

1 **Title: Mutation of an influenza virus polymerase 3' RNA promoter binding site inhibits**
2 **transcription elongation**

3 **Running title: Influenza virus polymerase 3' RNA promoter binding**

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Abstract

Influenza A virus encodes a viral RNA-dependent RNA polymerase (FluPol_A), which is responsible for transcribing and replicating the negative-sense viral RNA (vRNA) genome. FluPol_A transcribes vRNA using a host capped mRNA primer, and replicates it by synthesising a positive-sense complementary RNA (cRNA) intermediate which is copied back into vRNA. To carry out these functions, FluPol_A interacts with vRNA and cRNA using conserved promoter elements at the 5' and 3' termini. Recent structural studies have identified a new surface binding site for the 3' vRNA and cRNA promoters on FluPol_A, referred to as the Mode B site. However, the role of this binding site in FluPol_A function is unknown. In this study we used a combination of cell-based and biochemical assays to show that the Mode B site is important for both viral genome transcription and replication. Furthermore, we show that the Mode B site is not needed for initiating transcription *in vitro* but is required to synthesise a full-length product. This is consistent with a model in which the 3' terminus of the vRNA template binds in the Mode B site during elongation. Our data provide the first functional insights into the role of the Mode B site on FluPol_A, which advances our understanding of FluPol_A function and influenza A virus replication.

Importance

Influenza viruses are responsible for up to 650000 deaths per year through seasonal epidemics, and pandemics have caused tens of millions of deaths in the past. Most current therapeutics suffer from widespread resistance, creating a need for new drug

targets against influenza virus. The virus encodes an RNA-dependent RNA polymerase, which replicates and transcribes the vRNA genome. The polymerase interacts with vRNA and the complementary replicative intermediate cRNA using several specific binding sites, however, the functions associated with these binding sites remain unknown. Here, we functionally characterise a binding site for the 3' vRNA and cRNA promoters. Our data offer insight into the mechanism of viral genome transcription by the influenza virus polymerase, and may be applicable to other related viruses.

Keywords

Influenza virus, RNA polymerase, RNA promoter, promoter binding, transcription.

Introduction

Negative-strand RNA viruses cause several important human diseases such as Ebola, influenza and rabies. These viruses encode multifunctional RNA polymerases which are responsible for both transcribing and replicating their viral RNA (vRNA) genomes. In order to carry out these processes, viral polymerases must bind to the vRNA genome(1). Recently, structures of La Crosse and influenza virus RNA polymerases bound to vRNA have become available; however, we still know relatively little about how these polymerases interact with vRNA during their transcription and replication processes(2–6).

The best studied negative-strand RNA virus polymerases are those from influenza viruses. Influenza viruses have negative-sense RNA genomes divided into 7 to 8 non-

56 identical segments, which each consist of an open reading frame for one or more
57 proteins. These are flanked by non-coding regions which include conserved 5' and 3'
58 promoter elements of 13 and 12 nucleotides in length, respectively. Three of the viral
59 genome segments encode Polymerase Basic 1 (PB1), Polymerase Basic 2 (PB2) and
60 Polymerase Acidic (PA), which are subunits of the viral RNA-dependent RNA polymerase
61 (FluPol). FluPol associates with negative-sense vRNA by binding to the 5' and 3' vRNA
62 promoters. The circularised vRNA is also bound by nucleoprotein (NP), forming a viral
63 ribonucleoprotein (vRNP) complex. Influenza virions contain eight vRNPs, which are
64 released into the host cell upon infection and traffic to the nucleus(7). In the context of
65 a vRNP, FluPol transcribes vRNA to produce viral mRNA early in infection. FluPol initiates
66 viral genome transcription using fragments of host capped mRNAs as primers, through a
67 process known as cap-snatching. This produces a capped viral mRNA transcript, which is
68 then polyadenylated at the 3' end by polymerase stuttering(8). In addition to
69 transcription, FluPol carries out viral genome replication to produce vRNA. Viral genome
70 replication is a two-step process, and predominates over transcription later in the
71 course of an infection. In the first step of replication, vRNA is used as a template to
72 synthesise positive-sense complementary RNA (cRNA). Similar to vRNA, cRNA is bound
73 by FluPol and NP to form a complementary ribonucleoprotein (cRNP). FluPol then copies
74 cRNA back into vRNA with the 5' and 3' terminal cRNA sequences acting as the cRNA
75 promoter. Both steps of viral genome replication are initiated by FluPol in a primer-
76 independent manner, though the initiation mechanism for vRNA synthesis differs from
77 cRNA synthesis and requires FluPol dimerisation(6, 7).

