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

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Received 17 January 2005/Accepted 7 March 2005

Influenza virus RNA-dependent RNA polymerase is a heterotrimeric complex of PB1, PB2, and PA. We show that the individually expressed PB2 subunit can be assembled with the coexpressed PB1-PA dimer in vitro into a transcriptionally active complex. Furthermore, we demonstrate that a model viral RNA promoter can bind to the PB1-PA dimer prior to assembly with PB2. Our results are consistent with a recently proposed model for the sequential assembly of viral RNA polymerase complex in which the PB1-PA dimeric complex and the PB2 monomer are transported into the nucleus separately and then assembled in the nucleus.

Transcription (viral RNA [vRNA]→mRNA) and replication (vRNA→cRNA) of the eight segments of the negative-sense single-stranded influenza A virus genomic vRNA are catalyzed by the viral RNA-dependent RNA polymerase, which is a trimeric complex encoded by the three largest segments of the influenza virus genome—PB1, PB2, and PA (reviewed in references 9, 22, and 27). The PB1 subunit contains the conserved motifs characteristic of RNA-dependent RNA polymerases (2, 3) and binds to the vRNA and cRNA promoters (5, 14, 24). It also has endonuclease activity required to snatch capped primers from host pre-mRNAs for viral RNA transcription (25). PB2 is responsible for recognition and binding the cap structure of host mRNAs (8, 9, 22, 27). The exact role of PA is less well understood, but it has a central role in both transcription and replication (10, 11, 18, 20).

No crystal structure is available for the influenza virus polymerase complex, but a low-resolution three-dimensional structure, determined by electron microscopy, showed that it is very compact, with no obvious boundaries between subunits (1). Protein-protein interactions between the individual polymerase subunits have been identified, suggesting that PB1 is the core of the polymerase complex. The N-terminal region of PB1 interacts with the C-terminal region of PA, while the C-terminal region of PB1 interacts with the N-terminal of PB2 subunit. No direct protein-protein interactions have been demonstrated between PB2 and PA (15, 28, 30, 34, 35). All three polymerase subunits are required for transcription and replication activity both in vivo and in vitro (4, 10, 29), although, controversially, Honda et al. reported that recombinant dimeric complexes of PB1-PB2 and PB1-PA have distinct transcriptase and replicase activities, respectively, in vitro (17).

Viral polymerase performs both transcription and replication of vRNA in the nuclei of the infected cells (19). How viral polymerase subunits are transported from the cytoplasm to the nucleus and the detailed mechanism of assembly of the polymerase complex remain unclear. Recently, Fodor and Smith proposed a model in which PB1 and PA are transported into the nucleus as a dimeric complex. They suggested that PB2 enters the nucleus as a monomer and subsequently binds to the PB1-PA dimer in the nucleus (13). In order to further understand the process of viral polymerase assembly, we attempted the in vitro reconstitution of a functionally active viral RNA polymerase complex from recombinant subunits expressed in human embryonic kidney cells (293T). Single subunits, dimers, or a trimeric PB1-PB2-PA complex were purified from cells transfected with protein expression plasmids expressing the subunits of the influenza A/WSN/33 virus polymerase. To facilitate the purification, one of the expressed subunits was tagged at the C terminus with TAP—a tag containing a calmodulin binding site, a TEV cleavage site, and two protein A domains (31). We selected the C-terminal TAP tag construct because a previous report showed that an N-terminal green fluorescent protein tag of PB1 or PA interfered with RNA polymerase activity (13). In addition, by reconstituting recombinant RNPs in vivo and studying their activity (13), we have confirmed that the C-terminal TAP tag attached to any of the three subunits is compatible with both transcription and replication activity of the RNA polymerase (Fodor and Smith, unpublished data). TAP-tagged subunits, together with associated proteins, were purified from lysates of transfected cells using immunoglobulin G (IgG) Sepharose, which binds the protein A component of the TAP tag. Bound proteins were then released by cleavage with tobacco etch virus (TEV) protease. We have confirmed that there is no detectable nonspecific pull-down of any of the individual subunits (i.e., PB1, PB2, and PA) by IgG Sepharose in the absence of the TAP tag (data not shown). The samples were analyzed by silver staining of 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Figure 1A shows that PB1 can be copurified with both PAtap (lane 4) and PB2tap (lane 6), but no PB2 can be copurified with PAtap (lane 5). Purified PB1-PAtap and PB1-PB2tap dimers were then mixed with individually expressed PB2 and PA monomers, respectively, in an attempt to assemble a functionally active heterotrimeric complex. The assembly experiment was performed either in solution by mix-
ing purified dimers and monomers or by using a solid-phase assembly. In the latter approach, the dimers were first immobilized onto IgG Sepharose through the TAP tag, which then acted as a bait to pull down the third untagged subunit from a crude cell lysate of 293T cells containing the expressed monomers. Finally, the putative assembled complex was released from its solid phase (IgG Sepharose) with TEV protease, and its functionality was tested in transcription assays.

