Accepted Manuscript Posted Online
Journal of Virology
JVI

JVI Accepted Manuscript Posted Online 20 April 2016
J. Virol. doi:10.1128/JVI.00494-16
Copyright © 2016 Martínez-Alonso et al.
This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.

RNA-free and ribonucleoprotein-associated influenza virus polymerases directly bind the serine-5 phosphorylated carboxyl-terminal domain of host RNA polymerase II

Mónica Martínez-Alonso,[a] Narin Hengrung,[a,b]* Ervin Fodor[a]#

Sir William Dunn School of Pathology[a], Division of Structural Biology[b], University of Oxford, Oxford, United Kingdom

Running Head: Direct binding of influenza polymerase to Pol II CTD

#Address correspondence to Ervin Fodor, ervin.fodor@path.ox.ac.uk.
*Present address: Narin Hengrung, The Francis Crick Institute, Mill Hill Laboratory, London, United Kingdom

Accepted Manuscript Posted Online

ABSTRACT

Influenza viruses subvert the transcriptional machinery of their hosts to synthesise their own viral mRNA. Ongoing transcription by cellular RNA polymerase II (Pol II) is required for viral mRNA synthesis. By a process known as cap-snatching, the virus steals short 5′ capped RNA fragments from host capped RNAs and uses these to prime viral transcription. An interaction between the influenza A virus RNA polymerase and the C-terminal domain (CTD) of the large subunit of Pol II has been established, but the molecular details of this interaction remain unknown. We show here that influenza virus ribonucleoprotein (vRNP) complex binds to the CTD of transcriptionally engaged Pol II. Furthermore, we provide evidence that the viral polymerase binds directly to the serine-5 phosphorylated form of the Pol II CTD, both in the presence and absence of viral RNA, and show that this interaction is conserved in evolutionarily distant influenza viruses. We propose a model in which direct binding of the viral RNA polymerase in the context of vRNPs to Pol II early in infection facilitates cap-snatching, while we suggest that binding of free viral polymerase to Pol II late in infection may trigger Pol II degradation.

IMPORTANCE

Influenza viruses cause yearly epidemics and occasional pandemics that pose a threat to human health as well as represent a large economic burden to healthcare systems globally. Existing vaccines are not always effective as they may not exactly match the circulating viruses. Furthermore, there are a limited number of antivirals available, and development of resistance to these is a concern. New measures to combat influenza are needed, but before they can be developed it is necessary to better understand the molecular interactions between influenza viruses and their host cells. By providing further insights into the molecular details of how influenza viruses hijack the host transcriptional machinery, we aim to uncover novel targets for development of antivirals.

The segmented negative sense RNA genome of influenza A virus is transcribed and replicated by the viral RNA-dependent RNA polymerase that consists of three subunits, polymerase basic 1 (PB1), polymerase basic 2 (PB2) and polymerase acidic (PA) proteins (1-

Journal of Virology
JVI
Journal of Virology

Accepted Manuscript Posted Online

3). Transcription and replication of the viral RNA genome are carried out in the context of viral ribonucleoprotein (vRNP) complexes in which the 5′ and 3′ termini of viral RNA (vRNA) interact with the viral polymerase while the rest of the RNA is coated by nucleoprotein (NP) (4, 5). Influenza A virus is dependent on the host RNA polymerase II (Pol II) transcriptional machinery. Viral transcription requires 5′ capped primers, which are derived from host capped RNAs (6-9). Furthermore, active Pol II transcription is required for nuclear export of viral mRNAs (10). Previous studies from our group showed that Pol II co-immunoprecipitates with influenza A virus polymerase from infected cell lysates, and trimeric recombinant viral polymerase interacts with the serine-5 phosphorylated form of the C-terminal domain (CTD) of Pol II that is characteristic of initiating Pol II (11). Interaction between the viral polymerase and Pol II was confirmed by further studies (12-15). In addition, influenza virus polymerase was also shown to associate with Pol II promoter DNA (16).

Despite the clear functional and physical link between the viral and host transcriptional machineries, the details of this interaction remain poorly understood. In particular, it is not clear whether only free polymerase interacts with the CTD of Pol II or whether viral polymerase in the context of vRNPs can also interact. Although the influenza polymerase requires active Pol II to provide it with a source of capped RNA primers, the viral polymerase has also been linked to Pol II degradation. This occurs at late times during infection (17, 18), when free polymerase is present, and coincides with the shutdown of viral mRNA synthesis (18). Therefore, association of a free heterotrimeric polymerase to the CTD of Pol II might promote Pol II degradation, while binding of a fully assembled vRNP would more likely facilitate cap-snatching by positioning the viral polymerase next to a supply of nascent, host capped RNAs. Additionally, it is also unknown whether the interaction between the viral polymerase and Pol II CTD is direct or mediated by cellular factors. In fact, this issue remains controversial. While some reports point at cellular factors such as hCLE (19, 20) or cyclin T1/CDK9 (21) as mediators of the interaction between the viral polymerase and Pol II, other reports suggest that this interaction is direct (14). This study was designed to address these questions. Our results indicate that the viral polymerase can interact with the CTD of Pol II that is engaged in active transcription in RNA-free form as well as in the context of vRNPs raising the possibility that the interaction of the viral polymerase with Pol II could fulfil multiple functions.

