Influenza polymerase can adopt an alternative configuration involving a radical repacking of PB2 domains.

Highlights
- Flexibly linked influenza polymerase domains found to pack in a new configuration
- PB2 nuclear localization signal domain translocates 93 Å to pack on PA endonuclease
- Similar arrangement observed in influenza A, B, and C polymerases
- Multiple conformations accessible in solution depending on which viral RNA is bound

In Brief
Influenza polymerase is a multifunctional machine that both replicates and transcribes the viral RNA genome. Thierry et al. show that different functional states can occur by repacking of peripheral domains flexibly linked to the polymerase core depending on which kind of viral RNA is bound.

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Influenza Polymerase Can Adopt an Alternative Configuration Involving a Radical Repacking of PB2 Domains

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SUMMARY

Influenza virus polymerase transcribes or replicates the segmented RNA genome (vRNA) into respectively viral mRNA or full-length copies and initiates RNA synthesis by binding the conserved 3′ and 5′ vRNA ends (the promoter). In recent structures of promoter-bound polymerase, the cap-binding and endonuclease domains are configured for cap snatching, which generates capped transcription primers. Here, we present a FluB polymerase structure with a bound complementary cRNA 5′ end that exhibits a major rearrangement of the subdomains within the C-terminal two-thirds of PB2 (PB2-C). Notably, the PB2 nuclear localization signal (NLS)-containing domain translocates ∼90 Å to bind to the endonuclease domain. FluA PB2-C alone and RNA-free FluC polymerase are similarly arranged. Biophysical and cap-dependent endonuclease assays show that in solution the polymerase explores different conformational distributions depending on which RNA is bound. The inherent flexibility of the polymerase allows it to adopt alternative conformations that are likely important during polymerase maturation into active progeny RNPs.

INTRODUCTION

The eight single-stranded RNA segments of the influenza virus genome (vRNA) are individually packaged in rod-shaped ribonucleoprotein particles (RNPs). Within the RNP, the conserved 3′ and 5′ ends of each vRNA segment (the promoter) are bound to the viral-RNA-dependent RNA polymerase, and the rest of the vRNA is coated with nucleoprotein (NP). The polymerase is a heterotrimer composed of subunits PA, PB1, and PB2, and, in the context of the RNP, it performs both transcription and replication in the infected cell nucleus using the same template vRNA (Fodor, 2013; Ortín and Martín-Benito, 2015; Resa-Infante et al., 2011). Transcription of viral mRNA occurs through “cap snatching” (Plotch et al., 1981), whereby short capped oligomers, derived from host pre-mRNA, are bound by the PB2 subunit (Guilligay et al., 2008), cleaved by an endonuclease in the PA subunit (Olás et al., 2009), and then used to prime mRNA synthesis by the PB1 subunit. In contrast, replication involves unprimed synthesis of a full-length copy of the vRNA into cRNA and subsequently the inverse process back to progeny vRNA. Nascent replicates are co-translationally packaged with incoming, newly synthesized polymerase and NP into progeny vRNPs or cRNPs. Recent crystal structures of bat influenza A (bat FluA; Pflug et al., 2014) and human influenza B (FluB; Reich et al., 2014) polymerases gave the first structure-based insight into how the vRNA promoter is specifically bound and how RNA synthesis is performed. In particular, comparison of the FluA and FluB structures suggested a mechanism for cap snatching whereby in situ rotation of the PB2 cap-binding domain could direct cap-bound, host pre-mRNA first toward the endonuclease for cleavage and then into the polymerase active site to prime transcription (Reich et al., 2014). However, there are many open questions. For instance, what is the conformation of the polymerase in the active transcription initiation or elongation state? Is the polymerase in a different state for replication and does replication require accessory polymerases, as has been proposed (Jorba et al., 2009; York et al., 2013), and in what conformation are they? Is there a difference between cRNA- and vRNA-bound polymerase, and how are incoming apo-polymerase and NP incorporated into a nascent RNP? Here, we use a combination of X-ray crystallography, biochemistry, and solution biophysical techniques to characterize alternative functional configurations of the multifunctional influenza polymerase. Our findings highlight the high flexibility of the polymerase, particularly the PB2-C domains, and suggest that the polymerase can exist in a number of alternative states,
each of which may be important during different steps of transcription, replication, and progeny RNP assembly.

RESULTS

Structure of Isolated PB2-C from Influenza A/H5N1 Polymerase
One of the original FluB polymerase structures (denoted FluB2) lacked electron density for the entire PB2-C region (residues 250–757), whereas it was visible in the FluB1 structure (Reich et al., 2014), suggesting that PB2-C interacted less strongly with the core of the polymerase (PA/PB1/PB2-N). Consequently, we decided to express the entire PB2-C domain, which comprises the mid, cap-binding, cap-627 linker, 627, and nuclear localization signal (NLS) domains, to determine whether this behaved as a functional unit with perhaps an alternative structure. PB2 residues 247–736 of A/Vietnam/1203/2004(H5N1) (i.e., lacking the presumed flexible NLS peptide 737–759) (Figure 1A), were expressed in E. coli, purified, and co-crystallized with or without the cap analog m7GTP in two different crystal forms diffracting to 3.2 Å and 2.4 Å resolution, respectively (Table 1). Both structures are essentially the same and show continuous electron density for the mid, cap, cap-627 linker, and 627 domains but none for the NLS domain. A sequence alignment of PB2-C from influenza A, B, and C polymerases with superposed secondary structure is given in Figure S1.

While each of the mid, cap-binding, cap-627 linker, and 627 domains are individually little changed in structure, there is a major difference in the packing arrangement of the four domains in the isolated H5N1 PB2-C structure (Figure 1B) compared to that observed in the full promoter-bound polymerase (Figure 1C). Analysis of the relative domain movements highlights that the mid (251–322) and cap-627 linker domains (490–536) form a rigid unit, denoted mid-link module. The integrity of this module is maintained by the short anti-parallel β strand interactions between residues 287–289 (mid) and 528–530 (cap-627 linker), the packing of the three-stranded anti-parallel β sheet (496–514, cap-627 linker) on helix 306–316 (mid), and the hydrophobic packing of Met535, Met536 (cap-627 linker) with Met283 and Ile266 (mid). Relative to the mid-linker module, there are hinges in the regions 321–324 and 484–498, which allow the cap-binding domain to rotate (as previously described; Reich et al., 2014), and another hinge at 536–541, which allow the 627 domain to rotate by 62° (Table 2; Figure S1). The net result is that whereas PB2-C forms an arc in the promoter-bound FluA and FluB structures, the isolated PB2-C structure is straighter. In addition, the NLS domain, although not visible in the electron density, must have separated from the 627 domain (Figures 1B and 1C). The 424-loop (residues 420–427) of the cap-binding domain is well ordered in the H5N1 PB2-C structure with its tip packing on the interface between the mid and linker domain and Arg423 making a salt bridge with Glu520, both absolutely conserved residues. This helps to stabilize this particular orientation of the cap-binding domain.
Structures of H5N1 PB2-C have been obtained with and without m7GTP. The m7GTP is bound in the cap-binding site as expected (Guilligay et al., 2008), but the triphosphate is in an unusually bent conformation enabling the γ-phosphate to interact with His357, Lys339, Arg355 and the ribose 2′OH (Figure S2A). Due to the particular rotation of the cap-binding domain with respect to the mid-link module, the ribose and phosphates are juxtaposed to residues Asn510-Val511 of the last strand of mid-link three-stranded anti-parallel β sheet (496–514). This environment makes a much less accessible cap-binding site than that observed in previous structures of the full-length polymerase (Figure S2B). To test whether this precludes capped RNA binding, we attempted co-crystallization of H5N1 PB2-C with the dinucleotide cap-analog m7GpppG but no electron density for this ligand was observed (Table 1).

Table 1. Crystallographic Data Collection and Refinement Statistics, Related to Experimental Procedures

|                  | H5N1 PB2-C + m7GTP | H5N1 PB2-C + m7GTP | Flu B Polymerase c5′ End: 1–12 | Flu B Polymerase v5′ End: 1–12 | FluB Endonuclease PB2 NLS |
|------------------|---------------------|---------------------|-------------------------------|-------------------------------|--------------------------|
| **Data collection** |
| Space group      | P321                | H32                 | P1                           | P21                          | P21                      |
| **Cell dimensions** |
| a, b, c (Å)      | 97.3                | 123.0               | 193.7                        | 126.6                        | 35.4                     |
|                 | 97.3                | 123.0               | 210.0                        | 200.5                        | 80.8                     |
|                 | 126.2               | 229.9               | 210.6                        | 133.1                        | 37.9                     |
| α, β, γ (°)      | 90.0                | 90.0                | 117.7                        | 90.0                         | 90.0                     |
|                 | 90.0                | 90.0                | 92.8                         | 107.7                        | 97.1                     |
|                 | 120.0               | 120.0               | 113.7                        | 90.0                         | 90.0                     |
| **Number of crystals** | 1                   | 1                   | 1                            | 1                            | 1                        |
| Resolution (Å)  | 50.0-2.40           | 50.0-3.20           | 50.0-4.1                     | 50.0-3.40                    | 50.0-1.76                |
|                   | 4.3-1.4             | 3.55-3.40           | 1.76-1.70                    | 0.043 (0.559)                |
| **Refinement**   |
| Resolution (Å)  | 50.0-2.40 (2.46-2.40) | 50.0-3.20 (3.28-3.20) | 50.0-4.10 (4.15-4.10) | 50.0-3.40 (3.48-3.40) | 40.4-1.70 (1.78-1.70) |
| No. of reflections work/free | 26,428/1,145 | 10,884/467 | 188,032/7,760 | 83,996/2,595 | 21,181/1,098 |
| Rmerge           | 0.154 (1.31)        | 0.389 (1.66)        | 0.093 (0.626)                | 0.259 (1.38)                 | 0.043 (0.559)            |
| l/σ              | 12.2 (2.04)         | 8.21 (2.02)         | 5.49 (1.23)                  | 7.83 (1.66)                  | 12.68 (1.84)             |
| Completeness (%) | 100 (100)           | 99.9 (100.0)        | 92.9 (94.9)                  | 99.6 (99.6)                  | 98.2 (93.0)              |
| Redundancy (%)   | 11.11 (10.48)       | 10.80 (10.98)       | 1.75 (1.77)                  | 6.37 (6.11)                  | 2.72 (2.71)              |
| **Validation**   |
| Ramachandran (%) | 97.0                | 95.3                | 92.3                         | 94.7                         | 98.6                     |
| Favored         | 23.2                | 23.9                | 111.3                        | 32.6                         | 32.6                     |
| Outliers        | 0.23                | 0.47                | 1.29                         | 0.6                          | 0                        |
| Clash score     | 0.14                | 0.29                | 1.16                         | 0.53                         | 3.87                     |
| MolProbity score| 0.75                | 1.04                | 1.55                         | 1.29                         | 1.53                     |

*a*Outer-shell statistics in brackets.

*MolProbity: http://molprobity.biochem.duke.edu/*.

Structures of H5N1 PB2-C have been obtained with and without m7GTP. The m7GTP is bound in the cap-binding site as expected (Guilligay et al., 2008), but the triphosphate is in an unusually bent conformation enabling the γ-phosphate to interact with His357, Lys339, Arg355 and the ribose 2′OH (Figure S2A). Due to the particular rotation of the cap-binding domain with respect to the mid-link module, the ribose and phosphates are juxtaposed to residues Asn510-Val511 of the last strand of mid-link three-stranded anti-parallel β sheet (496–514). This environment makes a much less accessible cap-binding site than that observed in previous structures of the full-length polymerase (Figure S2B). To test whether this precludes capped RNA binding, we attempted co-crystallization of H5N1 PB2-C with the dinucleotide cap-analog m7GpppG but no electron density for this ligand was observed (Table 1).
found in the resultant map. Modeling, based on observed RNA binding to the cap-binding site in the full FluB1 polymerase structure (Reich et al., 2014), suggests that whereas a straightened triphosphate could be accommodated, the second nucleotide would clash with the last strand of the three-stranded anti-parallel β sheet. Thus, it is likely that the observed H5N1 PB2-C conformation sequesters the cap-binding site against the three-stranded β sheet in a way that prevents capped RNA binding.