To assay for polymerase activity of the putative assembled complex, both cap-independent and cap-dependent transcription activities were examined using ApG and globin mRNA as primers, respectively. Short synthetic RNA oligonucleotides corresponding to the 5' end (15 nucleotides [nt]) and 3' end (14 nt) of the wild-type influenza A virus vRNA promoter were used as described previously (10). Figure 1B shows that in both ApG-primed and globin mRNA-primed transcription assays, significant transcription was observed when monomeric PB2 was assembled with coexpressed PB1-PAtap dimer (lanes 6

FIG. 1. (A) Purification of TAP-tagged RNA polymerase subunits from 293T cells transfected with expression plasmids for various combinations of the three polymerase subunits, PB1, PB2, and PA, analyzed by silver staining of an 8% SDS-PAGE gel. The plasmids transfected into 293T cells in different combinations to generate monomers and dimers are indicated above the lanes. Sizes of protein standards in kilodaltons are shown on the left; positions of PB1, PAtap, PB1tap, PB2, and PB2tap are shown either on the right or on the left. The star indicates the position of PB2tap in lane 6. The circles indicate the positions of Hsp90 identified by both matrix-assisted laser desorption ionization–time of flight and liquid chromatography–tandem mass spectrometry. In lanes 4 to 7, the tagged subunit is in higher yield than the untagged subunit(s) because of copurification of the tagged monomer. (B) PB1-PA dimers can assemble with PB2 in vitro to produce functional polymerase (solid-phase assembly). Lanes 1 to 8: as assayed by in vitro ApG-primed transcription with all possible combinations of monomers, dimers, and 3P. Lanes 9 to 15: as assayed by in vitro globin mRNA-primed transcription with the all possible combinations of monomers, dimers, and 3P. The transcription products (TP) are indicated on the right with a line. The products were analyzed by 20% PAGE in 7 M urea followed by autoradiography. (C) The amount of assembled PB2 with the PB1-PAtap dimer, compared with coexpressed 3P complex, was analyzed by Western blotting using both an anti-PB2 antibody (rabbit polyclonal antibody from Inglis [7]) and an anti-PB1 antibody (rabbit polyclonal antibody from T. Jung). PAtap alone was used as a negative control for nonspecific binding of PB2 to IgG Sepharose beads (lane 1). The positions of PB1 and PB2 are shown on the right and size markers on the left in kilodaltons.
and 13). The transcription efficiency of the assembled complex varied in the different experiments in the range of 5 to 50% for in-solution assembly (six experiments) and 5 to 20% for solid-phase assembly (four experiments) compared with the activity of coexpressed 3P. The results were independent of which subunit was tagged, since similar results were obtained if PB1tap-PA was used instead of PB1-PAtap in the in-solution assembly experiments (results not shown). However, no activity was observed when monomeric PA was assembled with PB1-PB2 dimer (see lanes 7 and 14). Furthermore, no transcription activity was obtained with any monomers or the two dimeric complexes (lanes 1 to 5, 9 to 12), which confirms that all three subunits are required for transcriptase activity, contradicting the results of Honda et al. (17). A Western blot of the assembled complexes that were used in lanes 1, 6, and 8 of Fig. 1B confirmed that PB2 had assembled with the PB1-PAtap dimer in the solid-phase assembly (Fig. 1C, compare lane 2 with lane 1, negative control). However, the level of PB2 in the in vitro-assembled complex was significantly lower than in the in vivo-coexpressed 3P, in contrast to the level of PB1, which was similar in both samples (compare lane 2 with lane 3). This result is consistent with the lower transcriptional efficiency (5 to 20%) of the polymerase complex assembled in vitro. It suggests that the in vitro-assembled trimeric complex is fully active in transcription and the low activity observed (5 to 20%) is primarily due to the limiting amount of PB2 in the complex. The reason for the limited efficiency of in vitro assembly is unknown but could be explained by host factors interacting with either the PB1-PA dimer or PB2 monomer that may interfere with the assembly in vitro. Overall, our successful in vitro assembly implies that the trimeric polymerase complex may be assembled sequentially in vivo by first forming a PB1-PA dimer and then interacting with PB2 (see below).

It has been reported that the PB1-PA dimeric complex can bind to the 5′ terminus of vRNA in a gel shift experiment (23). To confirm this, we tested the promoter-binding capacity of the PB1-PA dimer, purified on IgG Sepharose, in a UV cross-linking assay with the 32P-labeled 5′ end of the vRNA promoter (15 nt) in the presence of unlabeled 3′ end (14 nt) as described previously (6, 10) (Fig. 2). As expected, the PB1-PA dimer (lane 4) showed very efficient promoter-binding activity compared with coexpressed 3P complex (lane 7), whereas none of the monomers or the dimers binds the promoter (lanes 1 to 3, 5, and 6). These results confirmed that the PB1-PA dimeric complex can bind the viral RNA promoter, and both PB1 and PA are cross-linked to it (lane 8), as expected (12). It follows that PB2 is not required for initial recognition and binding of the vRNA promoter.