Journal of Virology
JVI
Journal of Virology

Accepted Manuscript Posted Online

MATERIALS AND METHODS

RNA immunoprecipitation (RIP). RIP was performed as previously described (16, 18, 22) with some modifications. Briefly, HEK 293T cells about 50% confluent were mock infected or infected with influenza A/WSN/33 virus at a multiplicity of infection (MOI) of 5. Cells were harvested 4.5 hours post-infection (hpi) and cross-linked with 1% formaldehyde for 10 min at room temperature and the reaction was quenched by adding glycine to a final concentration of 125 mM. Cells were washed twice with cold PBS and lysed in Buffer A [50 mM Tris-HCl pH 8.0, 0.5% Igepal, 100 mM NaCl, 1 mM DTT, and 1 complete mini EDTA-free protease inhibitor cocktail tablet (Roche) per 10 mL of buffer]. Cells were sonicated for 12.5 min using Bioruptor (Diagenode) and cell lysates were clarified by centrifugation at 16200g for 5 min. Cell lysates were supplemented with $MgSO_4$ and $CaCl_2$ to a final concentration of 10 mM and 1 mM, respectively, and treated with RNase-free DNase (Promega, Cat. No. M610A) for 30 min at 37°C. The reaction was stopped by adding EDTA to a final concentration of 20 mM. These samples were immunoprecipitated overnight at 4°C with antibodies specific for PA (kind gift of T. Toyoda) and Pol II (RNA Pol II, clone CTD4H8, Millipore Cat. No. 05-623) and Protein G Sepharose (Sigma). Immunocomplexes were washed with 10 mM Tris-HCl pH 8.0, 0.1% Igepal, 1 mM PMSF and 1 mM EDTA, containing 150 mM NaCl (once), 1 M NaCl (three times), and 0.5 M LiCl (three times). Crosslinks were reversed in both immunocomplexes and input samples by the addition of Elution Buffer (1% SDS, 50 mM Tris-HCl pH 6.8, 200 mM NaCl, 1 mM EDTA) and heating at 65°C overnight. Protein G Sepharose was removed by centrifugation and samples were treated with proteinase K for 30 min at 45°C. RNA was extracted with phenol-chloroform and precipitated with ethanol in the presence of tRNA carrier. RNA samples were subjected to primer extension analysis of viral neuraminidase (NA) and NP segment-specific RNAs, performed as previously described (23) except that products were analysed on 6% polyacrylamide gels containing 7 M urea. The following primers were used: 5′-TCCAGTATGGTTTTGATTTCCG-3′ (for NA mRNA and cRNA), 5′-TGGACTAGTGGGAGCATCAT-3′ (for NA vRNA), 5′-ATCGTCCAATTCCACCAATC-3′ (for NP mRNA and cRNA), 5′-GAGCTCTCGGACGAAAAGG-3′ (for NP vRNA) and 5′-TCCCAGGCGGTCTCCCATCC-3′ (for 5S rRNA).

Design and synthesis of Pol II CTD mimic peptides. Peptides were chemically synthesised by Cambridge Peptides Ltd using solid phase peptide synthesis. Peptides were designed to contain 4 repeats of the heptapeptide consensus sequence of Pol II CTD (YSPTSPS) with

Journal of Virology JVI Journal of Virology

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

modifications representing different phosphorylation states of the CTD. Full amino acid sequences are shown in Table 1. All peptides were synthesised with C-terminal amidation, N-terminal biotinylation and contained a nine-atom polyethylene glycol spacer between the biotin moiety and the first amino acid. Peptides were HPLC purified to at least 90% purity. Quality control of the peptides was performed by mass spectrometry.

Pull-down assays with CTD mimic peptides. HEK 293T cells were mock infected or infected with influenza A/WSN/33 at a MOI of 5. Cells were harvested 4.5 hpi and lysed on ice for 10 min in Buffer B [10 mM HEPES (PAA, Cat. No. S11-001), 150 mM NaCl, 0.1% Igepal, 1x Halt protease inhibitor cocktail (Pierce, Cat. No. 78425)]. Debris was removed by centrifugation at 16200g for 5 min at 4°C and lysates were pre-cleared by incubation with streptavidin agarose resin (Pierce, Cat. No. 20347) for 2 h. Pol II CTD mimic peptides were bound to the beads for 2 h. Peptide-coated beads were washed three times in Wash Buffer [10 mM HEPES (PAA, Cat. No. S11-001), 150 mM NaCl, 0.1% Igepal, 1 mM PMSF], blocked with 1% BSA for 1 h and washed twice. Pre-cleared lysates were bound to the peptide-coated beads for 2 h at 4°C and unbound material removed by washing three times. Beads were split into two aliquots during the last wash, and either boiled for 5 min in Sample Buffer [250 mM Tris-HCl pH 6.8, 2% SDS, 20 mM DTT, 20% glycerol, 0.01% bromophenol blue] for protein analysis, or the RNA was extracted using Trizol (Ambion), precipitated in the presence of glycogen carrier and analysed by primer extension as described above. For detection of viral proteins by western blot, a custom made rabbit polyclonal antibody raised against the trimeric viral RNA polymerase (Eurogentec) (24) or a rabbit polyclonal antibody against NP (kind gift of P. Digard) were used.

Expression and purification of influenza virus polymerase. For recombinant production of influenza A/WSN/33 virus polymerase in a mammalian cell expression system, HEK 293T cells were grown to about 50% confluency and transfected with pCAGGS-based or pcDNA-based plasmids expressing each of the polymerase subunits (PB1, PA, and C-terminal TAP-tagged PB2) (25, 26) and a plasmid expressing a short 37 nucleotide (nt)-long vRNA-like template derived from segment 5 (27). Cells were harvested 48 h post transfection, washed with cold PBS and lysed on ice for 10 min in Buffer C [50 mM HEPES (PAA, Cat No S11-001), 200 mM NaCl, 25% glycerol, 0.5% Igepal, 1 mM β-mercaptoethanol, 1 mM PMSF, 1x Halt protease inhibitor cocktail (Pierce, Cat. No. 78425)]. Lysates were subjected to purification on IgG Sepharose 6 Fast Flow beads (GE Healthcare, Cat. No. 17-0969-01) and the polymerase was cleaved with AcTEV (Invitrogen) in Cleavage Buffer [10 mM HEPES

Accepted Manuscript Posted Online

(PAA, Cat No S11-001), 150 mM NaCl, 10% glycerol, 0.1% Igepal, 1 mM DTT, 1x Halt protease inhibitor cocktail (Pierce, Cat. No. 78425)]. AcTEV contains a His-tag that allows its removal by incubation with Ni-NTA agarose (Qiagen), and IgG was removed with Protein A Sepharose (Sigma). Recombinant RNA-free influenza A/NT/60/68 and influenza C/Johannesburg/1/66 virus polymerase was produced from baculovirus-infected Sf9 insect cells as described elsewhere (28). To produce the polymerase-vRNA complex, the above protocol was followed, but with the addition of an RNA-binding step before gel filtration. This was carried out by mixing the polymerase in a high salt buffer (2 M NaCl, 25 mM HEPES pH 7.5, 10% glycerol, 1 mM $MgCl_2$, 0.1 mM $MnCl_2$, 0.5 mM TCEP) with a two to three fold molar excess of two synthetic RNA oligonucleotides corresponding to the 5′ and 3′ termini of vRNA (5′-AGUAGAAACAAGGCC-3′ and 5′-GGCCUGCUUUUGCU -3′). The NaCl concentration of the mixture was then reduced to 0.5 M overnight by dialysis. After dialysis, the polymerase-RNA complex was purified away from unbound RNA by gel filtration as described previously (28).