78 FluPol has specific binding sites for the 5' and 3' vRNA and cRNA promoters which have
79 been identified by crystallography and cryo-EM studies. The 5' vRNA and cRNA
80 promoters both form similar hook structures, which bind with high affinity in a single
81 pocket formed by the PB1 and PA subunits(2, 6, 9). On the other hand, the 3' vRNA
82 promoter has three distinct binding sites. The 3' vRNA promoter can bind in the
83 polymerase active site, where it acts as a template for RNA synthesis, or in one of two
84 sites on the surface of FluPol(4, 6, 10)(Fig. 1A). The first of these surface binding sites to
85 be observed was the 'Mode A site' for FluPol_B, in which the 3' vRNA promoter is located
86 close to the entrance to the active site(4). More recently, another 3' vRNA binding site
87 termed the 'Mode B site' has been structurally characterised for FluPol_D(5, 6)(Fig. 1B).
88 The Mode B site is located in a groove further away from the polymerase active site
89 entrance, with all three FluPol subunits contributing to it, and is similar to 3' vRNA
90 promoter binding sites present in the related La Crosse orthobunyavirus and Machupo
91 mammarenavirus polymerases(3, 11). Structural studies have observed that the 3' cRNA
92 promoter can also bind in the Mode B site(5, 6)(Fig. 1C).

93 Despite detailed structural data describing 3' vRNA and cRNA promoter binding in the
94 Mode B site, no functional analysis has been able to identify a role for this site. Here, we
95 aimed to functionally characterise the Mode B site for FluPol_A using a combination of
96 cell-based and biochemical assays. We show that promoter binding in the Mode B site is
97 important for both replication and transcription of the viral genome, and identify that
98 mutating the Mode B site inhibits transcription elongation. These data support a model
99 in which the 3' vRNA promoter binds in the Mode B site during transcription elongation.

Our results provide the first functional insights into the role of the FluPol_A Mode B promoter binding site, and may be widely applicable to other negative-sense RNA viruses with similar 3' RNA promoter binding sites.

Results

Mutagenesis of the Mode B 3' RNA promoter binding site

In order to study the function of 3' RNA promoter binding in the Mode B site we mutated key residues identified in a structure of FluPol_A in complex with 3' cRNA promoter(6)(Fig. 1B, C). We targeted clusters of residues on the PB1 and PA subunits, as these interact most extensively with the 3' cRNA promoter in the Mode B site, and tested these mutants in FluPol_A minigenome assays (Fig. 2). We did not observe a strong inhibition of FluPol_A activity with any of the single alanine substitutions. We hypothesised that this could be because the Mode B site is an extended groove involving many contacts between the 3' promoter and FluPol_A, some of which may be redundant. In order to more effectively disrupt the Mode B site, we created three FluPol_A constructs with multiple point mutations, each targeting different regions of the Mode B site. Of these mutations, PB1_{K553A/Y557A/R560A} and PA_{Y464A/K488A/R490A/R496A} significantly inhibited mRNA, cRNA and vRNA accumulation by FluPol_A in minigenome assays. Interestingly, the PB1_{Q567A/Q569A/R571A} mutant had reduced mRNA synthesis but not vRNA or cRNA accumulation, indicating that these mutations specifically affect viral genome transcription.

