Elizaveta Elshina2, Aartjan J.W. te Velthuis2

2 Division of Virology, Department of Pathology, University of Cambridge, Addenbrooke’s Hospital, Hills Road, Cambridge CB2 0QQ, United Kingdom.

**Additional Main text**

**PB1 mutations D27N and T677A mutations affect influenza A virus polymerase activity.**

To investigate if the PB1 mutations D27N and T667A could induce IFN expressing by altering the influenza A virus RNA polymerase activity, we first identified the location of the residue and interactions with the template by superposing the pre-initiation complex of the bat influenza A virus RNA polymerase with the transcription initiation complex of the influenza B virus RNA polymerase (Fig. 7A). The model shows how the 3′ terminus of the template enters the template entry channel above the PB1 active site and that T667A sits at tip of a helix that interacts with the 3′ terminus as it enters the channel. By contrast, D27N resides deeper in the polymerase and close to the binding pocket of the 5′ terminus. These observations suggest that both mutations may affect polymerase activity by changing the interactions of the RNA polymerase with the template.

To investigate how the two PB1 mutations affect polymerase activity on a full-length viral RNA template, we expressed the wildtype or mutant PB1 subunits along with PB2, PA, NP and the segment 6 vRNA in HEK 293T cells. Twenty-four hours after transfection, we extracted the total RNA and analysed the replication (vRNA level) and transcriptional (mRNA level) activity by primer extension. Interestingly, both mutations showed activities that were significantly different from wildtype (Fig. 7B), with D27N and T667A showing lower and higher viral replication and transcription, respectively. Both PB1 mutants showed higher activities than the PB1 active site control (PB1a) that we used to subtract background RNA levels. Both mutants were expressed to similar levels, as assessed by western blot, suggesting that protein stability or expression levels could not account for the differences observed.

We next investigated if the activity of the mutants was sufficient to induce IFN induction. Analysis of the IFN levels showed that both the D27N and T677A mutant induced more IFN expression in HEK 293T cells. Correcting for the vRNA steady state levels, the D27N and T677A induced comparable IFN levels to each other, but higher levels compared to wildtype. This suggests that both mutants had an increased tendency to produce induce IFN expression, irrespective of the RNA synthesis rate.

Previous research has shown that aberrant RNA products, or mini viral RNAs (mvRNAs) {te Velthuis 2018}, produced during influenza A virus replication can trigger RIG-I activation and IFN production. To investigate if the PB1 mutants produced mvRNAs, we fractionated the total RNA from cells expressing the mutant polymerases and a segment 6 template to enrich for small RNA species (17-200 nt long) and performed an RT-PCR that amplified RNA species that contained both the 3′ and 5′ terminus of the template on equal amounts of RNA. This analysis revealed that both D27N and T677A expressing cells contained higher levels of mvRNAs than the wildtype control (Fig. 7C), suggesting that the mutations had altered the tendency of the influenza A virus polymerase to generate mvRNAs.

The D27N mutant produce low viral RNA levels , but higher mvRNA and IFN levels compared to wildtype, on the full-length segment 6 template. This suggests that the processivity of this mutant was impaired. To investigate this hypothesis, we replaced the segment 6 template with a short segment 5-derived 246-nt long template (Fig. 7D). Under these conditions, the activity of the D27N mutant was similar to wildtype, while mvRNA and IFN levels remained higher. In the same assays, the activity, mvRNA level and IFN induction of the T677A mutant was higher than wildtype. Overall, these results suggest that the D27N mutation reduces polymerase processivity and increases the likelihood for mvRNA formation, while the T677A mutation increases polymerase activity and the likelihood for mvRNA formation.

**Additional Discussion**

The D27N mutation in PB1 impairs polymerase activity on a full-length segment 6 template and results in mvRNA production on this template. However, on a shorter template, the mutation has little effect on polymerase activity. This suggests that D27N has a negative effect on polymerase processivity. Any mvRNAs or DIs that are produced as a result can be efficiently amplified and induce IFN signalling during viral infections. By contrast, T667A shows higher polymerase activity compared to wildtype and higher levels of mvRNA production and IFN induction. This suggests that the likelihood of mvRNA synthesis by T667A is higher than wildtype, which will lead to faster mvRNAs and/or DIs production and amplification and thus IFN signalling in an infected cell.