***In vitro* binding of purified viral polymerase to synthetic Pol II CTD mimic peptides.** Pol II CTD mimic peptides were bound to streptavidin agarose resin as described above for the pull-down assays. Viral polymerase, purified either from HEK 293T or Sf9 cells, was bound to the peptide-coated beads for 2 h at 4°C. Complexes were washed three times with Wash Buffer, and split into two aliquots during the last wash. For protein analysis, beads were boiled for 5 min in Sample Buffer and analysed by silver staining. For RNA analysis, the RNA in the bound complexes was extracted using Trizol (Ambion) and precipitated in the presence of glycogen. RNA was dephosphorylated for 10 min at 37°C with FastAP (Fermentas), and the enzyme was inactivated by heating at 75°C for 5 min. Dephosphorylated RNA was 5′ end labelled with [γ-32P]-ATP for 1 h at 30°C using T4 Polynucleotide Kinase (Fermentas). Both reactions were carried out in Tango buffer (Fermentas). Labelled RNA was mixed with formamide, heated at 95°C for 3 min and analysed on 20% polyacrylamide gels containing 7 M urea in TBE buffer, and visualised by autoradiography.

***In vitro* transcription assay.** The viral polymerase was immobilised on streptavidin resin coated with Pol II CTD mimic peptides as described above, and its transcriptional activity was evaluated with an [α-32P]GTP incorporation assay as previously described (29). Briefly, 1.75 µl of peptide-bound polymerase was incubated in a 3.5 µl reaction volume containing 1 mM ATP, 0.5 mM CTP, 0.5 mM UTP, 1 µCi [α-32P]GTP (Perkin Elmer), 10 ng β-globin mRNA (Sigma), 5 mM $MgCl_2$, 2 mM DTT, and 1 U/µl RNasin (Promega) for 2 h at 30 °C,

Journal of Virology JVI Journal of Virology

Accepted Manuscript Posted Online

mixed with 10 µl of formamide and heated at 95 °C for 3 min. Transcription products were analysed on 16% polyacrylamide gels containing 7 M urea in TBE buffer, and visualised by autoradiography.

RESULTS

Viral RNAs co-immunoprecipitate with Pol II. Although the viral polymerase in the absence of NP and viral RNA has been shown to interact with Pol II (11) it is unclear whether polymerase in the context of vRNP can also associate with Pol II. If the viral polymerase interaction with Pol II were to facilitate viral mRNA synthesis, viral polymerase in the context of vRNP would be expected to bind Pol II. To test this, HEK 293T cells were mock infected or infected with influenza A/WSN/33, harvested 4.5 hpi and subjected to RNA immunoprecipitation (RIP). Co-immunoprecipitated RNAs were analysed for NA (Fig. 1A) and NP (Fig. 1B) viral RNAs by primer extension. As expected, vRNA and cRNA could be immunoprecipitated with an antibody against PA (Fig. 1A and 1B). When Pol II complexes were analysed vRNA and mRNA was detected, as well as low amounts of cRNA. No 5S rRNA was immunoprecipitated with the PA- or Pol II-specific antibodies, and no RNAs could be detected in a control without primary antibody, confirming the specificity of the interactions. These results suggest that polymerase in the context of vRNPs also interacts with Pol II.

vRNPs can be pulled down from infected cell lysates with a Pol II CTD mimic peptide phosphorylated on serine-5. To further investigate the interaction between vRNPs and Pol II, a peptide pull-down assay was developed. Biotinylated Pol II CTD mimic peptides containing four copies of the conserved heptapeptide repeat (YSPTSPS) of Pol II CTD were chemically synthesised. Although the full-length human Pol II CTD consists of 52 heptad repeats, we reasoned that four repeats would be sufficient for the interaction, based on structural studies of other CTD-binding proteins (30). It was previously shown that the interaction of the viral polymerase with Pol II depends on the phosphorylation status of the CTD. In particular, the viral polymerase interacts with the initiating form of Pol II, which is phosphorylated on serine-5 in the CTD, but not with the elongating form, phosphorylated on serine-2 (11). Therefore, we used peptides phosphorylated on serine-2 (Ser2P) or serine-5 (Ser5P), an unphosphorylated peptide and a scrambled control peptide (Table 1). The results show that the peptide phosphorylated on serine-5 was able to pull-down vRNPs from infected

Journal of Virology
JVI
Journal of Virology

Accepted Manuscript Posted Online

cell lysates as indicated by the presence of viral polymerase and NP in the bound complexes (Fig. 2). The peptide phosphorylated on serine-2, the unphosphorylated and the scrambled control peptide bound only background levels of NP while no viral polymerase could be detected. Furthermore, RNA extracted from the complexes bound to the peptides was analysed by primer extension using a primer specific for the NA segment RNAs. The peptide phosphorylated on serine-5 was able to specifically pull-down NA vRNA (Fig. 2). No mRNA and cRNA could be detected. Taken together, these data further support the notion that viral polymerase in the context of vRNPs can interact with Pol II.

Influenza A virus polymerase binds directly and specifically to Pol II CTD phosphorylated on serine-5. To address the question of whether the viral polymerase interacts directly with the Pol II CTD, the set of peptides described above was incubated with recombinant viral polymerase expressed and purified from HEK 293T cells in the presence or absence of a 37 nt-long vRNA-like template. Short vRNA-like templates can be transcribed and replicated in cells in the absence of NP (23, 31). The viral polymerase bound specifically to the serine-5 phosphorylated CTD mimic peptide and did so only when vRNA was co-expressed (Fig. 3A, upper panel). To confirm that the vRNA template co-purified with the polymerase remained bound throughout the peptide binding assay, RNA was extracted from bound complexes, 5′ end labelled with [γ-32P]-ATP and analysed on a polyacrylamide gel. As expected, the 37 nt-long vRNA was present in the complexes bound to the CTD mimic peptide phosphorylated on serine-5 (Fig. 3A, lower panel). Only background binding of the polymerase to the other peptides was observed.

As we could not entirely exclude the possibility that a factor co-purifying from mammalian cells with the viral polymerase could have been involved in mediating the interaction with the CTD mimic peptide, we also tested viral polymerase produced in Sf9 insect cells, in the presence or absence of 15 and 14 nt-long RNA oligonucleotides mimicking the 5′ and 3′ ends of the vRNA promoter. As expected, these preparations of the viral polymerase also bound specifically to the CTD mimic peptide phosphorylated on serine-5. However, in the case of these highly pure preparations the presence or absence of vRNA did not affect the binding, as both RNA-free and RNA-bound forms interacted equally well (Fig. 3B, upper panel). We confirmed that the 5′ and 3′ ends of the vRNA promoter remained bound to the polymerase that bound to the Pol II CTD mimic peptide phosphorylated on serine-5 (Fig. 3B, lower panel). The differential requirement for vRNA promoter in CTD peptide binding by insect and mammalian cell derived viral polymerase may be due to the presence of contaminating

Journal of Virology
JVI
Journal of Virology

Accepted Manuscript Posted Online

host factors (either protein or RNA). In the mammalian system, we consistently find that co-expression of short vRNA-like templates reduces the amount of cellular proteins and RNA that co-purify with the viral polymerase (Fig. 3C). Therefore, the inability of viral polymerase expressed in mammalian cells without vRNA to bind to CTD peptide may be due to higher levels of contaminating inhibitory cellular factors. Altogether, these data show that the binding of the viral polymerase to the serine-5 phosphorylated CTD of Pol II is direct and both the RNA-free form and the vRNA promoter-bound form of the viral polymerase are able to bind Pol II.