Mode B site mutations affect both viral genome transcription and replication

Next, we wanted to determine if these mutations inhibit the ability of FluPol_A to carry out viral genome replication, transcription, or both. For this purpose, we took advantage of a previously described complementation assay which uses transcription-deficient FluPol_A mutants(12). FluPol_A synthesises mRNA using vRNA as a template, so if vRNA accumulation is inhibited then mRNA synthesis is always decreased, independently of whether FluPol_A transcription is also inhibited. To overcome this limitation, we expressed transcription-deficient mutant FluPol_A, which is able to support vRNA accumulation, then co-expressed each Mode B site FluPol_A mutant (Fig. 3). We used PB1_{K669A/R670A} as the transcription-deficient FluPol_A mutant for complementing PB1 mutants, and PA_{D108A} for complementing the PA mutant(13, 14). PB1_{K669A/R670A} and PA_{D108A} FluPol_A mutants are unable to efficiently synthesise mRNA, so mRNA signal observed when we co-express Mode B site FluPol_A mutants is the result of mRNA synthesis by those mutants. When we co-expressed the PB1_{K553A/Y557A/R560A} or the PB1_{Q567A/Q569A/R571A} mutant with PB1_{K669A/R670A} we observed only weak mRNA signals. This indicates that the PB1_{K553A/Y557A/R560A} and PB1_{Q567A/Q569A/R571A} mutations inhibit viral genome transcription. However, when we co-expressed PA_{Y464A/K488A/R490A/R496A} with PA_{D108A} we observed a robust mRNA signal, indicating that the PA_{Y464A/K488A/R490A/R496A} mutations affect viral genome replication but not transcription. Collectively, these data show that mutations in the Mode B site affect both viral genome transcription and replication.

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144 **Mode B site mutations disrupt 3' vRNA and cRNA promoter binding**

145 To confirm that the Mode B site mutations affect 3' RNA promoter binding, we
146 overexpressed affinity tagged FluPol_A with PB1_{K553A/Y557A/R560A}, PB1_{Q567A/Q569A/R571A} or
147 PA_{Y464A/K488A/R490A/R496A} mutations in HEK 293T cells and purified the recombinant FluPol_A
148 (Fig. 4A). We then incubated the purified FluPol_A with radiolabelled 3' vRNA or cRNA
149 promoter in the presence of the corresponding 5' RNA promoter, cross-linked the
150 promoter RNAs to FluPol_A, and determined the quantity of radiolabelled 3' RNA
151 promoter bound by SDS PAGE and phosphorimaging (Fig. 4B). All of the Mode B site
152 mutations significantly reduced FluPol_A binding to radiolabelled 3' vRNA and cRNA
153 promoters, with PB1_{Q567A/Q569A/R571A} and PA_{Y464A/K488A/R490A/R496A} having the strongest
154 effect. These results confirm that the mutations in the Mode B site reduce the ability of
155 FluPol_A to bind to both the 3' vRNA and 3' cRNA promoters.

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157 **Mode B site mutations in vRNPs inhibit transcription elongation**

158 To gain further insight into the role of the Mode B site we took advantage of the
159 transcription-specific phenotype of the PB1_{Q567A/Q569A/R571A} FluPol_A mutant. These
160 mutations do not affect viral genome replication, so PB1_{Q567A/Q569A/R571A} mutant RNPs
161 accumulate efficiently in cells and can be purified. We overexpressed affinity-tagged
162 FluPol_A with NP and a vRNA template in HEK 293T cells, then purified wild type and
163 PB1_{Q567A/Q569A/R571A} mutant RNPs (Fig. 5A). We then tested the *in vitro* transcription

activity of purified vRNPs by incubating them with a 5' capped RNA primer in the presence or absence of NTPs, and analysed the reaction products by primer extension. Using a radiolabelled primer specific for the 5' end of the mRNA product (NA 160*), we detected equal levels of mRNA product synthesised by wild type and PB1_{Q567A/Q569A/R571A} mutant vRNPs in vitro (Fig. 5B, C). We also performed reverse-transcription and qPCR of the reaction products using the same primer without a radiolabel (NA 160), which produced similar results to the primer extension assay (Fig. 5B, D).

To examine the ability of the PB1_{Q567A/Q569A/R571A} mutant vRNPs to synthesise full-length mRNA, we then reverse-transcribed reaction products and performed qPCR using a primer directed against the 3' end of the mRNA product (NA 1394). In contrast to results using the NA 160 primer, we found that PB1_{Q567A/Q569A/R571A} mutant vRNPs produced significantly reduced full-length mRNA signal compared to wild type (Fig. 5B, D).

Collectively, these data indicate that the Mode B site is not required for vRNPs to initiate mRNA synthesis, however, it is required for complete elongation of the mRNA product.

Discussion

FluPol_A has multiple surface binding sites for the 3' vRNA promoter, however, the functions associated with these sites are unknown. Here, we perform the first functional characterisation of the recently-identified Mode B site. We find that the Mode B site is necessary for both viral genome replication and transcription, and show that while it is

184 dispensable for initiating viral genome transcription, the Mode B site is required to
185 produce a full-length transcript.