Structure of FluB Polymerase with Bound 5′ cRNA

FluB polymerase was crystallized with a cRNA 5′ end 12-mer (5′-pAGCAGAAGCAGA-3′) giving a P1 crystal form diffracting to 4.1 Å resolution (Table 1). To solve the structure, the polymerase core (PA-C, PB1, and PB2-N) and then the PA endonuclease were placed by automatic molecular replacement. Subsequently, the PB2 mid and cap-binding domains could be unambiguously positioned manually in residual positive difference density. There are six polymerases in the asymmetric unit, arranged as a hollow spherical particle with 32-point symmetry (Figures S3A and S3B). Despite the moderate resolution, using map averaging and map sharpening, an essentially complete model of the FluB polymerase-c5′ complex could be obtained (Table 1).

The structure of the FluB-c5′ complex is remarkable in that, whereas the polymerase core (PA-C, PB1, and PB2-N) has relatively minor changes overall (see below), the PB2-C mid, cap-binding, cap-627 linker, and 627 domains are dramatically rearranged compared to the full FluB polymerase-promoter complex (Reich et al., 2014) (Figures 2A–2C). Furthermore, the altered organization of the PB2-C domains is essentially identical to that observed in the crystal structure of the equivalent part of FluA/H5N1 PB2-C in isolation (Figures 2A and 2B). The conservation of this alternative PB2-C multi-domain structure across FluA and B strains (and FluC, see below) suggests that it is of functional importance. Comparing the two FluB polymerase conformations confirms that the mid and cap-627 linker constitute a single rigid unit (root mean square deviation [RMSD] = 0.88 Å for 109 aligned Cα positions in the mid-link module, residues 252–322 and 498–536). It also reveals an additional hinge around PB2 residues 250–256 between PB2-N and PB2-C, about which the mid-link module rotates by 135.8° between its position in the new c5′-bound structure compared to the promoter-bound FluB1 structure (Table 2; Figure S1). The net consequence of all the relative rotations between the PB2 domains is that the 627 and NLS domains are translated by remarkable center of mass displacements of 67 Å and 93 Å respectively from their positions in the promoter-bound structures, the mid-link domain by 24 Å and the cap-binding domain by 27 Å (Figure 2D; Table 2). A striking example of the repacking of domains between the c5′ and promoter-bound conformations concerns the endonuclease and NLS domains, which intimately interact in the c5′-bound structure (see below). Another example is the complete change in interface mediated by the PB1 palm domain helix x10 (residues 280–297). In the promoter-bound conformation, this helix interacts with the β sheet of the 627 domain, whereas in the c5′ conformation, the same helix, slightly displaced, makes extensive interactions with the cap-binding domain (Figure 2E). Indeed, in the c5′ conformation, the cap-binding domain appears to be immobilized by several inter-domain interactions, whereas in the promoter-bound conformation, it can clearly rotate in situ consistent with the proposed cap-snatching mechanism (Reich et al., 2014). The 627′ domain is exposed on the periphery of the complex (Figures S3A and S3B) with host-specific residue 627 being highly accessible.

Interaction of the PA Endonuclease with the PB2 NLS Domain

Another remarkable feature of the FluB-c5′ complex is that the PA-Nter endonuclease is repositioned and interacts directly with the PB2 NLS domain (Figure 2A). Instead of being packed against the PB1-Cter/PB2-Nter helical bundle, via endonuclease helix x4 (PA residues 84–98), as observed in the promoter-bound structures (Pflug et al., 2014; Reich et al., 2014), the endonuclease is rotated in situ by 137°. This results in a different but less extensive interface with the PB1-PB2 helical bundle, which, however, is compensated by a completely novel interface between the endonuclease and the PB2 NLS domain with a substantial total buried surface area of 2.950 Å² (Figure 2A). As in the H5N1 PB2-C structure, the FluB NLS domain has separated from the 627 domain, the two domains being connected by an extended linker (677–693), most of which lacks electron density. In the FluB-c5′ complex, the compact, globular part of the NLS domain packs against endonuclease helix x6 (FluB PA residues 164–179) and the beginning of strand β7 (150–152) on the side of the nuclease. Most interestingly, residues 745–770 of

Table 2. PB2-C and PA Inter-domain Hinge Rotations and Translations Calculated by Comparing v3′-5′- or c5′-Bound Crystal Structures of FluB Polymerase

| Domain 1   | Domain 2      | Hinge Residues between Domain 1 and 2 | Rotation Angle around Hinge (°)¹ | Center of Mass Translation of Domain 2 (Å)²       |
|------------|---------------|---------------------------------------|-----------------------------------|-------------------------------------------------|
| PB2–N (1–251) | mid-link (252–322, 498–536) | PB2: 250–256                          | 136                              | 24                                              |
| Mid-link   | cap-binding (323–497) | PB2: 321–324                          | 62                               | 27                                              |
| Cap-binding | mid-link      | PB2: 484–498                          | –62                              | –                                               |
| Mid-link   | 627 (537–677) | PB2: 536–541                          | 59                               | 67                                              |
| 627       | NLS (696–740) | PB2: 677–700                          | 133                              | 93                                              |
| PA-C (200–726) | PA endonuclease (1–195) | PA: 190–200                           | 137                              | 2                                               |

¹Calculated using DynDom (Taylor et al., 2014).

²Center of mass translation of domain 2 between the v3′-5′- and c5′-bound conformations after superposing the polymerase core (PA-C, PB1, and PB2-N).
the bipartite NLS containing, extreme C-terminal peptide of PB2 (Tarendreau et al., 2007) (for FluB, 740-KRKRYSALSNDISQGIKRQRMTVESMGWALS-770, bipartite NLS underlined) form a long α helix that packs on the endonuclease domain (Figure S1). The helical conformation of the NLS containing peptide contrasts with the extended structure the same peptide makes when bound to α-importin (Pumroy and Cingolani, 2015; Tarendreau et al., 2007).

To study this interaction further, we co-crystallized a synthetic FluB PB2 29-mer NLS peptide (residues 742–770) with the FluB endonuclease domain PA (residues 1–197). The high-resolution (1.7 Å) structure so determined (Table 1) shows the same interaction between the endonuclease and NLS peptide as observed in the lower-resolution FluB-c5' complex (Figure 3A). The amphipathic helical NLS peptide runs roughly perpendicular to PA helix α3, strand i3 (in the vicinity of PA Glu81, corresponding to active site residue Glu80 in FluA), and helix α4 and makes a number of hydrophobic and specific polar interactions, resulting in a total buried surface area of 1,500 Å² (Figure 3A). There are charged polar interactions between PA residues Asp50, Glu78, and Arg169 with and PB2 residues Arg757, Asp750, and Thr761, respectively. Of particular note is the side chain of PB2 Gln758, which is completely buried and reaches inward to make multivalent hydrogen bonds with the main-chain carbonyl oxygens of PA Val79 and Glu81 and the hydroxyl of Tyr46, just behind the nuclease active site (Figure 3B). The normal two metal coordination of divalent cations (in this case magnesium ions, since no manganese was added) is observed in the endonuclease active site. The endonuclease C-terminal residues 190–194 fold back toward the active site, with the side chain of Glu193 approaching within hydrogen-bonding distance of metal coordinating His41. The electron density in the c5'-bound polymerase is compatible with this and indeed shows that the domain arrangement is essentially identical.

Figure 2. Structure of the FluB Polymerase-c5' RNA Complex

(A) Ribbon diagram of the FluB polymerase c5'-RNA complex structure with PA-Nter (endonuclease) colored forest green, the rest of PA (green), PB1 (cyan), PB2-N (red) and the PB2-C domains as in Figure 1A. The c5' RNA 12-mer is in violet. The unobserved linker between the 627 and NLS domains is shown dotted.

(B) View of the A/H5N1 PB2-C structure (with bound m7GTP) after superposition on the equivalent domains of the FluB polymerase-c5' RNA complex. The root-mean-square difference in Cα positions is 1.68 Å for 310/424 aligned Cαs over residues 254–670 in the mid, cap-binding, cap-627 linker and 627 domains, showing that the domain arrangement is essentially identical.

(C) Structure of the FluB polymerase-promoter complex (with the 3' and 5' vRNA respectively yellow and violet) after superposing via the PA1 subunit on the FluB polymerase-c5' RNA complex. Comparison of (A) and (C) shows that the polymerase core (PA-C, PB1 and PB2-N core) is largely unchanged apart from near the promoter binding site (see Figure S2C).

(D) Diagram comparing the positions of the PB2-C and PA endonuclease domains in the FluB c5' RNA (left) and promoter (right) complexes relative to the conserved PA-C, PB1 and PB2-N core (dotted ellipse). Table 2 gives the relative rotations and displacements of the domains.

(E) Involvement of PB1 helix α10 (residues 275–295, cyan) in interacting either with the cap-binding domain (orange) or the 627 domain (deep salmon) in respectively the c5' (left) or full promoter bound (right) FluB polymerase conformations. The two structures were superposed via the invariant polymerase core, here represented by PB2-N and the PB1 helix. In the c5' bound structure the cap-binding domain is immobilized by inter-domain interactions whereas in the promoter bound structure the cap-binding domain (represented by an ellipse) is free to rotate.

See also Figures S2–S4.
Figure 3. High-resolution Structure of FluB Endonuclease-NLS Peptide Complex

(A) Ribbon diagram of the FluB PA-Nter endonuclease (forest green) and PB2 NLS-peptide (yellow) with key residues as sticks and secondary structure elements labeled. The amphipathic helical NLS peptide runs roughly perpendicular to PA helix α3 (e.g., PB2 Ile751 and Ile755 stack on PA Tyr46), strand β3 (in the vicinity of PA Glu81, corresponding to active site residue Glu80 in FluA) and helix α4 (e.g., PB2 Val762 and Met765 make contacts with PA Met83, Ile87 and Val91). Two magnesium ions (magenta spheres) are present in the nuclease active site.

(B) Detail of the PB2 NLS peptide interactions with the endonuclease in the vicinity of the active site. Residues coordinating the two magnesium ions are shown as well as the hydrogen bonding interactions (red dotted lines) of PB2 Gln758 with PA Tyr46 and the main-chain carbonyls of Val79 and Glu81. These interactions are likely to be FluB specific, due to genera dependent sequence differences.

(C) Isothermal calorimetry data and curve fit to derive the affinity of the FluB NLS peptide for the endonuclease domain.

(D) Effect of different bound RNAs on FluB polymerase cap-dependent endonuclease activity. 7 M urea gel showing the endonuclease activity of FluB polymerase on 32P-capped 20-mer RNA with time (minutes) in the presence of no vRNA (panel 1), 1.2 μM 3' vRNA 1-18 (panel 2), 1.2 μM 5' v RNA 1-18 (panel 3), 1.2 μM 5' and 3' vRNA (panel 4) or 1.2 μM 5' cRNA 1-18 (panel 5). RNA markers are shown in the first and last lanes (lanes L).

See also Figure S5.
structure, where this folding back does not occur and the solvent-exposed linker is approximately nine residues. The conformation of this linker region may affect the accessibility of the endonuclease active site to substrate RNA.

**Structure of Influenza B Polymerase with Bound 5’ vRNA**

Co-crystallization of FluB polymerase with only a 5’ vRNA 12-mer (5’-pAGUAGUAACAAG-3’) gave a new monoclinic crystal form diffracting to 3.4 Å. There are two complexes in the asymmetric unit and both are in the conformation very like the FluB1 form originally described (Reich et al., 2014) and thus quite different from the structure determined with the c5’ bound (Figure S3C). This is surprising given that the v5’ or c5’ RNAs used, at the resolution of the structures obtained, appear to have the same stem-loop structure bound in the same way as previously described (Pflug et al., 2014). The two RNAs differ at 5/12 positions, two of which alter one of the stem base pairs (3-U:A-8 to vRNA to C:G in cRNA): one is a substitution in the loop (6-U to A), and two (11-AG to GA) are distal to the stem-loop and, at least for the vRNA, engage in base pairs with the 3’ end of the promoter when present. The higher-resolution v5’ structure clearly shows that bases 11–12 maintain stacking with PA His506 in the absence of the 3’ end, and the lower resolution c5’ density is compatible with this. On the other hand, the consequence of the absence of the 3’ end in both these structures, as well as the short 5’ end (12-mer), results in movements or refolding of regions of the polymerase that interact with the promoter, such as the PB1 β-ribbon (residues 185–207) and PB1 residues 670–681, and PB2 residues 82–91 also become disordered (Figure S3D). These changes are clear in the higher-resolution v5’ structure but are also compatible with the electron density in the c5’ structure.