Influenza A virus polymerase is transcriptionally active when bound to a Pol II CTD mimic peptide. If the binding of the viral polymerase to the CTD of Pol II were to facilitate cap-snatching for viral transcription, polymerase bound to Pol II CTD would be expected to be active in transcription. To test this hypothesis, recombinant viral polymerase was purified from HEK 293T cells co-expressing a 37 nt-long vRNA-like template and immobilised on streptavidin resin coated with the CTD mimic peptide phosphorylated on serine-5. The activity of the viral polymerase was tested *in vitro* using β-globin mRNA as a cap donor to prime viral transcription. Quantification of capped transcription products revealed that there was at least 35 fold higher activity obtained with the polymerase bound to the CTD mimic peptide phosphorylated on serine-5 compared with the negative control scrambled peptide (Fig. 3D). Only background levels of products were obtained if the cap-donor, UTP or ATP were omitted from the reaction. These results show that binding of the viral polymerase to Pol II CTD is compatible with viral transcription.

Binding of the viral polymerase to Pol II CTD is conserved in evolutionarily distant influenza C virus. If the interaction of the viral polymerase with Pol II CTD were required for viral transcription, it would be expected that this interaction would also occur with evolutionarily distant influenza viruses, such as influenza C virus. To test this hypothesis, recombinant influenza C virus RNA polymerase was expressed and purified from Sf9 cells in the absence of vRNA, and incubated with the set of CTD mimic peptides as described above. Influenza C virus polymerase bound specifically to the CTD mimic peptide phosphorylated on serine-5 (Fig. 3E), matching the binding pattern found for influenza A virus polymerase (Fig. 3A and 3B). This result shows that the interaction of the influenza virus polymerase with Pol II is evolutionarily conserved across influenza virus genera.

Journal of Virology
JVI
Journal of Virology

Accepted Manuscript Posted Online

DISCUSSION

In this study we investigated the molecular details of how the influenza virus transcriptional machinery interacts with cellular Pol II. We provide biochemical evidence that not only RNA-free trimeric viral polymerase, but also viral polymerase in the context of vRNPs can associate with Pol II. First, we showed that viral RNAs are present in complexes containing Pol II. Second, we were able to pull down vRNPs from influenza virus-infected cell lysates using Pol II CTD mimic peptides. We showed that viral polymerase, NP as well as vRNA were present in the pull-downs. vRNPs were pulled down specifically only with a Pol II CTD mimic peptide phosphorylated on serine-5, in agreement with previous data that the viral polymerase associates with the serine-5 phosphorylated form of the CTD (11).

In addition to demonstrating that vRNPs can interact with the CTD of Pol II, we also show here that the binding between the viral polymerase and the CTD is direct. Indeed, recombinant trimeric influenza A polymerase from two different viral strains (A/WSN/33 and A/NT/60/68, purified from a mammalian or an insect cell expression system, respectively), was able to bind specifically to a Pol II CTD mimic peptide that was phosphorylated on serine-5. We were not able to detect any binding when polymerase subunits were individually expressed and purified (data not shown). This result is in agreement with previous data showing that none of the individually expressed polymerase subunits, or combinations of two subunits, co-purified with a tagged version of Pol II CTD (11). However, this may be because individually expressed and purified viral polymerase subunits are misfolded or are in a conformation incompatible with CTD binding. Indeed, a yeast two-hybrid screen identified the PA subunit as an interactor of the large subunit of Pol II (14).

In terms of evolution, influenza A and B viruses are more closely related to each other than they are to influenza C viruses (32, 33). In fact, amino acid sequences of the polymerase subunits show the least conservation between influenza A and C viruses (38.4%, 23.3% and 25.4% identity for PB1, PB2 and PA/P3, respectively) (34). Therefore, we chose the viral polymerase of influenza C virus to test whether polymerase binding to the CTD of Pol II was a conserved feature amongst influenza viruses. Indeed, our results show that influenza C virus polymerase binds directly to the initiating form of Pol II, which suggests that influenza viruses have evolved a conserved mechanism to hijack the transcriptional machinery of the host cell. Hence, the interaction domain of the influenza virus RNA polymerase involved in binding to the CTD of Pol II is likely to be highly conserved between influenza virus genera

Journal of Virology
JVI
Journal of Virology

Accepted Manuscript Posted Online

and therefore drugs targeting this interaction domain could be active against different influenza virus types.

Our data show that both RNA-free viral polymerase and vRNPs associate with the CTD of Pol II. The association of vRNPs with the CTD likely provides the viral polymerase with a platform to carry out transcription, enabling the polymerase to access nascent host capped RNAs as well as splicing factors and factors required for mRNP assembly (35). The CTD of Pol II is dynamically modified during the transcription cycle, undergoing different phosphorylation states that correlate with Pol II progress through transcription. Thus, a hypophosphorylated CTD is a mark of pre-initiating Pol II that can bind at promoters, while serine-5 and serine-2 phosphorylation marks correspond to initiating and elongating Pol II, respectively (36, 37). Influenza virus vRNPs specifically target Pol II CTD when it is phosphorylated at serine-5, the form of Pol II that is involved in capping nascent transcripts. Therefore, the physical association of influenza vRNPs with Pol II early in infection is likely to promote cap-snatching by providing access to nascent cellular capped RNAs for viral mRNA synthesis (Fig. 4). The regulation of the interaction between the viral polymerase in the context of vRNPs, and the CTD, remains unclear. Thus, it is not known whether the viral polymerase is released from the CTD immediately after cap-snatching or remains associated with it while it completes mRNA synthesis.

What would be the function of RNA-free trimeric polymerase associating with Pol II? Such a polymerase, lacking template vRNA, would not be competent in cap-snatching, as the polymerase needs to be associated with both the 5′ and 3′ end of vRNA to efficiently cap-snatch (38-40). Influenza virus infection results in the degradation of the large subunit of Pol II at late stages of infection, and expression of the polymerase, in the absence of viral RNA and NP, has been shown to induce Pol II degradation (17). Expression of all three subunits of the viral polymerase was required for Pol II degradation. Neither the expression of individual polymerase subunits nor expression of combinations of two of them was sufficient to induce induced Pol II degradation, in agreement with the finding that all three subunits of the viral polymerase are required for Pol II interaction. Indeed, the ability of the polymerase to induce degradation of Pol II has been linked to its ability to interact with Pol II (18). Thus, we speculate that late in infection, as free viral polymerase accumulates in the infected host cell nucleus, the main role of binding of the polymerase to Pol II is to trigger Pol II degradation to inhibit host gene expression, including the expression of antiviral genes (Fig. 4). In fact, the ability of the viral polymerase to degrade Pol II has been linked to virulence (41). This model

Journal of Virology
JVI
Journal of Virology

Accepted Manuscript Posted Online

is consistent with the pattern of the accumulation of the different types of viral RNAs in infected cells. mRNA synthesis peaks early in infection followed by a sharp decline late in infection, most likely due to the exhaustion of a source of capped RNA primers. In contrast, vRNA replication, which is independent of Pol II, continues late into infection.