186 These data support a model in which vRNPs initiate transcription independently of the
187 Mode B site (Fig. 6A), then the 3' vRNA promoter binds in the Mode B site after it has
188 passed through the active site, as has been suggested previously(6)(Fig. 6B, C). Keeping
189 the 3' end of the vRNA template bound to FluPol throughout transcription would help to
190 maintain the structural integrity of the vRNP, which may be required for FluPol to move
191 along the vRNA template. This is consistent with previous studies which have shown
192 that FluPol is dependent on NP as an elongation factor to transcribe full-length vRNA
193 templates, such as those present in vRNPs(15). In addition, recent cryo-EM data
194 suggests that the double helical vRNP arrangement is maintained during transcription
195 elongation(16). Maintaining the structure of the vRNP during transcription would
196 presumably be necessary for a single vRNP to be capable of subsequent rounds of viral
197 genome transcription, or for viral genome replication later in the course of an infection.

198 It is likely that the Mode B site has a similar role in viral genome replication as it does in
199 transcription, however, our data do not rule out the possibility of additional functions.

200 For example, previous studies have speculated that the 3' cRNA promoter may bind in
201 the Mode B site during the initiation of vRNA synthesis(5, 6). Furthermore, the Mode B
202 site is located at a FluPol_A dimer interface, raising the possibility that dimerisation may
203 have some interplay with 3' cRNA promoter binding during viral genome replication(6).

204 Interestingly, by mutating different clusters of residues in the Mode B site we observed
205 different phenotypes; specifically, the PB1_{Q567A/Q569A/R571A} mutations only affect viral

genome transcription while the PB1_{K553A/Y557A/R560A} mutations affect viral genome transcription and replication. However, the activity of Mode B site FluPol_A mutants did not correlate with 3' vRNA or cRNA promoter binding, which could be due to other sites contributing to the total 3' vRNA or cRNA promoter binding observed. Although the Mode B site appears to be important for both transcription and replication by FluPol_A, these data would also be consistent with the two processes having differences which result in dependence on different amino acid residues in the Mode B site, or require different affinities for the 3' vRNA or cRNA promoter.

Our results provide the first insights into the function of a FluPol_A 3' RNA promoter binding site by showing that the Mode B site is required for both viral genome transcription and replication. More detailed functional analyses indicate that the Mode B site is important for transcription elongation by vRNPs, which improves mechanistic understanding of FluPol_A and influenza A virus replication. Our findings may be further applicable to other influenza viruses and negative-strand RNA viruses with similar RNA polymerases.

Materials and methods

Cells and plasmids

Human embryonic kidney (HEK) 293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM) + 10% fetal calf serum (FCS). Plasmids pcDNA-PB1, pcDNA-PB1_{K669A/R670A}, pcDNA-PB2, pcDNA-PB2-TAP (Tandem Affinity Purification), pcDNA-PA,

pcDNA-PA_{D108A}, pcDNA-NP, pPOLI-NA were derived from influenza A/WSN/33 virus and have been described previously(13, 14, 17–19). All mutations used in this study were generated by site-directed PCR mutagenesis from pcDNA plasmids and validated by Sanger sequencing.

FluPol_A minigenome assay

Approximately 0.2×10^6 HEK 293T cells in DMEM + 10% FCS were transfected with 200ng pcDNA-PB1, pcDNA-PB2, pcDNA-PA, pcDNA-NP and pPOLI-NA plasmids using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Where indicated, the wild type pcDNA plasmid was substituted for a mutant pcDNA plasmid. For complementation with transcription-deficient mutants, equal amounts of the mutant plasmid and pcDNA-PB1_{K669A/R670A} or pcDNA-PA_{D108A} were transfected. 20 hours post-transfection, total cellular RNA was extracted using TRI reagent (Sigma) and primer extension analysis was carried out.