**Comparison with Apo-FluC Polymerase Structure**

The crystal structure of the complete influenza C (FluC) heterotrimeric polymerase has recently been determined in the absence of any bound RNA (Hengrung et al., 2015). FluC polymerase PA, PB1, and PB2 subunits are 25.6%, 40.8%, and 25.2% identical to their FluB counterparts (with very similar numbers when compared to FluA). The apo-FluC polymerase structure is remarkably similar to the FluB polymerase-c5’ complex, with not only the core polymerase (PA-C, PB1, and PB2-N) being in the same overall configuration but also the position of the mid, cap-binding, and cap-627 linker domains being quasi-identical (Figures S4A and S4B). The access to the cap-binding site is similarly obscured by residues from the cap-627 linker. The only differences concern the orientations of the 627 domain and the PA endonuclease-PB2 NLS domain unit (Figures S4C and S4D). In the apo-FluC structure, the NLS domain packs against the endonuclease in the same way as in the FluB c5’ structure. However, the FluC NLS peptide is two residues shorter than in FluA and eight residues shorter than in FluB (Figure S1), so the helix bound to the endonuclease is correspondingly shorter (Figure S4D). Also, the entire apo-FluC endonuclease-NLS unit is rotated in situ by 94° compared with FluB c5’ structures (Figure S4C/D). Furthermore the FluC 627 domain is packed closer to the polymerase core and to the NLS domain than in the FluB c5’ structure, thus making the apo-FluC structure slightly more compact (Figure S4D). However, the close juxtaposition of the 627 and NLS domains in the apo-FluC structure is quite different to that observed in the FluA and FluB promoter-bound structures (which is the same as in the crystal structure of the isolated double domain; Tandreau et al., 2008), due to a large rotation about the flexible linker joining them. A priori, it is not clear whether these differences reflect the absence of any RNA in the FluC structure, sequence divergence between FluC and FluB polymerases, or different crystal-packing constraints.

**Context-Dependent Endonuclease Activity**

As the PB2 NLS peptide interacts with the endonuclease in both the FluB c5’ and apo-FluC structures, with, in the case of FluB, Gln758 making intimate interactions with active-site proximal residues, we studied biochemically the effect that this binding might have on nuclease activity. We first measured the affinity of the FluB synthetic NLS peptide to the endonuclease by isothermal calorimetry (ITC) (Figure 3C). The derived Kd of 16.5 µM is only moderate, but in the context of the heterotrimeric polymerase, this would be effectively enhanced due to the intra-molecular nature of the interaction. One question that arises is whether the same kind of interaction could occur in FluA polymerase? In avian and human FluA, the NLS peptide is invariant with sequence 736-KRKDDSILDSQTATKRIRMAINE-759 (residues conserved in human/avian FluA and FluB underlined) (Figure S1). This peptide is not only shorter but also there are many substitutions compared to FluB, notably Gln758 is replaced by Ile754 in FluA, and similarly the PA endonuclease has diverged in sequence, so it is not clear whether a similar interaction is made. Indeed, no clear interaction was detected between a synthetic H3N2 FluB PB2 22-mer NLS peptide (residues 738–759) and the FluA endonuclease in corresponding experiments, nor was it possible to co-crystallize a complex, so the question remains open.

We next examined the effect of NLS peptide binding on the activity of the isolated endonuclease domain. Using a quantitative fluorescence resonance energy transfer (FRET)-based solution method (Kowalski et al., 2012) (Figure S5), a small increase in the FluB endonuclease domain activity was detected upon titrating FluB NLS peptide, with peptide concentrations up to several times the Kd (Figure S5D). This increase was not observed with FluA endonuclease domain (whose intrinsic nuclease activity is estimated to be ~40 times higher than for FluB; Figures S5A and S5B), although as indicated above, the Kd for NLS peptide binding is not known in the FluA case, but it is likely to be higher than that for FluB.

These results show that NLS peptide binding in trans to the isolated domain does not have a marked effect on the domain nuclease activity, consistent with the metal-binding active site not being perturbed in the structure. However, depending on the relative disposition of the cap-binding and endonuclease domains and the accessibility of their RNA binding sites, the nuclease activity, in the context of the complete heterotrimer, could vary markedly. Therefore, we assayed the cap-dependent endonuclease activity of the full polymerase as a function of which kind of viral RNA is bound, reasoning that this might be a good probe of the polymerase solution conformation. Time courses of FluB polymerase nuclease activity, with either no
RNA or bound to v3’, v5’, c5’, or v3’+v5’ RNAs, were monitored over a 2-hr period using a radioactively labeled capped 20-mer as substrate. The results show that with no RNA, or just the v3’ end bound, there is very low activity in contrast to the maximal activity exhibited when both v3’ and v5’ (i.e., the full promoter) are bound (Figure 3D). In the latter condition, the polymerase cap-dependent nuclease activity is far higher (~100 times) than for the isolated endonuclease domain and furthermore only requires magnesium and not manganese, which is essential in the case of the domain (Datta et al., 2013) (Figure S5E). Both v5’ and c5’ alone activate the cap-dependent endonuclease activity (although less than the full promoter), but the c5’ less so than the v5’ (Figure 3D). As shown below, these observations correlate with biophysical results showing that the polymerase conformation is very different without bound RNA or with only the v3’ end bound as compared to when bound to v5’, c5’, or v3’+v5’. We propose that the endonuclease activity reflects the fraction of time spent by the polymerase with the cap-binding domain and nuclease domain suitably positioned to maximize efficient capped-RNA binding and cleavage (see Discussion).

**Small-angle X-Ray Scattering and Multi-angle Laser Light Scattering**

To further probe the solution structure of FluB polymerase with different bound RNAs, we undertook small-angle X-ray scattering (SAXS), multi-angle laser light scattering (MALLS) and crosslinking mass spectrometry experiments.

SAXS measurements were made on solutions of FluB polymerase with either no bound RNA (apo) or with only v3’, only v5’, or both v3’ and v5’ RNAs, as they eluted from an on-line size-exclusion column. There was a clear distinction between the elution time and profile of the different samples. The apo- and v3’-bound samples behaved similarly, eluting earlier but with an asymmetric profile, whereas the v5’-only- or v3’- and v5’-bound samples eluted at a later time with a symmetric profile (Figure 4A). Equivalent results were obtained by MALLS experiments with the apo-FluB sample eluting before the v5’- or c5’-only-bound samples, which behave equivalently (Figure 4B). The MALLS measurements allowed estimation of the molecular weight of the particles in the two elution peaks giving very similar values for each (237 kDa for apo and 226 kDa for v5’ or c5’ bound, somewhat less than the actual value of 262.7 kDa), indicating monomeric complexes and no aggregation in either peak. In contrast, calculation of the radius of gyration at each time point of the elution during the SAXS measurements showed that whereas the v5’-only or v3’- and v5’-bound samples had a radius of gyration of ~44 Å, uniform across the peak, the radius of gyration of the apo- or v3’-only-bound samples varied across the elution profile between ~48 Å and 56 Å (Figure 4A). Taken together, these results show that apo- or v3’-only-bound polymerase has a much more extended conformation in solution but with conformational heterogeneity likely due to flexibility, whereas binding of either the v5’ or c5’ RNA ends leads to compaction of the polymerase, which is not measurably changed upon further addition of the v3’ end.

The question arises as to whether the two distinct solution states observed by SAXS and MALLS, apo/v3’ bound or v5’/v5’-3’ bound, correspond to any of the known crystal structures. The radii of gyration calculated from the apo-FluC, apo-FluB (modeled on apo-FluC), c5’-bound, or v5’-3’-bound structures are respectively 41.5, 41.7, 43.3, and 41.9 Å. Thus, all crystal structures are slightly more compact than the v5’/v5’-3’-bound.
solution state and do not correspond at all to the apo/3'-bound solution state. Furthermore, at the level of the scattering curves, as expected by the closer correspondence of the radius of gyration, the c5'-bound structure fits best the v5'/v5'-3'-bound scattering curve at low resolution. However, none of the models (or even an average of the three) fit satisfactorily the data at higher resolution as highlighted by the clear secondary maximum at ~0.12 Å⁻¹ in the theoretical scattering curves of all models, which is smeared out in the experimental curve (Figure S6). These observations indicate that there is a greater variation in the conformations present in solution than those sampled in crystal structures, not only for the highly flexible apo- and v3'-bound forms but also for the more compact 5'-cRNA-bound forms.

**Crosslinking Mass Spectrometry of Influenza Polymerase Conformation in Solution**

Recombinant polymerase complexes with no bound RNA (Bat FluA and FluB) or 5'-3' vRNA bound (Bat FluA) were cross-linked using two amine-reactive, homo-bifunctional reagents of different length, disuccinimidyl-suberate (DSS) or disuccinimidyl-glutarate (DSG). Subsequently, crosslinked peptides were subjected to mass spectrometric analysis and stringently identified using the xQuest/xProphet software (Walzthoeni et al., 2012). High-confidence crosslinks with a linear-discriminant (Id) score (Walzthoeni et al., 2012) higher than 25 were selected for the analysis, resulting in a total of 234 unique crosslinks (see Figure S7 and Tables S1, S2, and S3 for more details on the datasets). Pooled crosslinks from the different samples were then mapped onto four different FluB polymerase structures: the crystallographically determined c5'- or v5'-3'-bound conformations, the v5'-3'-bound conformation with the alternative cap-binding domain orientation modeled on the bat FluA structure (Pflug et al., 2014), and a modeled apo-FluB conformation, obtained by superposing the FluB 627, NLS, and endonuclease domains on to the positions of the equivalent domains in the apo-FluC structure. Crosslinks were deemed to be satisfied (or violated) if the corresponding lysine-lysine distance in the apo-FluC structure (Pflug et al., 2014), and a modeled apo-FluB conformation, obtained by superposing the FluB 627, NLS, and endonuclease domains on to the positions of the equivalent domains in the apo-FluC structure. Crosslinks were deemed to be satisfied or violated if the corresponding lysine-lysine distance in the apo-FluC structure was shorter or longer (c.f. or violated) than 35 Å (see Experimental Procedures). Out of the 234 crosslinks, 231 satisfied in at least one of the apo, 5' cRNA or 5'-3' vRNA structures (Table S2). The percentage of crosslinks not satisfied by any structure (15%) is significantly higher than the expected false-positive rate of crosslink identification implemented in xProphet (5%). In agreement with the SAXS results, these data suggest that the high-scoring crosslinks observed as “violated” might be explained by uncharacterized alternative conformations co-existing in solution (Figures S7B–S7G).

Although most crosslinks were satisfied in more than one conformation (Table S2; Figure 5A), certain subsets of crosslinks uniquely support individual apo-, c5'-bound, or v5'-3'-bound conformations, suggesting that these conformations coexist in solution. These crosslinks involve the PA endonuclease, PB2-627, and PB2-NLS domains, i.e., the domains that show the most pronounced differences between the observed crystal structures. In particular, seven crosslinks from PB2-627 to PB2-mid and PB2-NLS domains are violated in v5'-3'-bound conformations but become satisfied in the c5'-bound or apo conformation due to the large displacements of the PB2-627 and PB2-NLS domains (Figure 5B). On the other hand, one crosslink from the PB2-627 to the PB2-NLS satisfied in v5'-3'-bound conformations gets violated in the c5'-bound and apo conformations. Likewise, the three different orientations of the PA endonuclease lead to satisfaction of different set of crosslinks (Figure 5C). Interestingly, the different conformations coexist in the sample of FluB reconstituted with vRNA: the crosslinks obtained from this sample corresponded not only to the 5'-3' vRNA conformation but also to the 5' cRNA and apo conformations.