It is not clear how binding of the polymerase to the CTD of Pol II would trigger Pol II degradation. However, the ubiquitin-proteasome system is likely to be involved. Our group reported that increasing amounts of ubiquitylated Pol II are present late in infection and the expression of the viral polymerase trimer is sufficient to trigger ubiquitylation of the serine-5 phosphorylated form of Pol II. Furthermore, the expression of a viral polymerase mutant with reduced Pol II-binding activity induced reduced levels of ubiquitylated Pol II (18). We also found that the viral polymerase interacts with several ubiquitin ligases (42). It is possible that the viral RNA polymerase, by binding the CTD of Pol II late in infection, recruits a ubiquitin ligase to mediate the ubiquitylation of Pol II and its subsequent degradation by the proteasome. Although this mechanism would lead to the specific degradation of serine-5 phosphorylated Pol II, given the dynamic nature of CTD phosphorylation, other forms of Pol II would be depleted as well. Indeed, a specific reduction in the hypophosphorylated form of Pol II has been reported in virus infected cells and also upon the expression of the viral polymerase heterotrimer (17, 18). The influenza virus polymerase has been shown to exist in multiple conformations depending on viral RNA binding (34, 40, 43, 44). The vRNP-bound polymerase associated with Pol II involved in cap-snatching would be in the conformation described for influenza A and B virus polymerases. However, the RNA-free polymerase triggering Pol II degradation might be in the apo conformation described for the influenza C virus polymerase. Only the apo conformation might be competent in recruiting ubiquitin ligases such that no degradation would occur as a result of viral polymerase binding in the context of vRNPs.

Induction of Pol II degradation is not unique to influenza virus. La Crosse and Schmallenberg virus, both members of the family *Bunyaviridae*, encode the NSs protein that is known to trigger a DNA damage response-like degradation of transcribing RNA polymerase II (45, 46). Perhaps the influenza virus RNA polymerase also acts by triggering a DNA damage response-like phenomenon.

Taken together, we show in this study that both vRNP bound and free RNA polymerase associates with Pol II and we propose that the two associations have different roles during the viral replication cycle (Fig. 4). On one hand, this interaction allows the virus to promote the

Journal of Virology
JVI
Journal of Virology

Accepted Manuscript Posted Online

transcription of its genes, on the other, it allows the virus to shut-off the host with important consequences for virulence.

ACKNOWLEDGEMENTS

We thank Tetsuya Toyoda and Paul Digard for antibodies. We also thank Frank Vreede for helpful discussions and Jane Sharps for providing technical advice.

FUNDING INFORMATION

This research was supported by a Marie Curie Intra European Fellowship within the 7th European Community Framework Programme (to M.M-A.), a Medical Research Council (MRC) programme grant MR/K000241/1 (to E.F.), and a Wellcome Trust Studentship 092931/Z/10/Z (to N.H.).

REFERENCES

1. **Fodor E.** 2013. The RNA polymerase of influenza a virus: mechanisms of viral transcription and replication. Acta Virol **57:**113-122.

2. **Resa-Infante P, Jorba N, Coloma R, Ortin J.** 2011. The influenza virus RNA synthesis machine: advances in its structure and function. RNA Biol **8:**207-215.

3. **Rodriguez-Frandsen A, Alfonso R, Nieto A.** 2015. Influenza virus polymerase: Functions on host range, inhibition of cellular response to infection and pathogenicity. Virus Res **209:**23-38.

4. **Arranz R, Coloma R, Chichon FJ, Conesa JJ, Carrascosa JL, Valpuesta JM, Ortin J, Martin-Benito J.** 2012. The structure of native influenza virion ribonucleoproteins. Science **338:**1634-1637.

5. **Moeller A, Kirchdoerfer RN, Potter CS, Carragher B, Wilson IA.** 2012. Organization of the influenza virus replication machinery. Science **338:**1631-1634.

6. **Gu W, Gallagher GR, Dai W, Liu P, Li R, Trombly MI, Gammon DB, Mello CC, Wang JP, Finberg RW.** 2015. Influenza A virus preferentially snatches noncoding RNA caps. RNA **21:**2067-2075.

7. **Koppstein D, Ashour J, Bartel DP.** 2015. Sequencing the cap-snatching repertoire of H1N1 influenza provides insight into the mechanism of viral transcription initiation. Nucleic Acids Res **43:**5052-5064.

Journal of Virology
JVI
Journal of Virology

Accepted Manuscript Posted Online

8. **Krug RM, Broni BA, Bouloy M.** 1979. Are the 5' ends of influenza viral mRNAs synthesized in vivo donated by host mRNAs? Cell **18:**329-334.

9. **Sikora D, Rocheleau L, Brown EG, Pelchat M.** 2014. Deep sequencing reveals the eight facets of the influenza A/HongKong/1/1968 (H3N2) virus cap-snatching process. Sci Rep **4:**6181.

10. **Amorim MJ, Read EK, Dalton RM, Medcalf L, Digard P.** 2007. Nuclear export of influenza A virus mRNAs requires ongoing RNA polymerase II activity. Traffic **8:**1-11.

11. **Engelhardt OG, Smith M, Fodor E.** 2005. Association of the influenza A virus RNA-dependent RNA polymerase with cellular RNA polymerase II. J Virol **79:**5812-5818.

12. **Mayer D, Molawi K, Martinez-Sobrido L, Ghanem A, Thomas S, Baginsky S, Grossmann J, Garcia-Sastre A, Schwemmle M.** 2007. Identification of cellular interaction partners of the influenza virus ribonucleoprotein complex and polymerase complex using proteomic-based approaches. J Proteome Res **6:**672-682.

13. **Rameix-Welti MA, Tomoiu A, Dos Santos Afonso E, van der Werf S, Naffakh N.** 2009. Avian Influenza A virus polymerase association with nucleoprotein, but not polymerase assembly, is impaired in human cells during the course of infection. J Virol **83:**1320-1331.

14. **Tafforeau L, Chantier T, Pradezynski F, Pellet J, Mangeot PE, Vidalain PO, Andre P, Rabourdin-Combe C, Lotteau V.** 2011. Generation and comprehensive analysis of an influenza virus polymerase cellular interaction network. J Virol **85:**13010-13018.