Primer extension

Primer extension analysis was carried out as described previously(20). Briefly, RNA was reverse-transcribed with Superscript III reverse transcriptase (Invitrogen) using ³²P-labelled primers against positive sense mRNA and cRNA and negative sense vRNA, with a primer against cellular 5S ribosomal RNA (rRNA) included as a loading control. Products were separated by 6% denaturing PAGE with 7M urea and visualised by

phosphorimaging on an FLA-5000 scanner (Fuji). RNA signals were quantified using ImageJ and normalised to the 5S rRNA loading control. Input vRNA signal, estimated from the '-PB2' control, was subtracted from all subsequent lanes. Data were analysed using Prism 8 (Graphpad).

Western blotting

Western blotting was carried out using specific polyclonal antibodies produced in rabbits. Commercially available antibodies were used to blot PB1 (Genetex) and actin (Sigma), and a custom-made antibody was used to blot PA(21). Goat anti-rabbit antibody conjugated to HRP was used in all cases as a secondary antibody, and detection was carried out using Amersham ECL Western Blotting Detection Reagents (GE).

Recombinant FluPol_A purification

Approximately 4×10^6 HEK 293T cells in DMEM + 10% FCS were transfected with 3 μ g pcDNA-PB1, pcDNA-PB2-TAP and pcDNA-PA using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Where indicated, the wild type pcDNA plasmid was substituted for a mutant pcDNA plasmid. For vRNP preparations, pcDNA-NP and pPOLI-NA were also transfected. 48 hours post-transfection, cells were lysed in 500 μ l of lysis buffer (50mM Tris-HCl pH 8.0, 200mM NaCl, 25% glycerol, 0.5% Igepal CA-630, 1mM DTT, 1mM PMSF, 1x Protease Inhibitor Cocktail tablet (Sigma)) and the lysate was cleared by centrifugation at 17000xg for 5min at 4°C. The supernatant was diluted

in 2mL 150mM NaCl and incubated with washed IgG sepharose beads (GE), which bind to the two protein A domains in PB2-TAP. Beads were washed 2x in wash buffer (10mM Tris-HCl pH 8.0, 150mM NaCl, 10% glycerol, 0.1% Igepal CA-630, 1mM PMSF) and 1x in cleavage buffer (10mM Tris-HCl pH 8.0, 150mM NaCl, 40% glycerol, 0.1% Igepal CA-630, 1mM DTT, 1mM PMSF), before overnight cleavage at 4°C with AcTEV protease in 200µl cleavage buffer. Cleavage of PB2-TAP removes the protein A domains, leaving a calmodulin binding domain (CBD). Beads were cleared by centrifugation at 17000xg for 1min and elutants were analysed by SDS PAGE and silver staining using SilverXpress (Invitrogen).

RNA cross-linking assay

Purified FluPol_A preparations in cleavage buffer (see above) were incubated for 10min at 30°C with 1mM DTT, 2U/µl RNasin (Promega), 0.5µM vRNA (5'-pAGUAGAAACAAGGCC-3') or cRNA (5'-pAGCAAAAGCAGGCC-3') 5' RNA promoter, and 5x10⁴ cpm ³²P-labelled vRNA (5'-GGCCUGCUUUUGCU-3') or cRNA (5'-GGCCUUGUUUCUACU-3') 3' RNA promoter in a total volume of 10µl. The mixtures were then irradiated with 254nm UV light in a UV stratalinker (Stratagene) for 10min in a 96 well plate, and separated by 8% SDS PAGE. Cross-linked complexes were visualised by phosphorimaging on an FLA-5000 scanner (Fuji), and images were analysed using ImageJ and Prism 8 (Graphpad).

vRNP activity assay

Purified vRNP preparations in cleavage buffer (see above) were incubated with 1mM ATP, 0.5mM UTP, 0.5mM CTP, 0.5mM GTP, 5mM MgCl₂, 1mM DTT, 2U/μl RNasin (Promega) and 25nM synthetic capped RNA (m7GpppAAUCUAUAAUAG-3') for 4 hours at 30°C in a total reaction volume of 10μl. RNA was extracted using TRI reagent (Sigma) and primer extension or qPCR analysis of the reaction products was carried out.