Because the PB1-PA dimer binds to the vRNA promoter (Fig. 2), it was of interest to examine whether the addition of the vRNA promoter to the PB1-PA dimer prior to assembly with PB2 could enhance the functional assembly of a heterotrimeric complex. To test this, we first immobilized the PB1-PAtap dimer on IgG Sepharose through the C-terminal TAP tag of PA and then assembled it with the vRNA promoter (a mixture of the 5′ and 3′ strands of the vRNA promoter) and monomeric PB2 in different orders, as shown in Fig. 3A (i and ii). Each step of the assembly was carried out by mixing washed PB1-PAtap-bound IgG Sepharose with either crude cell lysate containing monomeric PB2 or promoter RNAs in an RNA binding buffer (containing 5 mM MgCl2 and 1 U/μl RNase inhibitors [Promega]) followed by incubation at 20°C for 15 min. The beads were thoroughly washed between each assembly step. Finally, the assembled complexes were released with TEV protease. The resulting complexes were tested for activity by the ApG-primed and globin-primed transcription assays in vitro, without the addition of further promoter RNAs (Fig. 3B). No activity was obtained with the PB1-PA dimer assembled with promoter vRNA (lanes 1 and 5), whereas the PB1-PB2 dimers assembled with vRNA promoter either before (lanes 2 and 6) or after (lanes 3 and 7) PB2 binding were all capable of transcribing the vRNA promoter both in cap-independent and cap-dependent manners. However, the transcriptional activity of the assembled complexes was in the range of 1 to 5% compared to the activity of coexpressed 3P complex (four experiments). The reasons for the low efficiency observed here (1 to 5%) compared to that observed before (5 to 20%) are most likely related to (i) the small amounts of promoter RNA assembled in vitro and (ii) potential RNA degradation during extensive washing steps prior to the functional assay. Interestingly, the activity of the complex assembled in the order of PB1-PA dimer, vRNA promoter, and PB2 monomer (Fig. 3A, i) was reproducibly twofold higher than that of the complex assembled in the order of PB1-PA dimer, PB2 monomer, and vRNA promoter (Fig. 3A, ii). The twofold difference in activity is likely to reflect a twofold difference in the efficiency of assembly, since identical components, although in differing orders, were used for each step in the assembly. These results showed that the PB1-PA dimeric complex can bind viral RNA promoter either prior to or subsequent to the assembly with PB2 to form an active polymerase complex.

We attempted to assemble a functional PB1-PA dimeric complex in vitro using individually expressed TAP-tagged PB1 and PA in 293T cells. Although we found that PB1 could pull down PA from cell lysates efficiently or vice versa (data not shown), the resulting dimeric complexes failed, under our conditions, to bind the RNA promoter in a UV cross-linking assay, and they also failed to assemble with PB2 (data not shown). Perhaps our failure to assemble a functional PB1-PA dimeric complex in vitro is due to the presence of host factors (e.g., Hsp90 [26]) which bind to individual subunits and might interfere with the assembly. Alternatively, host factors, required for
assembly of the PB1-PA dimeric complex, may be depleted in our partially purified polymerase preparations.

Efforts have been made before to reconstitute the viral RNA polymerase complex from monomeric subunits in vitro. Assembly was carried out either by a thioredoxin renaturation protocol of SDS-polyacrylamide gel-purified polymerase subunits from virion RNP complex (33) or by mixing the three individual subunits expressed in a baculovirus expression system in a urea solution and then dialyzing against a reconstitution buffer (21). However, the authenticity of the former report (33) remains in doubt, since their results were not confirmed in our laboratory (G. Brownlee, unpublished data). Moreover, the authors of the latter report could not themselves exclude “an imperfect reconstitution under the conditions employed,” since no cap-dependent transcription was observed (21).

In summary, we have developed an in vitro reconstitution system of influenza A virus polymerase complex from coexpressed PB1-PA dimer and individually expressed PB2 monomer. We have also demonstrated that the PB1-PA dimer can bind to the influenza vRNA promoter prior to its assembly with PB2 to form a functional polymerase. This raises the possibility that the PB1-PA dimeric complex could be present
FIG. 4. Model for assembly of the influenza virus RNA polymerase complex. In vivo evidence from reference 13 is showed by dotted arrows. Bold arrows emphasize the in vitro evidence for polymerase assembly described in this paper. The promoter is shown binding to both the PB1-PA dimer and the trimeric complex (see the text for full details).

We thank Inglis and T. Jung for antibody, B. Thomas and A. Akoulitchev for liquid chromatography/tandem mass spectrometry data, A. Willis for matrix-assisted laser desorption ionization–time of flight data, and F. Vreede for helpful discussions. This study was supported by MRC grants (G9523972 and G9901312 to G.G.B. and a senior nonclinical fellowship, G117/457, to E.F.) and an ORS award to T.D.

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