**DISCUSSION**

During influenza replication in the infected cell nucleus, active polymerase complexes are bound to and operate on vRNA (or cRNA) within the context of ribonucleoprotein particles. Newly synthesized polymerase subunits are imported into the nucleus as PA-PB1 heterodimers by RanBP5 (Deng et al., 2006) or separate PB2 monomers by importin α (Tarendeau et al., 2007), which then heterotrimerize in the nucleus (Deng et al., 2005; Huet et al., 2010). It is plausible that polymerase trimers (or possibly PA-PB1 heterodimers, before PB2 associates) first encounter viral RNA upon binding to the nascent c5' or v5' ends that emerge from replicating vRNPs or cRNPs, respectively. These ends can bind with high affinity as a stem loop to the allosteric 5' binding site formed by the PA and PB1 subunits (Pflug et al., 2014). Polymerases so bound might then nucleate assembly of progeny RNPs by directing sequential addition of incoming nucleoproteins on to the elongating replicate RNA. This model is consistent with results showing that interactions between polymerases are required to promote efficient replication (Jorba et al., 2008; Jorba et al., 2009; York et al., 2013). A detailed mechanistic understanding of all these processes requires structural information on the polymerase in its various functional states. So far, structures of bat FluA and FluB polymerase bound to the v3'-v5' promoter show a similar conformation, which has been interpreted to be the pre-transcription initiation configuration, competent for cap snatching and cap-dependent priming (Reich et al., 2014). However, there are indications both from crystallography (Reich et al., 2014) and from low-resolution electron microscopy of native RNPs (Arranz et al., 2012; Moeller et al., 2012) that the polymerase, particularly the modular PB2 subunit, is highly flexible and can adopt a variety of conformations. Here, we have used a variety of structural (crystallography and SAXS), biochemical (endonuclease activity), and biophysical (MALLS and crosslinking mass spectrometry) methods to characterize the conformation of influenza polymerase when it is RNA free or bound to v3', v5', c5', or v3'+v5' RNAs, all of which are likely to be relevant at some stage in the maturation or activity of the polymerase.

The most remarkable result is the concordance between crystal structures of FluA/H5N1 PB2-C and c5' RNA bound FluB polymerase that reveals a completely different stable packing of the PB2 mid, cap-binding, cap-627 linker, and 627 domains compared to the full promoter-bound structures. Furthermore, a similar arrangement of PB2 domains is
observed in the apo-FluC polymerase structure (Hengrung et al., 2015). An unexpected common feature of the c5'-FluB and apo-FluC polymerase structures is that the PB2 NLS domain packs against the PA endonuclease, with the C-terminal NLS peptide forming an α helix that participates in this interaction. We also report a high-resolution crystal structure of the FluB endonuclease-NLS peptide binary complex, which reveals the details of this interaction. However, biochemical assays show that this interaction in itself does not inhibit the activity of the isolated endonuclease domain. We also show that the cap-dependent nuclease activity of the trimeric polymerase is considerably enhanced when v5', c5', or v3'+v5' RNA are bound (in line with previous observations, e.g. Li et al., 2001) and correlates with the compact solution conformation of these states as detected by MALLS and SAXS. In contrast, both apo- and v3'-RNA-bound polymerases have poor cap-dependent nuclease activity, comparable to that of the isolated nuclease domain, again correlating with the extended conformation of these states in solution. Finally, both SAXS and crosslinking mass spectrometry results show

Figure 5. Cross-links Confirm the Crystallographically Observed Polymerase Conformations
(A) Crosslinks satisfied in the four different conformations of FluB polymerase (v5'-3' bound as in the FluB1 structure, v5'-3' bound with rotated cap-binding domain as observed in the bat FluA structure, c5'-bound and modeled apo-FluC conformation). Satisfied crosslinks are colored blue. The PB1 subunit and the PA-C domain are colored gray. The PA endonuclease and PB2-C domains are color-coded according to the domain structure.
(B) Crosslinks originating from the PB2-627 domain that are satisfied in at least one of the apo, c5'-bound, or v5'-3' bound conformations. Satisfied crosslinks are colored blue, violated are red.
(C) Crosslinks originating from PA nuclease that are satisfied in at least one of the apo, c5'-bound, or v5'-3' bound conformations. See also Figure S7.
that in solution, a variety of states coexist not restricted to the known crystal structures.

Our interpretation of these results is as follows. First, they suggest that whatever the RNA bound (or not), multiple polymerase conformations exist in equilibrium in solution, although apo/v3'-bound polymerases are preferentially extended, whereas v5'-, c5'-, or v3'+v5'-bound polymerases are preferentially compact. The extended apo/v3'-bound states presumably correspond to the situation where all flexibly linked domains (notably those of PB2-C and the endonuclease) are dangling in solution on their extended linkers, whereas the more compact states involve significant interfaces between these domains and with the polymerase core as observed in the various crystal structures. Second, crystallization generally favors more compact, less flexible conformations that are stabilized by crystal contacts and possibly oligomerization as well (as in the case of the c5'-bound FluB and apo-FluC structures). Crystal conformations may thus represent minority species in solution, as seems particularly likely for the apo-FluC structure, since MALLS and SAXS both suggest that the apo state should be highly extended. The cap-dependent endonuclease activity of the polymerase correlates well with the solution behavior in that apo/v3'-bound polymerases have low activity and v5'-, c5'-, or v3'+v5'-bound polymerases have much higher activity. Interestingly, the apo-FluC and c5'-bound FluB both have conformations in which the environment of the cap-binding site (including the position of the linker connecting the endonuclease to the rest of PA) disfavors capped RNA binding. This is likely representative of conformations in which the relative disposition of the cap-binding and endonuclease domains does not allow favorable coupling of strong capped RNA binding with nuclease cleavage; the nuclease activity then diminishes toward that of the isolated domain, which is weak mainly because of poor intrinsic RNA binding and a fast RNA off-rate (Datta et al., 2013). Interestingly, we find that c5'-, v5'-, or v3'+v5'-RNA-bound polymerases are increasingly active in cap-dependent endonuclease activity, respectively. This can be explained, assuming that the v3'+v5'-RNA-bound crystal structure is the optimal one for cap-dependent endonuclease activity, by an increasing propensity of the c5'-, v5'-, or v3'+v5'-RNA-bound polymerases, respectively, to be in this state. This would be consistent with the fact that c5'-bound polymerase preferentially crystallizes in a different conformation than v5'- or v3'+v5'-RNA-bound polymerase. Exactly why the conformation of the distal PB2-C region, which is distant from the promoter binding site, depends on which RNA is bound is still obscure. There is no striking overall difference in the core polymerase (PA-C, PB1, and PB2-N) between the FluB c5'-, v5'-, or v3'+v5'-RNA-bound polymerase crystal structures, except local perturbations around the RNA binding site as described above. Furthermore, the total buried surface area of the mobile domains (PB2-C and endonuclease) with the polymerase core (PA-C, PB1, and PB2-N) is very similar for the promoter or c5'-bound structures (6,621 Å² or 5,995 Å², respectively). However, the NLS domain/endonuclease interaction provides an additional 2,950 Å² apparently favoring the c5'-bound conformation, although it must be remembered that these numbers do not directly reflect binding energy. We propose that differential RNA binding more or less rigidifies the core polymerase to promote interfaces either between PB1 and the 627 domain and the endonuclease with the PB1-Cter/PB2-Nter helical bundle, in the case of v5' or v5'+v3', or PB1 and the cap-binding domain and the endonuclease with the NLS domain, but always allowing some probability of interconversion. With no RNA or only v3' RNA bound (i.e., without any v5' RNA bound), increased flexibility/instability around the promoter binding site could propagate to more distal regions of the core and destabilize both types of potential interface with PB2-C, allowing flexibly linked domains to be mobile in solution. In this connection, we point out that recent studies of the structurally and functionally related La Crosse bunyavirus polymerase have revealed considerable disorder in the apo state and also highlighted the role of vRNA 5' end binding in stabilizing the polymerase active site (Gerlach et al., 2015).

Finally, it is interesting to consider the functional significance of the c5'-bound polymerase conformation. The conservation of an alternative PB2-C domain arrangement across FluA, B, and C genera reinforces the conclusion that it is functionally important. The conformation is characterized by immobilization of the cap-binding domain against PB1 and packing of the NLS domain against the endonuclease such that cap snapping is disfavored. The most likely possibility is that this conformation corresponds to an incoming apo-polymerase binding to a nascent c5' end emerging from a replicating vRNP. Such a polymerase would not be required to do cap snatching but could be involved in initiating and guiding progeny cRNP formation by interacting with incoming apo-NP. Only at the end of replication would the polymerase bind the c3' end to complete progeny RNP formation, at the same time activating the polymerase by a switch in conformation. A mechanistic model of this kind has recently been proposed to describe RNP replication by the related bunyavirus polymerase (Gerlach et al., 2015). Alternatively, this conformation could represent that of the replicase rather than of the transcriptase, which again would not be expected to do cap snatching, but could receive a 3' end template donated by another RNP, as suggested in some models of replication (Jorba et al., 2009). In this scenario, the parental and incoming polymerase heterotrimers could initially form an asymmetric dimer, with the incoming polymerase possibly being activated by binding to small virus-generated RNAs (svRNAs) that correspond to truncated 5' end vRNAs and that accumulate in infected cells (Perez et al., 2012; Umbach et al., 2010). Clearly, further careful experiments are required to clarify the role of these alternative polymerase conformations. Finally, it is possible that particular interacting host factors (or indeed a second interacting polymerase, NP or NEP) could stabilize one or more interacting with incoming apo-NP. Only at the end of replication would the polymerase bind the c3' end to complete progeny RNP formation, at the same time activating the polymerase by a switch in conformation. A mechanistic model of this kind has recently been proposed to describe RNP replication by the related bunyavirus polymerase (Gerlach et al., 2015). Alternatively, this conformation could represent that of the replicase rather than of the transcriptase, which again would not be expected to do cap snatching, but could receive a 3' end template donated by another RNP, as suggested in some models of replication (Jorba et al., 2009). In this scenario, the parental and incoming polymerase heterotrimers could initially form an asymmetric dimer, with the incoming polymerase possibly being activated by binding to small virus-generated RNAs (svRNAs) that correspond to truncated 5' end vRNAs and that accumulate in infected cells (Perez et al., 2012; Umbach et al., 2010). Clearly, further careful experiments are required to clarify the role of these alternative polymerase conformations. Finally, it is possible that particular interacting host factors (or indeed a second interacting polymerase, NP or NEP) could stabilize one or other polymerase conformation, thus preferentially promoting for example transcription, vRNA to cRNA replication, or the reverse. This is plausible given that several well-known host-specific variants are located in PB2-C domains, particularly the 627 and NLS domains (Caudwell et al., 2014). Related to this is the intriguing possibility that the intra-molecular, inter-subunit binding of the PB2 NLS peptide to the PA endonuclease could play a role in the release of PB2 from the nuclear import factor importin α (which also binds the NLS peptide) and thus promote assembly of the heterotrimer through the interaction of PB2 with the PA-PB1 heterotrimer. The specific, but genera-dependent,
interaction between the PB2 NLS and the endonuclease could not also partly explain why transplanting of an unrelated NLS does not rescue the replication defect of mutations in the PB2 NLS (Resa-Infante et al., 2009).

EXPERIMENTAL PROCEDURES

Protein Production, Crystallization, and Structure Determination

Residues 247–736 from the A/Vietnam/1203/2004(H5N1) PB2 subunit were expressed in E. coli. Influenza B/Memphis/13/03 polymerase, purified as described previously (Reich et al., 2014), was mixed with either nucleotides 1–12 of the vRNA 5′ end (5′-pAGAGUAGAACAG-3′) or cRNA 5′ end (5′-pAGCAGAAGCAGA-3′). Residues 1–197 of FluB PA were expressed in E. coli, and purified endonuclease was mixed with synthetic FluB PB2 NLS peptide (residues 742–770). Crystallization trials were performed with a Cartesian robot and diffraction data measured on European Synchrotron Radiation Facility (ESRF) MX beamlines. Structures were solved by molecular replacement with PHASER and refined using REFMAC5 within the CCP4i package (Winn et al., 2011).

Endonuclease Activity Assays

FluA or FluB endonuclease domain activity was assayed as a function of bound NLS peptide using a FRET-based method (Kowalinski et al., 2012) with substrate 6-FAM-3′-CCUCUCUAUUUULCCUCAGUU-3′-BHQ1 (iBA). For cap-dependent endonuclease activity, FluB polymerase was mixed with a 32P-labeled capped 20-mer RNA (5′-AAUCUAUAUAGCAUUAUC-3′) and no RNA or v3′, c5′, v5′, or v3′+v5′ RNA and the time course of degradation monitored on a 20% acrylamide-7 M urea gel.