Strand-specific quantitative PCR

qPCR analysis was carried out as described previously(22). Briefly, RNA was reverse-transcribed with Superscript III reverse transcriptase (Invitrogen) using strand-specific primers against the 5' end (NA 160; 5'-TCCAGTATGGTTTTGATTCCG-3') or 3' end (NA 1394; 5'-CCAGATCGTTCGAGTCCGTTTTTTTTTTTTTTTTTTGAACAAACTAC-3') of NA segment mRNA. Quantitative PCR was carried out on a QuantStudio 5 RT-PCR machine (Applied Biosystems) with 2μl cDNA and 1x qRT-PCR Brilliant III SYBR Master Mix (Agilent) in a 20μl reaction. Forward and reverse primers for NA 160 (5'-AGCAAAAGCAGGAGTTTAAATGAATCCAAACC-3'; 5'-CCGGTTTGAATTGAATGGCTAATCCATAT-3') or NA 1394 (5'-TGAATAGTGATACTGTAGATTGGTCT-3'; 5'-CCAGATCGTTCGAGTCGT-3') were present at 0.75μM. Data presented are $2^{(-\Delta Ct)}$, normalised to the wild type sample.

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A.P.W. and E.F. designed and conceived the experiments. A.P.W. and J.S. performed the experiments and A.P.W. analysed the data. A.P.W. and E.F. wrote the paper.

We have no conflicts of interest to declare.

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Figure legends

FIG 1 RNA binding sites of FluPol_A. (A) Locations of RNA binding sites modelled on FluPol_A (PDB: 6QNW). The 5' vRNA promoter binds in a single site (red; PDB: 4WRT), while the 3' vRNA promoter can occupy the Mode A site (grey; PDB: 4WRT) or the Mode B site (navy; PDB: 6ABF). The 3' cRNA promoter can also occupy the Mode B site (gold; PDB: 6XQ3). (B) Close-up view of the 3' vRNA promoter bound in the Mode B site of FluPol_A, with key interacting residues on the PB1 and PA subunits visible. 3' vRNA promoter residues C2-A5 (navy; PDB: 6ABF) are overlaid on FluPol_A (PDB: 6XQ3). (C) Close-up view of 3' cRNA promoter residues U4-U7 (gold) bound in the Mode B site of FluPol_A, with key interacting residues on the PB1 and PA subunits visible (PDB: 6XQ3).

392

393 FIG 2 Mutagenesis of the Mode B site in FluPol_A. The effect of single and multiple
394 Mode B site mutations on FluPol_A activity was determined using minigenome assays.
395 Primer extension gels are shown (top) with quantitation from n=3 independent
396 transfections (middle). Western blots were carried out to confirm the expression of
397 mutant FluPol_A PB1 and PA subunits (bottom). Data are mean \pm s.e.m., analysed by one-
398 way ANOVA. *P<0.05, **P<0.01, ***P<0.001.

399

400 FIG 3 The Mode B site is important for both viral genome transcription and
401 replication. Transcription activity of Mode B site FluPol_A mutants was determined by
402 complementation with transcription-deficient FluPol_A PB1 and PA subunits in
403 minigenome assays. Primer extension gels (top) and quantitation from n=3 independent
404 transfections (bottom) are shown. Data are mean \pm s.e.m., analysed by one-way ANOVA.
405 *P<0.05, **P<0.01, ***P<0.001. mRNA signals were compared by two-tailed unpaired t-
406 test: *P<0.05, **P<0.01, ***P<0.001.

407

408 FIG 4 Mode B site mutations reduce 3' vRNA and cRNA promoter binding by FluPol_A.
409 (A) Mode B site FluPol_A mutants were purified from HEK 293T cells and run on SDS
410 PAGE, size markers and FluPol_A subunit bands are labelled. An unknown band, possibly
411 Hsp90, is labelled with an asterisk(18). Note that the PB1_{Q567A/Q569A/R571A} mutant FluPol_A
412 subunit migrated slightly faster than wild type, possibly due to the mutations

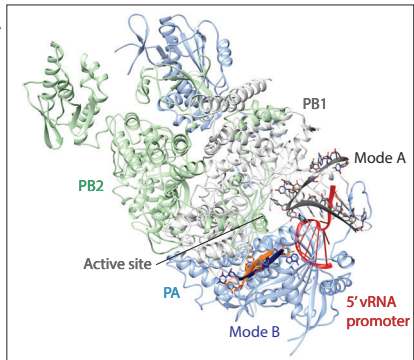
introduced. (B) 3' vRNA and cRNA promoter binding was determined by cross-linking radiolabelled 3' RNA promoter to FluPol_A and running SDS PAGE, followed by phosphorimaging (top). 3' viral RNA promoters cross-link to multiple FluPol_A subunits, so cross-linked products migrate as multiple bands. Quantitation is from n=3 independent replicates (bottom), each with different recombinant FluPol_A preparations. Data are mean \pm s.e.m., analysed by one-way ANOVA. *P<0.05, **P<0.01, ***P<0.001.