15. **Loucaides EM, von Kirchbach JC, Foeglein A, Sharps J, Fodor E, Digard P.** 2009. Nuclear dynamics of influenza A virus ribonucleoproteins revealed by live-cell imaging studies. Virology **394:**154-163.

16. **Chan AY, Vreede FT, Smith M, Engelhardt OG, Fodor E.** 2006. Influenza virus inhibits RNA polymerase II elongation. Virology **351:**210-217.

17. **Rodriguez A, Perez-Gonzalez A, Nieto A.** 2007. Influenza virus infection causes specific degradation of the largest subunit of cellular RNA polymerase II. J Virol **81:**5315-5324.

18. **Vreede FT, Chan AY, Sharps J, Fodor E.** 2010. Mechanisms and functional implications of the degradation of host RNA polymerase II in influenza virus infected cells. Virology **396:**125-134.

Journal of Virology
JVI
Journal of Virology

Accepted Manuscript Posted Online

19. **Perez-Gonzalez A, Rodriguez A, Huarte M, Salanueva IJ, Nieto A.** 2006. hCLE/CGI-99, a human protein that interacts with the influenza virus polymerase, is a mRNA transcription modulator. J Mol Biol **362:**887-900.

20. **Rodriguez A, Perez-Gonzalez A, Nieto A.** 2011. Cellular human CLE/C14orf166 protein interacts with influenza virus polymerase and is required for viral replication. J Virol **85:**12062-12066.

21. **Zhang J, Li G, Ye X.** 2010. Cyclin T1/CDK9 interacts with influenza A virus polymerase and facilitates its association with cellular RNA polymerase II. J Virol **84:**12619-12627.

22. **Bier K, York A, Fodor E.** 2011. Cellular cap-binding proteins associate with influenza virus mRNAs. J Gen Virol **92:**1627-1634.

23. **Turrell L, Lyall JW, Tiley LS, Fodor E, Vreede FT.** 2013. The role and assembly mechanism of nucleoprotein in influenza A virus ribonucleoprotein complexes. Nat Commun **4:**1591.

24. **Hutchinson EC, Charles PD, Hester SS, Thomas B, Trudgian D, Martinez-Alonso M, Fodor E.** 2014. Conserved and host-specific features of influenza virion architecture. Nat Commun **5:**4816.

25. **Deng T, Sharps J, Fodor E, Brownlee GG.** 2005. In vitro assembly of PB2 with a PB1-PA dimer supports a new model of assembly of influenza A virus polymerase subunits into a functional trimeric complex. J Virol **79:**8669-8674.

26. **Fodor E, Crow M, Mingay LJ, Deng T, Sharps J, Fechter P, Brownlee GG.** 2002. A single amino acid mutation in the PA subunit of the influenza virus RNA polymerase inhibits endonucleolytic cleavage of capped RNAs. J Virol **76:**8989-9001.

27. **Paterson D, te Velthuis AJ, Vreede FT, Fodor E.** 2014. Host restriction of influenza virus polymerase activity by PB2 627E is diminished on short viral templates in a nucleoprotein-independent manner. J Virol **88:**339-344.

28. **York A, Hengrung N, Vreede FT, Huiskonen JT, Fodor E.** 2013. Isolation and characterization of the positive-sense replicative intermediate of a negative-strand RNA virus. Proc Natl Acad Sci U S A **110:**E4238-E4245.

29. **Brownlee GG, Sharps JL.** 2002. The RNA polymerase of influenza a virus is stabilized by interaction with its viral RNA promoter. J Virol **76:**7103-7113.

30. **Fabrega C, Shen V, Shuman S, Lima CD.** 2003. Structure of an mRNA capping enzyme bound to the phosphorylated carboxy-terminal domain of RNA polymerase II. Mol Cell **11:**1549-1561.

Journal of Virology
JVI
Journal of Virology

Accepted Manuscript Posted Online

31. **Resa-Infante P, Recuero-Checa MA, Zamarreno N, Llorca O, Ortin J.** 2010. Structural and Functional Characterization of an Influenza Virus RNA Polymerase-Genomic RNA Complex. J Virol **84:**10477-10487.

32. **Gammelin M, Altmuller A, Reinhardt U, Mandler J, Harley VR, Hudson PJ, Fitch WM, Scholtissek C.** 1990. Phylogenetic analysis of nucleoproteins suggests that human influenza A viruses emerged from a 19th-century avian ancestor. Mol Biol Evol **7:**194-200.

33. **Yamashita M, Krystal M, Palese P.** 1989. Comparison of the three large polymerase proteins of influenza A, B, and C viruses. Virology **171:**458-466.

34. **Hengrung N, El Omari K, Serna Martin I, Vreede FT, Cusack S, Rambo RP, Vonrhein C, Bricogne G, Stuart DI, Grimes JM, Fodor E.** 2015. Crystal structure of the RNA-dependent RNA polymerase from influenza C virus. Nature **527:**114-117.

35. **York A, Fodor E.** 2013. Biogenesis, assembly, and export of viral messenger ribonucleoproteins in the influenza A virus infected cell. RNA Biol **10:**1274-1282.

36. **Egloff S, Murphy S.** 2008. Cracking the RNA polymerase II CTD code. Trends in Genetics **24:**280-288.

37. **Eick D, Geyer M.** 2013. The RNA polymerase II carboxy-terminal domain (CTD) code. Chem Rev **113:**8456-8490.

38. **Cianci C, Tiley L, Krystal M.** 1995. Differential activation of the influenza virus polymerase via template RNA binding. J Virol **69:**3995-3999.

39. **Rao P, Yuan W, Krug RM.** 2003. Crucial role of CA cleavage sites in the cap-snatching mechanism for initiating viral mRNA synthesis. EMBO J **22:**1188-1198.

40. **Thierry E, Guilligay D, Kosinski J, Bock T, Gaudon S, Round A, Pflug A, Hengrung N, El Omari K, Baudin F, Hart DJ, Beck M, Cusack S.** 2016. Influenza Polymerase Can Adopt an Alternative Configuration Involving a Radical Repacking of PB2 Domains. Mol Cell **61:**125-137.

41. **Llompart CM, Nieto A, Rodriguez-Frandsen A.** 2014. Specific residues of PB2 and PA influenza virus polymerase subunits confer the ability for RNA polymerase II degradation and virus pathogenicity in mice. J Virol **88:**3455-3463.

42. **York A, Hutchinson EC, Fodor E.** 2014. Interactome analysis of the influenza A virus transcription/replication machinery identifies protein phosphatase 6 as a cellular factor required for efficient virus replication. J Virol **88:**13284-13299.