Biophysical Methods

The Kc for FluB NLS peptide binding to the endonuclease was derived from ITC measurements performed at 25°C using an ITC200 Micro-calorimeter (Micro-Cal). SEC-MALLS was performed on FluB polymerase with or without 12-mers of v5′ or c5′ RNA bound. SAXS measurements were performed on ESRF beamline BM29 on FluB polymerase with either no bound RNA or with only v3′, only v5′ or both v3′ and v5′ RNAs as they eluted from an online size-exclusion column. Automatic data processing used the ATLAS package (Petoukhov and Svergun, 2007).

Crosslinking Mass Spectrometry

Crosslinking was performed using recombinant FluA or FluB polymerase or reconstituted FluA-vRNA polymerase complex by addition of isotope-labeled DSS or DSG as described previously (Leitner et al., 2014). Protein digestion was performed at 37°C using LysC for 4 hr and trypsin for 12 hr, and cross-linked peptides were enriched using gel filtration. Fractions were analyzed by liquid-chromatography-based mass spectrometry using a nanoAcquity ultra-performance liquid chromatography column connected to a LTQ Orbitrap Velos Pro instrument (Thermo Scientific). Mass spectrometry data were processed using the xQuest/xProphet. Identified crosslinks were mapped to known polymerase structures and analyzed using Xlink Analyzer (Kosinski et al., 2014).

ACCESSION NUMBERS

The accession numbers for the structure factors and coordinates are wwPDB: 5FMM (H5N1 PB2-C form 1), 5FMQ (H5N1 PB2-C form 2), 5EPI (FluB c5′), 5FMZ (FluB v5′), and 5FML (FluB endonuclease with PB2 NLS). The mass spectrometry proteomics data reported in this paper have been deposited to the ProteomeXchange Consortium (Vizcaino et al., 2014) with the dataset identifier PRIDE partner repository: PXD002234.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2015.11.016.

AUTHOR CONTRIBUTIONS

E.T., supervised by D.H., expressed and crystallized the H5N1 PB2-C and solved the structure with the help of A.P. D.G. crystallized FluB polymerase with c5′ or v5′ RNA; performed endonuclease assays with full-length polymerase; cloned, expressed, and first crystallized the FluB endonuclease; and provided samples for biophysical experiments. S.G. crystallized FluB endonuclease with the NLS peptide and performed ITC and FRET assays. A.R. performed and analyzed SAXS measurements. N.H. and K.E.O provided FluC polymerase co-ordinates prior to publication. F.B. and T.B. performed crosslinking mass spectrometry with samples provided by D.G., A.P., and F.B. Crosslinking data were analyzed by J.K., T.B., F.B., and M.B, who supervised the crosslinking mass spectrometry. S.C. did crystallographic analysis of the FluB c5′, v5′, and endonuclease-NLS complexes, overall directed the project, and wrote the paper with input from the other authors.

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REFERENCES

Arranz, R., Coloma, R., Chichón, F.J., Conesa, J.J., Carrascosa, J.L., Valpuesta, J.M., Ortín, J., and Martín-Benito, J. (2012). The structure of native influenza virion ribonucleoproteins. Science 338, 1634–1637.

Cauldwell, A.V., Long, J.S., Moncorge, O., and Barclay, W.S. (2014). Viral determinants of influenza A virus host range. J. Gen. Virol. 95, 1193–1210.

Datta, K., Wolkerstorfer, A., Szolar, O.H., Cusack, S., and Klumpp, K. (2013). Characterization of PA-N terminal domain of influenza A polymerase reveals sequence specific RNA cleavage. Nucleic Acids Res. 41, 8289–8299.

Deng, T., Sharps, J., Fodor, E., and Brownlee, G.G. (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.

Deng, T., Engelhardt, O.G., Thomas, B., Akoultchev, A.V., Brownlee, G.G., and Fodor, E. (2006). Role of ran binding protein 5 in nuclear import and assembly of the influenza virus RNA polymerase complex. J. Virol. 80, 11911–11919.

Dias, A., Bouvier, D., Crépin, T., McCarthy, A.A., Hart, D.J., Baudin, F., Cusack, S., and Ruigrok, R.W. (2009). The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. Nature 458, 914–918.

Fodor, E. (2013). The RNA polymerase of influenza a virus: mechanisms of viral transcription and replication. Acta Virol. 57, 113–122.

Gerlach, P., Malet, H., Cusack, S., and Reguera, J. (2015). Structural Insights into Bunyavirus Replication and Its Regulation by the vRNA Promoter. Cell 167, 1287–1279.

Guiligay, D., Tarenteau, F., Resa-Infante, P., Coloma, R., Crepin, T., Sehr, P., Lewis, J., Ruigrok, R.W., Ortin, J., Hart, D.J., and Cusack, S. (2008). The structural basis for cap binding by influenza virus polymerase subunit PB2. Nat. Struct. Mol. Biol. 15, 500–506.
Hengrung, N., El Omari, K., Sema Martin, I., Vreede, F.T., Cusack, S., Rambo, R.P., Vonhein, C., Bricogne, G., Stuart, D.I., Grimes, J.M., and Fodor, E. (2015). Crystal structure of the RNA-dependent RNA polymerase from influenza C virus. Nature 527, 114–117.

Huet, S., Avilov, S.V., Ferbittz, L., Daigle, N., Cusack, S., and Ellenberg, J. (2010). Nuclear import and assembly of influenza A virus RNA polymerase studied in live cells by fluorescence cross-correlation spectroscopy. J. Virol. 84, 1254–1264.

Jorba, N., Area, E., and Ortín, J. (2008). Oligomerization of the influenza virus studied in live cells by fluorescence cross-correlation spectroscopy. J. Virol. 82, 1254–1264.

Kosinski, J., von Appen, A., Ori, A., Karius, K., Müller, C.W., and Beck, M. (2015). Xlink Analyzer: software for analysis and visualization of cross-linking data in the context of three-dimensional structures. J. Struct. Biol. 199, 177–183.

Kovalinski, E., Zubieta, C., Wolkerstorfer, A., Szolar, O.H., Ruigrok, R.W., and Cusack, S. (2012). Structural analysis of specific metal chelating inhibitor binding to the endonuclease domain of influenza pH1N1 (2009) polymerase. PLoS Pathog. 8, e1002831.

Li, M.L., Rao, P., and Krug, R.M. (2001). The active sites of the influenza cap-dependent endonuclease are on different polymerase subunits. EMBO J. 20, 2078–2087.

Moeller, A., Kirchdoerfer, R.N., Potter, C.S., Carragher, B., and Wilson, I.A. (2012). Organization of the influenza virus replication machinery. Science 338, 1631–1634.

Ortin, J., and Martin-Benito, J. (2015). The RNA synthesis machinery of negative-stranded RNA viruses. Virology 479-480, 532–544.

Perez, J.T., Zlatev, I., Aggarwal, S., Subramanian, S., Sachidanandam, R., Kim, B., Manoharan, M., and tenCate, B.R. (2012). A small-RNA enhancer of viral polymerase activity. J. Virol. 86, 13475–13485.

Petrokhov, M.V., and Svergun, D.I. (2007). Analysis of X-ray and neutron scattering from biomacromolecular solutions. Curr. Opin. Struct. Biol. 17, 562–571.

Plotch, S.J., Bouloy, M., Umanan, I., and Krug, R.M. (1981). A unique cap(m7GpppX)m-dependent influenza virus endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. Cell 23, 847–858.

Pumroy, R.A., and Cingolani, G. (2015). Diversification of importin-α isoforms in cellular trafficking and disease states. Biochem. J. 466, 13–28.

Reich, S., Guilligay, D., Pfug, A., Malet, H., Berger, I., Crépin, T., Hart, D., Lunardi, T., Nanai, M., Ruigrok, R.W., and Cusack, S. (2014). Structural insight into cap-snatching and RNA synthesis by influenza polymerase. Nature 516, 361–366.

Resa-Infante, P., Jorba, N., Zamarreno, N., Fernández, Y., Juárez, S., and Ortín, J. (2008). The host-dependent interaction of alpha-importins with influenza PB2 polymerase subunit is required for virus RNA replication. PLoS ONE 3, e3904.

Taylor, D., Cavley, G., and Hayward, S. (2014). Quantitative method for the assignment of hinge and shear mechanism in protein domain movements. Bioinformatics 30, 3189–3196.

Vizcaino, J.A., Deutsch, E.W., Wang, R., Csordas, A., Reisinger, F., Rios, D., Dianes, J.A., Sun, Z., Farrar, T., Bandeira, N., et al. (2014). ProteomeXchange provides globally coordinated proteomics data submission and dissemination. Nat. Biotechnol. 32, 223–226.

Waltzthoeni, T., Claassen, M., Leitner, A., Herzog, F., Bohn, S., Förster, F., Beck, M., and Aebersold, R. (2012). False discovery rate estimation for cross-linked peptides identified by mass spectrometry. Nat. Methods 9, 901–903.

Win, M.D., Ballard, C.C., Cowtan, K.D., Dodson, E.J., Emsley, P., Evans, P.R., Keegan, R.M., Krissinel, E.B., Leslie, A.G., McCoy, A., et al. (2011). Overview of the CCP4 suite and current developments. Acta Crystallogr. D Biol. Crystallogr. 67, 235–242.

York, A., Hengrung, N., Vreede, F.T., Huiskonen, J.T., and Fodor, E. (2013). Isolation and characterization of the positive-sense replicative intermediate of a negative-strand RNA virus. Proc. Natl. Acad. Sci. USA 110, E4238–E4245.
Supplemental Information

Influenza Polymerase Can Adopt an Alternative Configuration Involving a Radical Repacking of PB2 Domains

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Figure S2

A

R355
N429
H357
K339
K376
E361
V511
N510

627
Cap NLS
Mid-link

424-loop

B

627
424-loop
Cap
m^7GTP

Mid-link

NLS
424-loop
Cap
627
m^7GTP
Mid-link
Figure S3

A

627 domain

B

PA endonuclease

C

PB2 NLS

D

PB1 β-ribbon (v5'+v3')(v5')

3' end RNA (v5'+v3')

PB1 (v5'+v3')(v5')

α20

697

668

80

93

PB2 (v5'+v3')(v5')

5' end RNA (v5'+v3')(v5')
Figure S4

A

B

C

PB1

β-ribbon

5′ cRNA

627

Cap-627 linker

Cap

Mid

Endo

NLS

Cap-627 linker

Cap

Mid

Endo

NLS
Figure S5

A

FluB

Fluorescence (r.u.) vs. Time (s)

B

FluA

Fluorescence (r.u.) vs. Time (s)

C

y = 6.0326x + 11711
R² = 0.9957

y = 4.6858x + 7207.1
R² = 0.9932

D

Flu B
Flu A

NLS peptide concentration (μM)

E

FluB Endo
FluA Endo

Mn2+
- + - + - + - +

1µM 10µM 100µM 1µM L

Capped 20mer RNA
Supplemental Figure Legends

Figure S1. Sequence alignment of PB2-C for influenza A, B and C polymerases. Related to Figs. 1 and 2.

Sequence alignment done with ClustalW (Chenna et al., 2003) of PB2-C region for influenza A/Vietnam/1203/H5N1, B/Memphis/12/97/ and C/AnnArbor/1/50. The secondary structure derived from the A/Vietnam/1203/H5N1 PB2-C and B/Memphis/12/97/ c5’ bound structures are indicated on top and at the bottom respectively. Hinges between the domains (which are marked with the standard domain colours, as in Figure 1) are shown by yellows arrows (see Table 2).

Figure S2. m\textsuperscript{7}GTP binding to the cap-binding domain in different contexts. Related to Fig. 1.

A. Detail of m\textsuperscript{7}GTP binding to the isolated H5N1 PB2-C showing proximity of cap-627 linker domain residues 510-511.

B. m\textsuperscript{7}GTP binding environment in the isolated H5N1 PB2-C conformation (left) or modelled into the promoter bound FluB conformation (right).

Figure S3. Features of the FluB polymerase c5’ and v5’ RNA bound structures. Related to Fig. 2.