FIG 5 Mode B site mutations inhibit transcription elongation by vRNPs *in vitro*. (A) Mode B site mutant RNPs were purified from HEK 293T cells and run on SDS PAGE, size markers and protein bands are labelled. (B) Schematic of the viral mRNA product with annealing positions of the NA 160 (top and middle) and NA 1394 primers (bottom), not to scale. A red asterisk denotes the ³²P-labelling used in the primer extension assay (top), and positions of the reverse qPCR primers are shown (middle and bottom). (C) Recombinant RNP preparations were used for capped RNA-primed *in vitro* transcription assays, in presence or absence of NTPs. mRNA products were visualised by primer extension with the NA 160* primer. (D) *In vitro* transcription products were analysed by reverse-transcription with the NA160 or NA 1394 primers followed by qPCR. RNP preparation concentrations were adjusted to normalise input vRNA levels. Quantitation of mRNA signal is from n=3-4 independent reactions, using 2 different recombinant RNP preparations. Data are mean \pm s.e.m., analysed by one-way ANOVA. *P<0.05, **P<0.01, ***P<0.001. Individual mRNA signals were compared by two-tailed unpaired t-test: *P<0.05, **P<0.01, ***P<0.001.

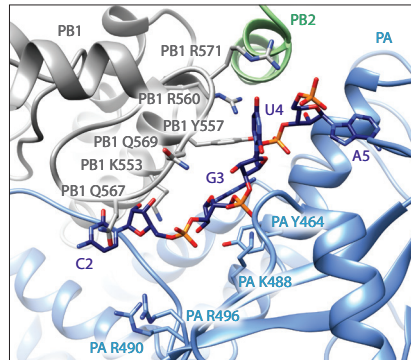
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436 FIG 6 Model for the function of the Mode B site during viral genome transcription. (A)
437 vRNPs initiate transcription of the viral genome using a capped mRNA primer, derived
438 from host mRNAs by the cap snatching function of FluPol. (B) FluPol extends the nascent
439 capped mRNA using vRNA as a template. After it is copied, the 3' end of the vRNA
440 template exits the FluPol active site. (C) The 3' end of the vRNA template binds in the
441 Mode B site, where it remains during transcription elongation.

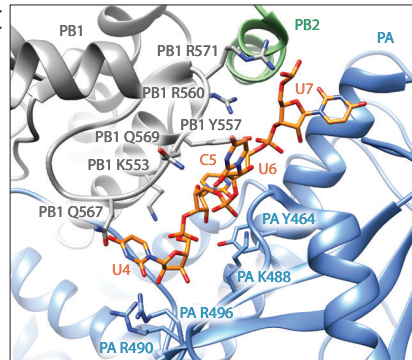
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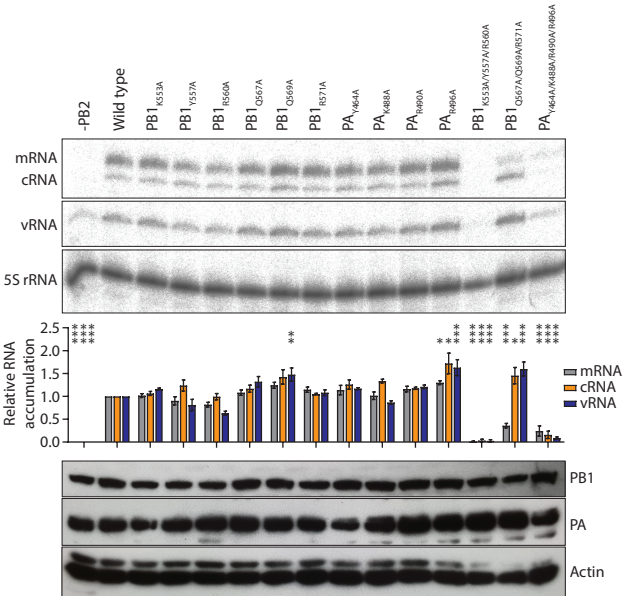


