therapeutic intervention is of great interest.

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