A. Ribbon diagram of the c5’ bound FluB oligomer, which is a hexamer with 32 point symmetry, viewed along the 3-fold (left) or 2-fold (right) axis. Each heterotrimer has a distinct colour. The externally positioned 627 domains are in slightly different orientations or, in one case, absent in the electron density.

B. Omit electron density for the FluB polymerase c5’ RNA bound structure in the region of the endonuclease (green Cα trace) and NLS domain (red Cα trace) interaction contoured at 1.0 σ (2Fo-Fc, brown mesh) and 2.7 σ (Fo-Fc, blue mesh).

C. Structure of the FluB v5’ RNA bound complex.

D. Differences in the vRNA binding site between the FluB v5’ RNA only bound structure (blue PB1, red PB2, magenta v5’ RNA) compared to the FluB v5’+ v3’ RNA bound structure (PDB 4WRT, cyan PB1, salmon PB2, violet v5’ RNA, yellow v3’ RNA). PB1 residues 671-676 interact with the v3’ RNA backbone in the v5’+ v3’ structure. In the v5’ only structure this region refolds and interacts with the PB1 β-ribbon which swings down when there is no v5’+ v3’ duplex.
Figure S4. Comparison of FluB polymerase c5’ bound structure with apo-FluC structure. Related to Fig. 2.

A. Ribbon diagram of the FluB polymerase c5’ RNA complex structure with PA-Nter (endonuclease) coloured forest green, the rest of PA (green), PB1 (cyan), PB2-N (red) and the PB2-C domains as in Figure 1A. The c5’ RNA 12-mer is in violet. The unobserved linker between the 627 and NLS domains is shown dotted.

B. Ribbon diagram of the apo-FluC polymerase structure in the same orientation as A after superposition of the PB1 subunit.

C. The mobile PB2-C and endonuclease domains of the FluB c5’ RNA complex (left) and apo-FluC structure (right) showing that the mid, cap and cp-627 linker are in the same configuration whereas the 627 and NLS-endonuclease unit are orientated differently.

Figure S5. Endonuclease assays. Related to Fig. 3

A. **Real-time fluorescence-based endonuclease reaction measurements.** The cleavage of the double labeled RNA by 10 μM FluB endonuclease is measured by the increase of florescence resulting from the release of 6-FAM dye at the 5’ RNA quenched by the BHQ-1 dye at the RNA-3’ (see methods). Because of the low endonuclease activity the plateau is reached only after 3 h. The reaction in the absence or the presence of increasing amounts of FluB NLS peptide are coloured as indicated. A control with 20 μM NLS peptide and EGTA 60 mM is included (light blue line).

B. The same for FluA endonuclease but with tenfold less protein concentration (1 μM). The plateau is reached after 30 min of reaction because of the much higher activity of the FluA endonuclease (see also panel E).

C. Same reactions as in panel A. Fits to the initial linear part corresponding to the steady state of the reaction. The initial velocity is calculated from the slope of the fitted lines. The fit parameters of the reactions in the absence or the presence of 100 μM NLS peptide are shown. The R² values in all fits were above 0.99.

D. Effect of NLS peptide concentration on FluA and FluB endonuclease activity. The bars show the variation of activity by increasing the concentration of the NLS peptide up to 100 μM in FluA (red) and FluB (blue). The activity values are normalised to the NLS peptide free reaction, which is set at 100. They are derived from the experiments
shown in A and B. The standard deviations come from two triple independent experiments. The activity background is shown by the 60 mM EGTA control.

E. Comparison of FluA and FluB endonuclease domain nuclease activity. The degradation of a $^{32}$P-capped 20-mer RNA is analyzed on a 7 M urea gel in the presence of 1 µM (lanes 1-2), 10 µM (lanes 3-4) or 100 µM (lanes 5-6) FluB endonuclease domain, or 1 µM of H3N2 FluA endonuclease domain (lanes 7-8) after 2 h of incubation at 30 °C. For the domain, MnCl$_2$ is required to trigger nuclease activity (Datta et al., 2013; Dias et al., 2009). An RNA ladder is shown in the right lane (L).

Figure S6. Comparison of the experimental SAXS scattering curves to model curves calculated from the various crystallographic structures. Related to Fig. 4.

Corrected and scaled, experimental SAXS scattering curves are plotted for the four samples, FluB polymerase with no RNA or v3′ RNA (upper two sets of data) and v5′ or v3′+ v5′ (lower two sets of data), each with symbols as indicated, the two pairs being displaced on the vertical axis for clarity. For each pair of datasets, the same three theoretical curves calculated from atomic models using CRYSOL (Svergun et al., 1995) are drawn: c5′ bound structure (blue), FluC-like apo-structure (green) and v3′+ v5′ structure (red). The models fit the v5′ or v3′+ v5′ data better at low resolution, since the corresponding radii of gyration are similar, but no model correctly fits the higher resolution data although the secondary maximum is in a similar position.

Figure S7. Crosslinking mass-spectrometry results. Related to Fig. 5.

A. Overview of crosslinking results for FluA and FluB polymerases. Inter-protein (blue) and intra-protein crosslinks (magenta) are indicated by lines between the three polymerase subunits (bars) of Influenza A (top figures) and Influenza B (bottom figure). Only crosslinks with a linear-discriminant score larger than 25 are shown. Polymerase subunit domain annotations and domain colours are taken from (Pflug et
al., 2014) (Flu A) and (Reich et al., 2014) (Flu B). The crosslink diagrams were drawn using xVis (Grimm et al., 2015).

B. Crosslinks that are not satisfied by known polymerase structures. Out of 230 crosslinks identified in total, 34 (15%) are not satisfied in any of the conformations, suggesting the existence of additional conformational states in solution other than those seen so far in crystal structures. They are shown (in red) mapped onto the 5′ cRNA structure and grouped by the domain they originate from. Nineteen of the unsatisfied crosslinks originate from the PB1 β-ribbon, the PB1 β-hairpin, and the PB1-Cter/PB2-Nter helical bundle, which are coloured black.

C. These crosslinks potentially correspond to a hypothetical non-RNA bound form. This suggests yet another conformation in which the PB1-Cter/PB2-Nter bundle separates from the polymerase core while the PB1 β-ribbon and the PB1 β-hairpin flexibly move around the region previously occupied by the same bundle (indicated by arrows coloured according to the respective domains). Since the PB1-Cter/PB2-Nter bundle crosslinks to several regions, it may remain flexible after separation.

D. The mobility of this bundle would agree with the tetrameric cryo-EM structure of FluA polymerase in which the PB2-arm and the PB2-mid domains are deleted (Chang et al., 2015). The panel shows 5′ cRNA structure fitted to the cryo-EM density; the part of PB2 absent in the construct used is coloured grey. At permissive iso-surface threshold the cryo-EM map is suggestive of a different conformation of the bundle (encircled) that otherwise remains unassigned.

E. The crosslinks originating from PB2-C and the PA endo (i.e. the domains that re-locate between the apo, 5′ cRNA, 5′-3′ vRNA conformations, coloured black) may correspond to transitional conformational states or result from the overall flexibility of the PB2-C.
F. Five of the violated crosslinks originate from the NLS helix, which can apparently detach from PA endonuclease domain in solution.

G. Two crosslinks remain enigmatic, which is within the expected false positive discovery rate of the mass spectrometry data (5%)
### Supplemental Tables

**Table S1. Summary of cross-link data. Related to Figure 5.**

|                | All crosslinks | Unique crosslinks | Unique inter-subunit crosslinks | Unique intra-subunit crosslinks | Unique monolinks |
|----------------|----------------|-------------------|---------------------------------|---------------------------------|------------------|
| Flu A - DSS - no RNA | 260            | 167               | 32                              | 59                              | 76               |
| Flu A - DSS - no RNA, interval mode* | 164            | 105               | 12                              | 29                              | 64               |
| Flu A - DSG - no RNA       | 78             | 53                | 4                               | 3                               | 46               |
| Flu A - DSS - RNA          | 163            | 114               | 6                               | 44                              | 64               |
| Flu A - DSG - RNA          | 87             | 62                | 3                               | 12                              | 47               |
| Flu B - DSS - no RNA       | 401            | 269               | 44                              | 138                             | 87               |
| Flu B - DSG - no RNA       | 153            | 111               | 15                              | 43                              | 53               |

* In interval mode, the crosslinker was applied stepwise by adding ten times 0.2 mM pulses of DSS every three minutes.
Table S2. Agreement of the conformations with crosslinks from XL-MS. Related to Figure 5.

|                | apo | 5′ cRNA | 5′-3′ vRNA (Flu B-like) | 5′-3′ vRNA (Flu A-like Cap domain) | All conformations‡ |
|----------------|-----|----------|-------------------------|-----------------------------------|-------------------|
| Satisfied*     | 192 | 177      | 172                     | 170                               | 197               |
| Conformation specific | 13  | 1        | 3‡                      | N/A                               |                   |
| Violated*      | 39  | 53       | 44                      | 46                                | 29                |
| All†           | 231 | 230      | 216                     | 216                               | 230               |
| Satisfied %    | 83% | 77%      | 80%                     | 79%                               | 86%               |
| Satisfaction score# | 85% | 79%      | 82%                     | 82%                               | 87%               |

*Crosslinks are regarded as satisfied (violated) if in the given structure the distance between Ca linked residues is below (above) 35 Å.
†All crosslinks that map to the given structure. Some crosslinks do not map to the structure because the corresponding residues are not resolved in the crystal structure. However, prior to mapping, some unobserved residues for which the position was unambiguous, were modelled to increase crosslink coverage.
‡Crosslinks satisfied in any conformation or violated in all conformations.
§Crosslinks specific to both FluA and FluB 5′-3′ vRNA conformation, which are treated together as these conformations are very similar.
# See methods.

Table S3. Listing of determined cross-links (See Excel file). Related to Figure 5.
Supplemental Experimental Procedures

A/H5N1 PB2-C construct, expression and purification

Residues 247-736 from the A/Vietnam/1203/2004 (H5N1) PB2 subunit (Genbank: AY818126.1) were cloned via AatII and NotI sites into the vector pET9, which also contains coding sequence for an N-terminal cleavable hexa-histidine tag. The PB2-C construct was expressed at 16°C in E. coli (BL21 RIL). The pelleted cells were re-suspended in buffer A (50 mM Tris pH8, 300 mM NaCl, 5% (v/v) glycerol and 5 mM β-mercaptoethanol) supplemented with protease inhibitors (Roche, complete mini, EDTA-free) and lysed by sonication. Cell debris was pelleted (30 min, 4° C, 58000 g) and the clear extract was loaded onto a metal ion affinity column (Qiagen, NiNTA agarose). Bound proteins were eluted with buffer B (50 mM Tris pH 8, 200 mM NaCl, 5% (v/v) glycerol, 5 mM β-mercaptoethanol and 500 mM imidazole). Target protein was dialyzed into buffer C (50 mM Tris pH 8, 200 mM NaCl, 5% (v/v) glycerol and 1 mM β-mercaptoethanol) and digested by tobacco etch virus (TEV) protease overnight at 4°C. The dialyzed sample was loaded onto a heparin column (GE Healthcare, HiTrap Heparin HP) and eluted with a linear gradient of buffer C supplemented with 1.5 M NaCl, concentrated (Amicon Ultra, 10 kDa molecular mass cut-off) and finally purified by size exclusion chromatography (GE Healthcare, Superdex75 10-300 GL) in buffer C. PB2-C protein was concentrated as above and protein concentration was determined by measuring the absorbance at 280 nm using the extinction coefficient 39,500 M⁻¹ cm⁻¹. Concentrated protein was aliquotted and stored at -80°C.