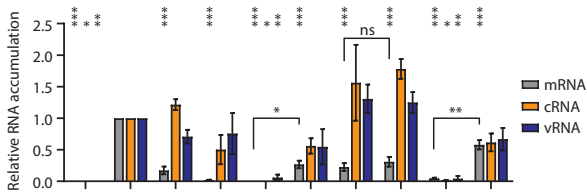
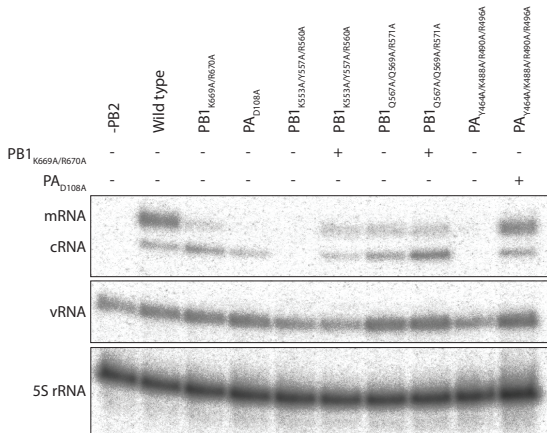
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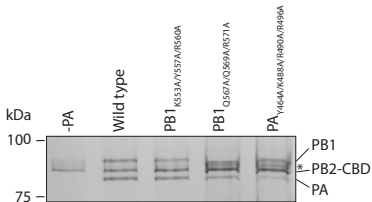
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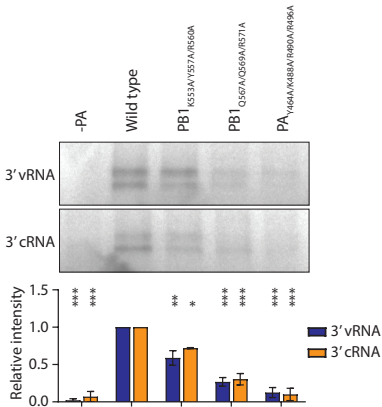


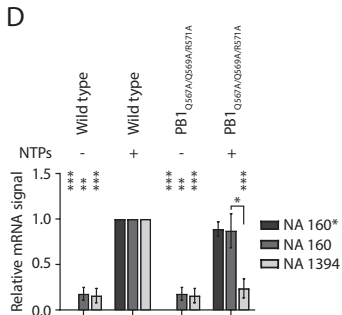
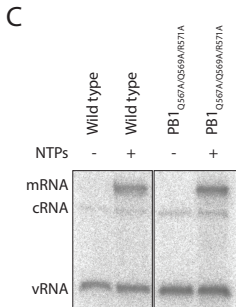
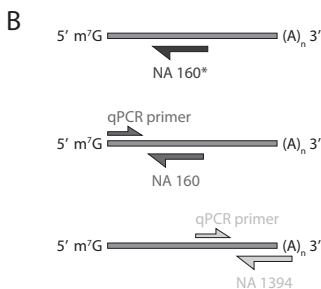
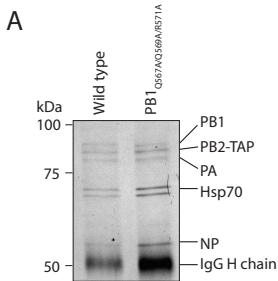


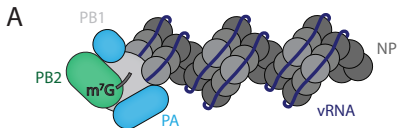
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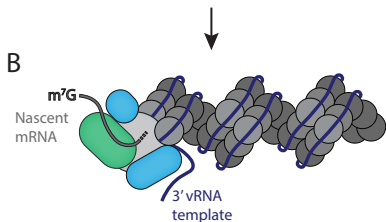
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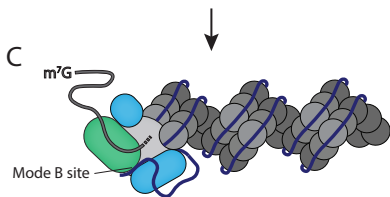




vRNPs initiate transcription using a capped mRNA primer



As the capped mRNA is extended, the 3' end of the vRNA template emerges from the FluPol active site



The 3' vRNA promoter binds in the Mode B site during transcription elongation