43. **Pflug A, Guilligay D, Reich S, Cusack S.** 2014. Structure of influenza A polymerase bound to the viral RNA promoter. Nature **516:**355-360.

Journal of Virology
JVI
Journal of Virology

Accepted Manuscript Posted Online

44. **Reich S, Guilligay D, Pflug A, Malet H, Berger I, Crepin T, Hart D, Lunardi T, Nanao M, Ruigrok RW, Cusack S.** 2014. Structural insight into cap-snatching and RNA synthesis by influenza polymerase. Nature **516:**361-366.

45. **Barry G, Varela M, Ratinier M, Blomstrom AL, Caporale M, Seehusen F, Hahn K, Schnettler E, Baumgartner W, Kohl A, Palmarini M.** 2014. NSs protein of Schmallenberg virus counteracts the antiviral response of the cell by inhibiting its transcriptional machinery. J Gen Virol **95:**1640-1646.

46. **Verbruggen P, Ruf M, Blakqori G, Overby AK, Heidemann M, Eick D, Weber F.** 2011. Interferon antagonist NSs of La Crosse virus triggers a DNA damage response-like degradation of transcribing RNA polymerase II. J Biol Chem **286:**3681-3692.

Journal of Virology
JVI
Journal of Virology

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

FIGURE LEGENDS

FIG 1 Viral RNAs co-immunoprecipitate with Pol II. HEK 293T cells were infected with influenza A/WSN/33 virus, harvested 4.5 h post-infection and subjected to RIP. RNAs from cell lysates (Input) and immunoprecipitates (IP) were analysed by primer extension using NA (panel A) and NP (panel B) segment-specific primers. A primer specific for 5S rRNA was used as a control. Note that the sample used for analysis of the input corresponds to 1/10 of that used for the immunoprecipitations.

FIG 2 vRNPs from infected cell lysates bind to serine-5 phosphorylated Pol II CTD mimic peptide *in vitro*. HEK 293T cells were infected with influenza A/WSN/33 virus or mock infected, harvested 4.5 h post-infection and lysed. Differentially phosphorylated Pol II CTD mimic peptides were immobilised on streptavidin agarose resin and incubated with the lysates. Bound complexes were analysed by silver staining (upper panel), western blot (second and third panels) using antibodies against the viral polymerase (3P) and NP, and primer extension of viral RNAs derived from the NA segment (lower panel). Background binding to streptavidin agarose resin without peptide was also analysed, and total cell lysates (Input) were included. S2P, Ser2P; S5P, Ser5P; UP, unphosphorylated; SCB, scrambled; B, beads; I, input.

FIG 3 Purified recombinant viral RNA polymerase binds to serine-5 phosphorylated Pol II CTD mimic peptide *in vitro*. (A) Recombinant viral polymerase from influenza A/WSN/33 (H1N1) virus was expressed in and purified from HEK 293T cells, in the presence (+) or absence (-) of a 37 nt-long vRNA-like template. Peptides mimicking different phosphorylation states of Pol II CTD (Ser2P, Ser5P, unphosphorylated) and a scrambled peptide control were immobilised on streptavidin agarose resin and incubated with purified viral polymerase. Complexes bound to the peptides were analysed by silver staining (upper panel) and RNA was detected by 5′ end labelling with [γ-32P]-ATP (lower panel). (B) Recombinant viral polymerase from influenza A/NT/60/68 (H3N2) virus was expressed in and purified from Sf9 insect cells, in the presence (+) or absence (-) of 15 and 14 nt-long RNAs corresponding to the 5′ and 3′ ends of the vRNA promoter, respectively. The polymerase was incubated with the Pol II CTD mimic peptides, and bound complexes were analysed as in panel (A). (C) Input samples of recombinant viral polymerase from panel (A) analysed for the presence of contaminating host proteins and RNA. Silver staining shows higher levels of contaminating host proteins co-purifying with the viral polymerase if vRNA is absent (left panel). Labelling of RNA with [γ-32P]-ATP shows that in the absence of

Accepted Manuscript Posted Online

vRNA there are higher levels of contaminating cellular RNA present as represented by the strong smear (right panel). (D) *In vitro* transcription by recombinant viral polymerase from influenza A/WSN/33 (H1N1) virus expressed in and purified from HEK 293T cells in the presence of a 37 nt-long vRNA-like template, using β-globin mRNA as a cap donor. Transcription products of input polymerase are shown as a positive control (lane 1). Lanes 2 to 5 show the transcriptional activity of the polymerase captured by Pol II CTD mimic peptides immobilised on steptavidin agarose resin. Transcription products are synthesised when the polymerase is bound to a Pol II CTD mimic peptide phosphorylated on serine-5 (lanes 2 and 4). A scrambled peptide with no detectable polymerase bound is included as a negative control (lanes 3 and 5). No transcription products are obtained in absence of β-globin mRNA cap donor, UTP or ATP (lanes 6 to 8). Lanes 4 and 5 show the result of a 2-fold dilution of the viral polymerase, compared to lanes 2 and 3. (E) Influenza C/Johannesburg/1/66 virus recombinant polymerase was expressed in and purified from Sf9 insect cells. The polymerase was incubated with the peptides as above, and bound complexes were analysed by silver staining. S2P, Ser2P; S5P, Ser5P; UP, unphosphorylated; SCB, scrambled; B, beads; I, input.

FIG 4 Model for the dual role of the interaction of the influenza virus RNA polymerase with the CTD of the large subunit of Pol II. Early in infection (left) binding of the viral polymerase in the context of vRNP to the Pol II CTD facilitates cap-snatching from nascent host capped RNA. The viral polymerase (3P) is shown in a surface model representation in the 'transcription pre-initiation' state with the PB2 cap-binding and PA endonuclease domains aligned for cap-snatching (PDB: 4WSB). Late in infection (right) binding of the free viral polymerase (3P) to the Pol II CTD triggers Pol II degradation. The viral polymerase is shown in the apo conformation, with the cap-binding pocket of PB2 blocked (PDB: 5D98). PB1, dark yellow; PB2, green; PA/P3, blue.