H5N1 PB2-C crystallization, data collection and structure solution

PB2-C was concentrated to 12 or 16 mg/mL in buffer C and mixed with 5 mM m⁷GTP (Sigma-Aldrich). Crystallization trials, performed by vapour diffusion (sitting drop) at 20° C using a Cartesian robot, yielded two different crystal forms. Clusters of plates were obtained with protein at 16 mg/mL in 0.1 M tri-sodium citrate pH 5, 10 % polyethylene glycol.
glycol monomethyl ether 5,000 (w/v). A semi hexagonal shape was obtained with protein at 12 mg/mL in 0.1 M tri-sodium citrate pH 5.8 and 1 M ammonium di-hydrogen phosphate. For data collection, both crystal forms were flash-frozen in well solutions supplemented with 25% glycerol. Diffraction data were collected at 100 K with an X-ray wavelength of 0.9763 Å on beamline ID29 of the European Synchrotron Radiation Facility equipped with a Pilatus 6M-F detector. All X-ray data were integrated and scaled with XDS (Kabsch, 2010). Initial phases were obtained by molecular replacement with the PB2-C domains extracted from bat FluA polymerase (PDB 4WSB) (Pflug et al., 2014). All refinements were performed with Refmac5 (Murshudov, 1997) or Phenix (Afonine et al., 2012). Plates and semi hexagonal shape crystals gave structures with resolutions of 2.4 Å and 3.2 Å respectively. The lower resolution structure shows good electron density for the 627 domain, whereas in the higher resolution structure, the domain is nearly in the same position but has very high B-factors. Ramachandran and other validation statistics were calculated with MolProbity (Chen et al., 2010). All structure figures were drawn with Pymol (DeLano, 2002), electron density with Bobscript (Esnouf, 1999) and buried surface areas calculated with PISA (http://www.ebi.ac.uk/pdbe/prot_int/pistart.html)(Krissinel and Henrick, 2007).

Crystallisation and structure determination of FluB polymerase with c5′ or v5′ RNA.

FluB polymerase, purified as described (Reich et al., 2014), was concentrated to 9 mg/mL (37 µM) in 500 mM NaCl, 50 mM HEPES-HCl pH 7.5, 5% glycerol and 2 mM TCEP and mixed with 40 µM of either nucleotides 1-12 of the vRNA 5′ end (5′-pAGUAGUAACAAG-3′, IBA GmbH) or nucleotides 1-12 of the cRNA 5′ end (5′-pAGCAGAAGCAGA-3′, IBA GmbH). Hanging drop vapour diffusion crystallization trials were performed at 4°C for both complexes. Crystals of influenza B polymerase in complex with the 5′ vRNA grew in 0.1 M KCl, 10 mM MgCl₂, 50 mM Tris-HCl pH 8.5 and 30% PEG400. Crystals of influenza B polymerase in complex with the 5′ cRNA appeared in 100
mM tri sodium citrate pH 5.6, 10 or 100 mM NaCl and 12% MPD. All crystals were cryo-protected with an additional 20% glycerol in their respective crystallization solutions and flash-frozen in liquid nitrogen. Data collection was performed on ESRF beamlines ID29 or ID23-1 using a Pilatus 6M-F detector. Structures were solved by molecular replacement using PHASER (McCoy et al., 2007) starting with the known FluB polymerase structures (PDB 4WSA) or individual domains therefrom. 6-fold NCS averaging of the c5’ map was performed with RESOLVE (Terwilliger, 2002) and refinement was done with Refmac5 (Murshudov, 1997), using local NCS restraints as each of the six copies had small differences. Subsequently a $P_{2_1}2_12_1$ form ($a=327.94$, $b=347.97$, $c=352.54$) diffracting to 6.0 Å resolution was obtained with a cRNA 5' 14-mer. This crystal form contains the same symmetrical oligomer of six polymerases in the asymmetric unit, but was not pursued further due to the low resolution (data not shown).

**Crystallisation and structure determination of FluB endonuclease-NLS peptide.**

Synthetic, codon optimized DNA (Shinegene) coding for residues 1 to 197 of FluB virus PA (B/Memphis/13/03) was cloned between the NcoI and XhoI sites in pETM11 vector, with an addition of a short linker (coding for MGSMA) between the TEV site and the start of the endonuclease coding sequence. Protein was expressed overnight in *E. coli* strain BL21 RIL at 20° C with 0.5 mM IPTG induction.

The bacterial pellet was re-suspended in 300 mM NaCl, 50 mM Tris pH 8.0, 15 mM imidazole, 5 mM β-mercaptoethanol and Complete EDTA-free protease inhibitor (Roche) for lysis. Cleared lysate was purified by affinity chromatography using Ni-sepharose resin. The His-tag was cleaved with TEV protease (1/100) overnight at 4° C followed by a second Ni-sepharose purification step where the PA domain no longer bound. The protein was finally purified on Superdex 75 column (GE Healthcare) in 150 mM NaCl, 20 mM Tris pH 8.0 and 5
mM β-mercaptoethanol and concentrated to around 23 mg/mL for crystallization trials and activity assays.

For crystallization trials, 500 µM of FluB PA 1-197 was mixed with 500 µM of a peptide corresponding to amino acids 742-770 of FluB polymerase PB2 subunit (742-KRYSAKSNDISQGIKRQRMTVESMGWALS-770, synthesized by Proteogenix) and sitting drops were set in a high throughput screen at 20°C. Crystals grew in 0.1 M MES pH 6.5, 25% PEG6000 or PEG8000 within a few days and were flash frozen in liquid nitrogen for data collection with 20% glycerol in the crystallization solution for cryo-protection. Data were collected in fully automatic mode on ESRF MASSIF beamline ID30A1 (Svensson et al., 2015). The structure was solved by molecular replacement using the previously determined structure of FluB endonuclease (PDB 4WSA).

**ITC measurements.**

ITC measurements were performed at 25 °C using an ITC200 Micro-calorimeter (MicroCal, Inc). Experiments consisted of 26 injections of 1.5 µL of peptide solution (1.6 or 1.0 mM) into the sample cell containing 65 or 60 µM of protein in 20 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM β-mercaptoethanol and 2% DMSO. The peptide used for ITC experiments was dissolved into the same buffer as the protein and the pH was adjusted to match that of the protein solution. Binding isotherms were fit by nonlinear regression using the identical and independent binding site model with Origin Software version 7.0 (MicroCal, Inc). The initial data point was routinely excluded of the fitting. The thermodynamic binding parameters correspond to the average of two independent experiments, and the estimated errors are about 5%.

**Endonuclease activity assays**

For the real-time quantitative endonuclease activity assays 500 nM of synthetic double labeled RNA, 6-FAM-5′-CUCCUCUUUUUCCCUAGUU-3′-BHQ1 (IBA), was
mixed with respectively 10 µM or 1 µM of FluB or FluA endonuclease domain and increasing amounts of specific NLS synthetic peptide for each virus: FluB (742-KRYSLNDSQGIKQRMTVESMGWALS) or FluA (738-KRDSSILTDQSQTATKIRMAIN) (ProteoGenix). The reaction buffer was 20 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM β-mercaptoethanol, 2 mM MnCl₂ and 10 mM MgCl₂. The fluorescence increase upon the RNA cleavage was measured in a TECAN (infinite M200 pro) at 26 °C using 465 nm excitation and 520 nm emission wavelengths. All reactions were measured until the plateau was reached (Fig. S5AB). The initial reactions velocities were determined by the slope of the linear part of the reaction start where the linear fitting quality was above R²=0.99 (Fig. S5CD).

For full-length FluB polymerase endonuclease activity (Fig. 4D), 1 µM of FluB polymerase was mixed with a capped 20-mer synthetic RNA (5′-AAUCUAAUAAUGCAUUAUCC-3′) ³²P labelled by capping as described (Reich et al., 2014) and either 1.2 µM of nucleotides 1-18 of the 5′ end of the vRNA (5′-pAGUAGUAACAAGAGGGUA-3′) or 1-18 of the 3′ end of the vRNA (5′-pUAUACCUCUCUUCUGCU-3′) or both, or 1-18 of the 5′ end of the cRNA (5′-pAGCAGAAGCAGAGGUA-3′) (all RNAs purchased from IBA GmbH), in a buffer containing 150 mM NaCl, 50 mM Hepes pH 7.5, 5 mM MgCl₂, 2 mM TCEP and incubated at 30 °C. Samples were taken at 0, 10 min, 20 min, 30 min, 60 min, 120 min and analyzed on a 20% acrylamide-7 M urea gel.

For FluB endonuclease domain nuclease activity (Fig. S5E), 1 µM, 10 µM or 100 µM of FluB PA (residues 1-197) were mixed with ³²P labelled 20-mer capped RNA in a buffer containing 150 mM NaCl, 50mM Hepes pH 7.5, 5 mM MgCl₂, 2 mM TCEP and no or 2.5 mM MnCl₂. As a control, 1 µM of H3N2 FluA PA (residues 1-209) was mixed with ³²P-
20mer capped RNA in the same conditions. After 2h incubation at 30 °C, samples were analyzed on a 20% acrylamide-7 M urea gel.

**SEC-MALLS**

Size-exclusion chromatography (SEC) combined with on-line detection by multiangle laser light scattering (MALLS) and refractometry allows measurement of the absolute molecular mass of biological particles in solution independently of their dimensions and shape (Wyatt, 1998). SEC was performed with a S200 Superdex column (GE Healthcare) at 20°C with a flow rate of 0.5 ml/min, using FluB polymerase with or without bound 12-mers of v5’ or c5’ RNA at ~ 5 mg/ml in 0.5 M NaCl, 50 mM Hepes pH 7.5, 5% glycerol and 2mM TCEP. MALLS detection was with a DAWNHELEOS II detector (Wyatt Technology) using a laser emitting at 690 nm and protein concentration was measured using differential refractive-index measurement with an Optilab T-rEX detector (Wyatt Technology) and a refractive-index increment (dn/dc) of 0.185 ml/g for protein and 0.175 ml/g for nucleic acids. Weight-averaged molar masses were calculated with ASTRA (Wyatt Technology).

**Small-angle X-ray scattering**

Small angle X-ray scattering (SAXS) measurements were performed on the ESRF BioSAXS beamline BM29 (Pernot et al., 2013) in Grenoble (France) on solutions of FluB polymerase (~ 9 mg/ml in 0.5 M NaCl, 50 mM Hepes pH 7.5, 5% glycerol and 2mM TCEP) with either no bound RNA (apo) or with only v3’, only v5’ or both v3’ and v5’ RNAs. Samples were measured in the flow cell of the BioSAXS sample changer (Round et al., 2015) as they were eluted from an on-line size-exclusion column (Superose 6, GE Healthcare) (Round et al., 2013). Consecutive one second frames were collected for the duration of the elution using a Pilatus 1M detector (Dectris). Individual frames were processed automatically and independently within the EDNA framework, yielding individual radially averaged curves of normalized intensity versus scattering angle q=4πsinθ/λ. Additional data reduction within
EDNA utilizes the automatic data processing tools of EMBL-Hamburg ATSAS package (Petoukhov and Svergun, 2007), to combine time frames. The ISPyB BioSAXS interface (De Maria Antolinos et al., 2015) was used to check for regions of stable radius of gyration (Rg) corresponding to the elution of a single species. All frames from these regions were then averaged to yield the scattering curve for each species. Theoretical scattering profiles for each crystal structure were calculated with CRYSOL (Svergun et al., 1995).

**Reconstitution of influenza polymerase-vRNA complex for crosslinking**

A T7 transcribed min-panhandle of 39 bases including the conserved ends of influenza vRNA (5′-pppAGUAGUAACAGAGGGUAUUGUAUACCUCUUCUGCUUC-3′) was mixed with BatFluA polymerase with a protein/RNA ratio of 1:1.1 in 50 µl of buffer containing 50 mM Hepes pH 7.5, 350 mM NaCl, 3% glycerol and 1.5 mM TCEP.

**Crosslinking of reconstituted influenza polymerase / RNA-polymerase complex**

Chemical crosslinking reactions were performed as described (Walzthoeni et al., 2012). In brief, 50 µg of recombinant FluA or FluB influenza polymerase or reconstituted FluA-vRNA polymerase complex (1 µg/µl) was crosslinked by addition of isotope labeled DSS (disuccinimidyl-suberate, Creative Molecules) or DSG (disuccinimidyl-glutarate, Creative Molecules) crosslinkers (both at 2 mM final concentration) at 35 °C and centrifuged at 600 rpm for 30 minutes. The crosslinking reaction was quenched by addition of ammonium bicarbonate (final concentration 100 mM) at 35 °C and 600 rpm for 10 min. To perform carbamidomethylation of cysteines prior to protein digestion, crosslinked samples were denatured by addition of urea (final concentration 4 M) and subsequently treated with 10 mM DTT for 30 min at 37 °C followed by 15 mM IAA at room temperature in the dark for 30 min. Protein digestion was performed using 1:100 (w/w) LysC (Wako Pure Chemical Industries) for 4 h at 37 °C and finalized with 1:50 (w/w) trypsin (Promega) at 37 °C for 12
hours after the urea concentration was diluted to 1.5 M. After digestion, samples were acidified with 10% (vol/vol) trifluoroacetic acid and desalted using Macro SpinColumns (Harvard Apparatus) according to the manufacturer’s procedure. Purified peptide mixtures were dried in a vacuum concentrator and stored at 20 °C until further use.