**Additional Methods**

Structural analysis

To locate the PB1 mutations in the influenza A virus RNA polymerase structure relative to the template and active site, we superposed the bat influenza A virus RNA polymerase structure (PDB 4WSB), which shows the 3′ terminus of the template on the surface of the RNA polymerase, with the influenza B virus transcription initiation complex (PDB 5MSG), which shows the 3′ terminus of the template in the template entry channel that leads towards the active site. The structural alignment was performed in Pymol 1.8.7 using motifs A and C.

Plasmids and antibodies.

Plasmids pcDNA-PB1, pcDNA-PA, pcDNA-PB2, pcDNA-NP, pPolI-NA {Fodor 2002}, and pcDNA-PB1a {Vreede 2004} have been described previously. To construct plasmids expressing mutant PB1 proteins D27N and T677A, the plasmid pcDNA-PB1 was subjected to site-directed mutagenesis. PB1 expression was analysed by western blot using antibody GTX125923 (GeneTex).

Primer extension and RT-PCR

To analyse the activity of the PB1 mutations in cell culture, plasmids expressing the proteins PA, PB2, NP and PB1 of influenza A/WSN/33 were transfected into HEK 293T cells together with a plasmid expressing either the wildtype NA vRNA or a 246-nt long segment 5-based template {te Velthuis 2018}. Twenty-four hours post transfection, the RNA was extracted using Trizol (Invitrogen), and the steady state RNA levels assessed using reverse transcription with 32P-labelled oligonucleotides against the viral RNA species and ribosomal 5S RNA as described previously {te Velthuis 2016}{te Velthuis 2018}. 32P-derived signals were imaged using phosphorimaging on a Typhoon scanner and analysed using Prism (GraphPad). In all experiments, the apparent RNA levels were background corrected using the PB1a mutant and normalised to the 5S rRNA loading control. RT-PCRs were performed using the protocol and primers described previously {te Velthuis 2018}.

Luciferase-based interferon expression assay

To measure the induction of the IFN-beta promoter in the presence of viral replication and transcription, RNP reconstitution assays were carried out in the presence of a plasmid expressing *Renilla* luciferase from a CMV promoter and a plasmid expressing Firefly luciferase from the IFN-beta promoter {te Velthuis 2018}. Twenty-four hours post transfection, cells were harvested, lysed and analysed using a DualGlo luciferase kit (Promega) according to the manufacturer’s instructions. Samples were analysed using a GloMax (Promega).

**Additional References**

**Additional Figure legend**

Mutations D27N and T677A in the PB1 subunit affect polymerase activity *in vitro*. **A**) Structural model of bat influenza A virus RNA polymerase (PDB 4WSB) superposed with the structure of the influenza B virus RNA polymerase (PDB 5MSG). The locations of PB1 D27 and T677 (both red) of the influenza A virus polymerase relative to the 5′ (blue) and 3′ (orange) termini of the template and the PB1 active site (grey; PB1 act) are indicated. The PA endonuclease (green; PA endo) and PB2 cap binding domain (pink; PB2 cap) are also indicated for reference. Part of the fingers subdomain of PB1 is not shown to reveal the template in the entry channel. **B**) Influenza A virus RNA polymerase activity on a neuraminidase (NA) encoding vRNA template in HEK 293T cells. Steady state RNA levels were measured by primer extension, denaturing PAGE, and phosphorimaging. A PB1 active site control (PB1a) was used as negative control and background correction. The 5S rRNA signal was used as loading control. Other panels show western blot analysis of PB1, NP and GAPDH expression. Graph shows the average steady state RNA levels of three biological repeats. **C**) Panel shows PAGE analysis of RT-PCR of short (17-200 nt long) influenza virus RNAs or mini viral RNAs (mvRNAs). The length of the mini viral RNAs was extended by the adapters (adapt) present in the primers. Graph shows the IFN-beta promoter activity in transfected HEK 293T cells as measured using a dual luciferase reporter assay. **D**) Influenza A virus polymerase activity on a segment 5-derived 246 nt long vRNA template. The top panel shows the steady state levels of vRNA template were analysed by primer extension and denaturing PAGE. The other two panels show the PB1 and tubulin expression levels analysed by western blot. The top graph shows the average steady state RNA levels of three biological repeats. The second graph shows the IFN-beta promoter activity in transfected HEK 293T cells as measured using a dual luciferase reporter assay. Error bars indicate standard deviation. P-values were determined by two-sided t-test.