Journal of Virology
JVI
Journal of Virology

Accepted Manuscript Posted Online

631 **TABLES**

632 TABLE 1 Design of Pol II CTD mimic peptides with different phosphorylation states.

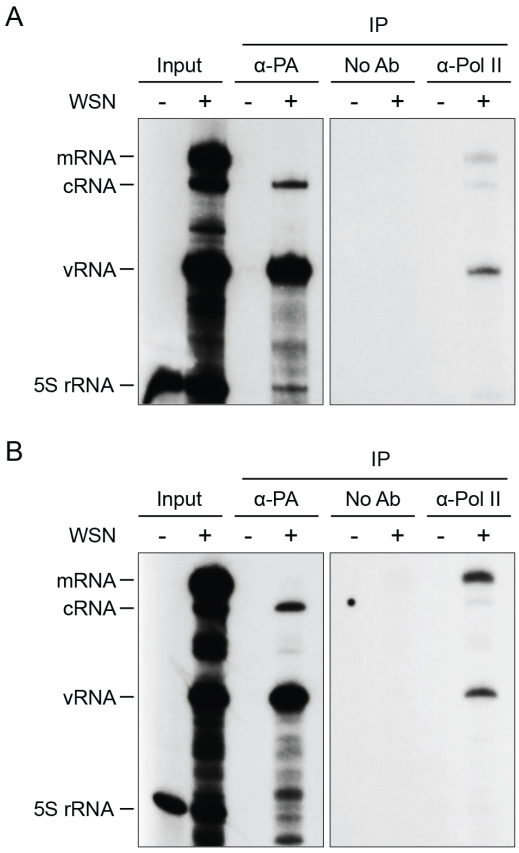
Peptide	Sequence
Ser2P	Y(pS)PTSPSY(pS)PTSPSY(pS)PTSPSY(pS)PTSPS[a]
Ser5P	YSPT(pS)PSYSPT(pS)PSYSPT(pS)PSYSPT(pS)PS[a]
Unphosphorylated	YSPTSPSYSPTSPSYSPTSPSYSPTSPS
Scrambled	PSSSTPSSYTPSPSSSPTSYSPYYTSPP

633 [a](pS) indicates phosphoserine.

Journal of Virology
JVI
Journal of Virology

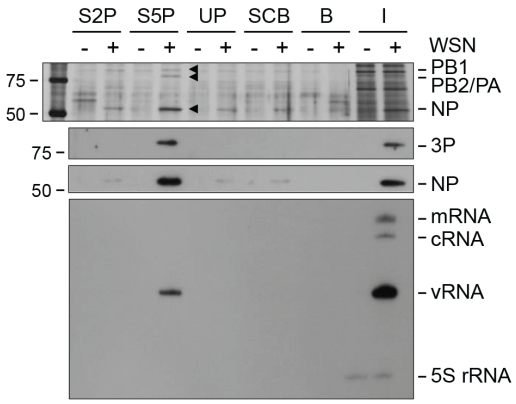
Accepted Manuscript Posted Online
Journal of Virology
JVI
Journal of Virology

Figure 1



Accepted Manuscript Posted Online

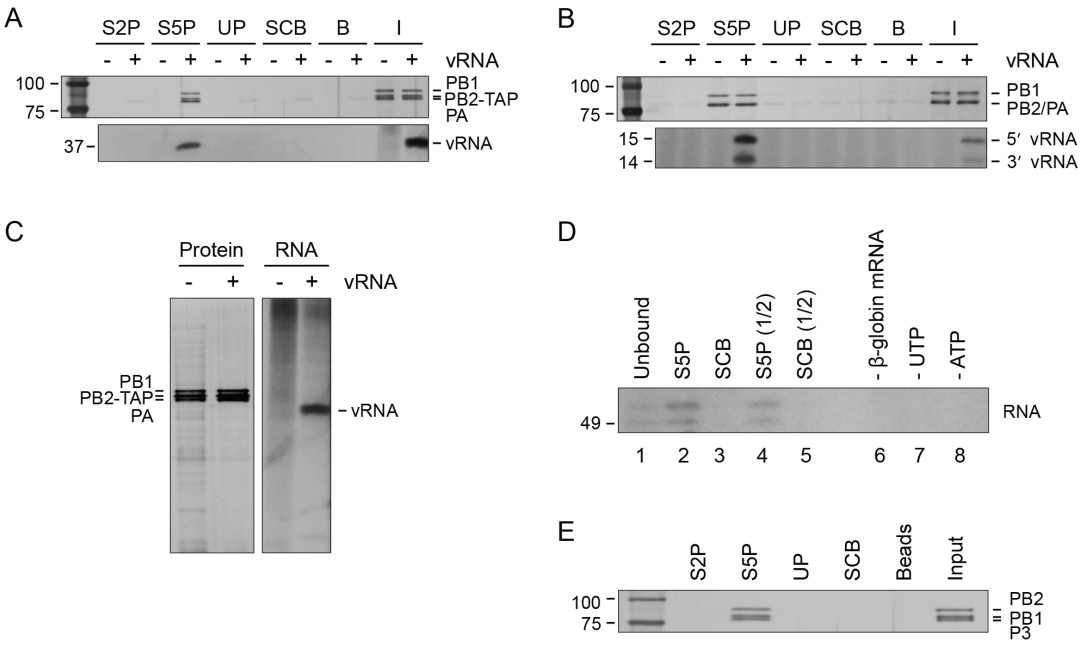
Figure 2



Journal of Virology
JVI
Journal of Virology

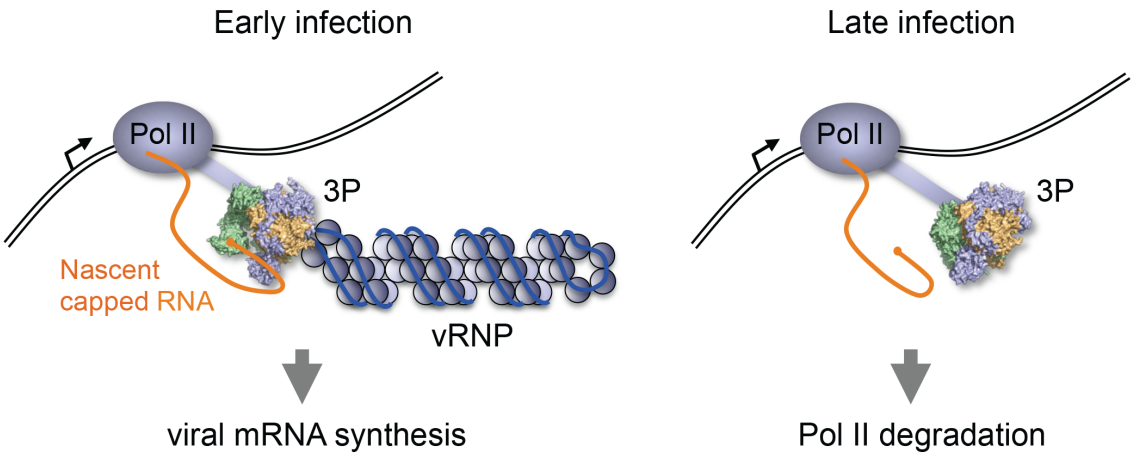
Accepted Manuscript Posted Online
Journal of Virology
JVI
Journal of Virology

Figure 3



Accepted Manuscript Posted Online

Figure 4



Journal of Virology
JVI
Journal of Virology