**Enrichment of crosslinked influenza polymerase peptides by size exclusion chromatography**

Crosslinked peptides were enriched using gel-filtration as described before (Leitner et al., 2014). In brief, purified peptide mixtures were resuspended in buffer containing 30% (v/v) acetonitrile / 0.1% (v/v) trifluoroacetic acid. Reconstituted peptides were fractionated using a Superdex Peptide PC 3.2/30 column (GE) on an Äktamicro LC system (GE) at a flow rate of 50 μl/min. Based on the 215 nm absorbance profile, fractions eluting between 0.9 and 1.4 mL were collected and dried in a vacuum concentrator. Fractions were reconstituted in 20-40 μL buffer containing 5% (v/v) acetonitrile and 0.1% (v/v) formic acid prior to mass spectrometry analysis.

**Mass spectrometry based analysis of crosslinked peptides**

All fractions were analysed in technical duplicates by liquid-chromatography based mass spectrometry. Crosslinked peptide fractions were separated using a BEH300 C18 (75 μm x 250 mm, 1.7 μm) nanoAcquity UPLC column (Waters) with a stepwise 60 min gradient from 3% to 85% (v/v) acetonitrile in 0.1% (v/v) formic acid at a flow rate of 0.3 μl/min. The nano-liquid chromatography system was connected to a LTQ Orbitrap Velos Pro instrument (Thermo Scientific), which was operated in data-dependent mode. Crosslinked peptides were fragmented in collision induced dissociation mode using one survey MS scan followed by maximum 20 fragmentation scans (TOP20) of the most abundant ions analyzed. Single and double charged ions were excluded from MS/MS analysis. Important MS parameters were: full MS: AGC = 10⁶, maximum ion time = 500 ms, m/z range = 375-1600, resolution =
30,000 FWHM; MS2: AGC = 30,000, maximum ion time = 10 ms, minimum signal threshold = 100, dynamic exclusion time = 30 s, isolation width = 2 Da, normalized collision energy = 40, activation Q = 0.25. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Vizcaino et al., 2014) via the PRIDE partner repository with the dataset identifier PXD002234.

Identification of crosslinked peptides from fragmentation spectra and crosslink analysis

Raw mass spectrometry data was processed using the xQuest/xProphet packages (Rinner et al., 2008; Walzthoeni et al., 2012). Raw data files were converted to mzXML data format using Mass Matrix file converter. Converted data files were searched by xQuest against a database containing the influenza polymerase subunit sequences. Posterior probabilities were calculated by xProphet. The xQuest search results were filtered for: false-discovery rate = 0.05, delta score = 0.95, MS1 tolerance window = -4 to 7 ppm, ld-score equal or larger than 25. Fragmentation spectra were checked manually to confirm they contained a convincing series of crosslinked fragment ions and common fragment ions identified for both crosslinked peptide instances. A complete listing of determined cross-links is given in Table S3. The crosslinks were mapped to the structures and analyzed using Xlink Analyzer (Kosinski et al., 2015). To map crosslinks of FluA polymerase to the FluB structure, the crosslinks were re-numbered based on sequence alignments (justified due to high sequence and structural similarity between influenza A and B polymerase subunits (Reich et al., 2014)). Crosslinks were deemed to be satisfied (or violated) if the lysine-lysine inter-Cα distance in the structures was smaller (longer) than 35 Å. This threshold is in line with various studies that mapped crosslinking MS data sets to known structures (Erzberger et al., 2014; Herzog et al., 2012; Kahraman et al., 2013). The longer threshold accounts for local flexibility of domains and loops, particularly apparent in the influenza polymerase structures (e.g. the PB1 β-ribbon).
The satisfaction score in Table S2 was calculated as:

\[
\text{score} = \sum \left\{ \begin{array}{ll}
1 & \text{if } d \leq 35 \text{ Å} \\
1 - \frac{d - 35}{40 - 35} & \text{if } 35 \text{ Å} < d \leq 40 \text{ Å} \\
0 & \text{if } d > 40 \text{ Å}
\end{array} \right.
\]

summing over all crosslinks mapped to a structure with distance between crosslinked residues \(d\). The satisfaction score allows taking into account crosslinks that only slightly violate the 35 Å distance threshold.
Supplemental References

Afonine, P.V., Grosse-Kunstleve, R.W., Echols, N., Headd, J.J., Moriarty, N.W., Mustyakimov, M., Terwilliger, T.C., Urzhumtsev, A., Zwart, P.H., and Adams, P.D. (2012). Towards automated crystallographic structure refinement with phenix.refine. Acta crystallographica Section D, Biological crystallography 68, 352-367.

Chang, S., Sun, D., Liang, H., Wang, J., Li, J., Guo, L., Wang, X., Guan, C., Boruah, B.M., Yuan, L., et al. (2015). Cryo-EM Structure of Influenza Virus RNA Polymerase Complex at 4.3 A Resolution. Molecular cell.

Chen, V.B., Arendall, W.B., 3rd, Headd, J.J., Keedy, D.A., Immormino, R.M., Kapral, G.J., Murray, L.W., Richardson, J.S., and Richardson, D.C. (2010). MolProbity: all-atom structure validation for macromolecular crystallography. Acta crystallographica Section D, Biological crystallography 66, 12-21.

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T.J., Higgins, D.G., and Thompson, J.D. (2003). Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 31, 3497-3500.

Datta, K., Wolkerstorfer, A., Szolar, O.H., Cusack, S., and Klumpp, K. (2013). Characterization of PA-N terminal domain of Influenza A polymerase reveals sequence specific RNA cleavage. Nucleic Acids Res.

De Maria Antolinos, A., Pernot, P., Brennich, M.E., Kieffer, J., Bowler, M.W., Delageniere, S., Ohlsson, S., Malbet Monaco, S., Ashton, A., Franke, D., et al. (2015). ISPyB for BioSAXS, the gateway to user autonomy in solution scattering experiments. Acta crystallographica Section D, Biological crystallography 71, 76-85.

DeLano, W.L. (2002). PyMOL Molecular Graphics System. available online at http://wwwpymolsourceforgenet.

Dias, A., Bouvier, D., Crepin, T., McCarthy, A.A., Hart, D.J., Baudin, F., Cusack, S., and Ruigrok, R.W. (2009). The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. Nature 458, 914-918.

Erzberger, J.P., Stengel, F., Pellarin, R., Zhang, S., Schaefer, T., Aylett, C.H., Cimermancic, P., Boehringer, D., Sali, A., Aebersold, R., et al. (2014). Molecular architecture of the 40S eIF1-eIF3 translation initiation complex. Cell 158, 1123-1135.

Esnouf, R.M. (1999). Further additions to Molscript version 1.4, including reading and contouring of electron density maps. Acta Crystallogr 55, 938-940.

Grimm, M., Zimniak, T., Kahraman, A., and Herzog, F. (2015). xVis: a web server for the schematic visualization and interpretation of crosslink-derived spatial restraints. Nucleic Acids Res 43, W362-369.

Herzog, F., Kahraman, A., Boehringer, D., Mak, R., Bracher, A., Walzthoeni, T., Leitner, A., Beck, M., Hartl, F.U., Ban, N., et al. (2012). Structural probing of a protein phosphatase 2A network by chemical cross-linking and mass spectrometry. Science 337, 1348-1352.

Kabsch, W. (2010). Integration, scaling, space-group assignment and post-refinement. Acta crystallographica Section D, Biological crystallography 66, 133-144.

Kahraman, A., Herzog, F., Leitner, A., Rosenberger, G., Aebersold, R., and Malmstrom, L. (2013). Cross-link guided molecular modeling with ROSETTA. PloS one 8, e73411.

Kosinski, J., von Appen, A., Ori, A., Karius, K., Muller, C.W., and Beck, M. (2015). Xlink Analyzer: software for analysis and visualization of cross-linking data in the context of three-dimensional structures. Journal of structural biology 189, 177-183.

Krissinel, E., and Henrick, K. (2007). Inference of macromolecular assemblies from crystalline state. Journal of molecular biology 372, 774-797.
Leitner, A., Walzthoeni, T., and Aebersold, R. (2014). Lysine-specific chemical cross-linking of protein complexes and identification of cross-linking sites using LC-MS/MS and the xQuest/xProphet software pipeline. Nature protocols 9, 120-137.

McCoy, A.J., Grosse-Kunstleve, R.W., Adams, P.D., Winn, M.D., Storoni, L.C., and Read, R.J. (2007). Phaser crystallographic software. J Appl Crystallogr 40, 658-674.

Murshudov, G.N. (1997). Refinement of macromolecular structures by the maximum-likelihood method. Acta crystallographica Section D, Biological crystallography 53, 240-255.

Perrot, P., Round, A., Barrett, R., De Maria Antolinos, A., Gobbo, A., Gordon, E., Huet, J., Kieffer, J., Lentini, M., Mattenet, M., et al. (2013). Upgraded ESRF BM29 beamline for SAXS on macromolecules in solution. Journal of synchrotron radiation 20, 660-664.

Petoukhov, M.V., and Svergun, D.I. (2007). Analysis of X-ray and neutron scattering from biomacromolecular solutions. Current opinion in structural biology 17, 562-571.

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

Reich, S., Guilligay, D., Pflug, A., Malet, H., Berger, I., Crepin, T., Hart, D., Lunardi, T., Nanao, M., Roigrok, R.W., et al. (2014). Structural insight into cap-snatching and RNA synthesis by influenza polymerase. Nature 516, 361-366.

Rinner, O., Seebacher, J., Walzthoeni, T., Mueller, L.N., Beck, M., Schmidt, A., Mueller, M., and Aebersold, R. (2008). Identification of cross-linked peptides from large sequence databases. Nature methods 5, 315-318.

Round, A., Brown, E., Marcellin, R., Kapp, U., Westfall, C.S., Jez, J.M., and Zubieta, C. (2013). Determination of the GH3.12 protein conformation through HPLC-integrated SAXS measurements combined with X-ray crystallography. Acta crystallographica Section D, Biological crystallography 69, 2072-2080.

Round, A., Felisaz, F., Fodinger, L., Gobbo, A., Huet, J., Villard, C., Blanchet, C.E., Pflug, P., McSweeney, S., Roessle, M., et al. (2015). BioSAXS Sample Changer: a robotic sample changer for rapid and reliable high-throughput X-ray solution scattering experiments. Acta crystallographica Section D, Biological crystallography 71, 67-75.

Svensson, O., Monaco, S., Popov, A.N., Murizzo, D., and Bowler, M.W. (2015). The fully automatic characterization and data collection from crystals of biological macromolecules. Acta Cryst D 71.

Svergun, D.I., Barberato, C., and M.H.J., K. (1995). CRYSOL - a Program to Evaluate X-ray Solution Scattering of Biological Macromolecules from Atomic Coordinates. JApplCryst 28, 768-773.

Terwilliger, T.C. (2002). Statistical density modification with non-crystallographic symmetry. Acta crystallographica Section D, Biological crystallography 58, 2082-2086.

Vizcaino, J.A., Deutsch, E.W., Wang, R., Csordas, A., Reisinger, F., Rios, D., Dianes, J.A., Sun, Z., Farrah, T., Bandeira, N., et al. (2014). ProteomeXchange provides globally coordinated proteomics data submission and dissemination. Nature biotechnology 32, 223-226.

Walzthoeni, T., Claassen, M., Leitner, A., Herzog, F., Bohn, S., Forster, F., Beck, M., and Aebersold, R. (2012). False discovery rate estimation for cross-linked peptides identified by mass spectrometry. Nature methods 9, 901-903.

Wyatt, P.J. (1998). Submicrometer Particle Sizing by Multiangle Light Scattering following Fractionation. Journal of colloid and interface science 197, 9-20.