The influenza virus polymerase complex, consisting of the PA, PB1, and PB2 subunits, is required for the transcription and replication of the influenza A viral genome. Previous studies have shown that PB1 serves as a core subunit to incorporate PA and PB2 into the polymerase complex by directly interacting with PA and PB2. Despite numerous attempts, largely involving biochemical approaches, a specific interaction between PA and PB2 subunits has yet to be detected. In the current study, we developed and utilized bimolecular fluorescence complementation (BiFC) to study protein-protein interactions in the assembly of the influenza A virus polymerase complex. Proof-of-concept experiments demonstrated that BiFC can specifically detect PA-PB1 interactions in living cells. Strikingly, BiFC demonstrated an interaction between PA and PB2 that has not been reported previously. Deletion-based BiFC experiments indicated that the N-terminal 100 amino acid residues of PA are responsible for the PA-PB2 interaction observed in BiFC. Furthermore, a detailed analysis of subcellular localization patterns and temporal nuclear import of PA-PB2 binary complexes suggested that PA and PB2 subunits interacted in the cytoplasm initially and were subsequently transported as a dimer into the nucleus. Taken together, results of our studies reveal a previously unknown PA-PB2 interaction and provide a framework for further investigation of the biological relevance of the PA-PB2 interaction in the polymerase activity and viral replication of influenza A virus.
inhibiting influenza virus replication (9). The X-ray structure of the binding pocket for PB1N25 in PA protein has been recently solved by cocrystallography of truncated PA protein (residues 257 to 716) with PB1N25 (14), further validating the importance of the PA-PB1 interaction and polymerase complex assembly in influenza virus replication. Because the RNA polymerase represents a therapeutic target for anti-influenza virus drug development, coupled with recent evidence that it plays a role in viral pathogenesis and transmission of influenza H5N1 virus from poultry to humans (41), a better understanding of protein-protein interactions in the assembly and nuclear import of the polymerase complex is important.

Various techniques, including yeast two-hybrid, coimmunoprecipitation, glutathione S-transferase pull down, and recently developed tandem affinity purification (TAP), have been utilized to detect and study protein-protein interactions in the formation of influenza virus polymerase complex (4, 5, 10, 30, 36, 38, 39, 45, 48). These techniques have been widely used to study protein-interaction networks and are particularly effective in detecting strong protein-protein interactions. Despite their strengths, there are several limitations that have been noticed. For example, in the TAP-based approach, protein-protein interactions may not be detected, as the assay requires cellular disruption and stringent purification steps which might perturb native complexes and prevent the detection of weak or transient interactions. This challenge is also applicable to the popular glutathione S-transferase pull-down and coimmunoprecipitation assays. The yeast two-hybrid technique is capable of detecting weak and transient interactions, but this system requires the fusion proteins to be imported to the nucleus, which may limit sensitivity for probing interactions which occur in the cytoplasm.

Bimolecular fluorescence complementation (BiFC) has emerged as a promising technique to detect in vivo weak or otherwise-transient protein-protein interactions (24). BiFC is based on the principle that N- and C-terminal fragments of fluorescent proteins (green fluorescent protein [GFP] and its derivatives) alone do not fluoresce. However, if fused to interacting proteins the two nonfunctional halves can be brought into close proximity and form a functional fluorophore as a result of the specific protein interactions. Thus, through BiFC, the specific protein-protein interactions can be visualized, quantified, and localized within live cells. It is worthwhile to mention that BiFC can be used not only to detect protein-protein interactions but also to effectively measure spatial and temporal changes in protein complexes responding to small molecule inhibitors that activate or inhibit particular cellular pathways (18–20, 27, 28, 46, 47).

The purpose of the experiments described in this report was to develop and utilize BiFC to study in vivo protein-protein interactions in the assembly of the influenza virus A polymerase complex. The PA-PB1 interaction has been selected as a proof-of-concept study, as this interaction has been well-established and interaction domains have been characterized. We also sought to determine if additional interactions could be detected with this recently developed approach.

**MATERIALS AND METHODS**

BiFC plasmid construction. A list of BiFC plasmids expressing fusion proteins of influenza A virus polymerase is summarized in Fig. 1. Overlapping PCR was used to produce these BiFC fusion constructs (21). PA, PB1, and PB2 expression plasmids derived from influenza H1N1 A/WSN/33 virus reverse genetics systems were kindly provided by Erich Hoffmann at St. Jude Children’s Research Hospital, Memphis, TN (16). For the construction of BiFC plasmids, sequences encoding the amino (residues 1 to 173 [VN]) or carboxyl (residues 155 to 238 [VC]) fragments of Venus fluorescence protein were fused to the C terminus of PA polymerase proteins (PA, PB1, and PB2). The L indicates a linker sequence inserted between Venus fragments and proteins of interest. A Δ represents a stretch of residues that is deleted in the fusion constructs, while a solid line indicates that polymerase proteins with internal deletions are fused in frame with protein linkers and Venus fragments downstream. PA−VC fusion protein expresses the N-terminal domain of PA and VC fragment of Venus.

![Fig. 1. Schematic diagram of BiFC fusion proteins used in this study. Venus fragments VN (N-terminal 173 residues) and VC (C-terminal 83 residues) are fused in frame to the C terminus of WT or mutant polymerase proteins (PA, PB1, and PB2). The L indicates a linker sequence inserted between Venus fragments and proteins of interest. A Δ represents a stretch of residues that is deleted in the fusion constructs, while a solid line indicates that polymerase proteins with internal deletions are fused in frame with protein linkers and Venus fragments downstream. PA−VC fusion protein expresses the N-terminal domain of PA and VC fragment of Venus.](http://jvi.asm.org/)

**TABLE 1**

<table>
<thead>
<tr>
<th>BiFC Plasmid</th>
<th>PA Fragment</th>
<th>PB1 Fragment</th>
<th>VC Fragment</th>
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<td>PA-VN</td>
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3946 HEMERKA ET AL. J. VIROL. ing was also the same for all the images shown in this study.
Photoshop (version 6). All the images were taken under similar experimental equipted with an IX81 microscope. Digital images were processed with Adobe Fluoview FV300 confocal system (Olympus America Inc., Melville, NY) laboratories, Burlingame, CA). Fluorescent imaging of fixed cells was done using a Fluoview FV300 confocal system (Olympus America Inc., Melville, NY) equipped with an IX51 microscope. Digital images were processed with Adobe Photoshop (version 6). All the images were taken under similar experimental conditions (i.e., exposure time, magnification, and intensification), and processing was also the same for all the images shown in this study.  

Luciferase assays. To monitor differences in transfection efficiencies between samples, a Renilla luciferase vector (0.25 µg) was cotransfected into COS-1 cells with selected BiFC construct pairs or control plasmids. The total amount of transfected DNA in each sample was held constant by using an empty vector lacking a coding region to compensate. At 24 h posttransfection, cells were harvested, subjected to a luciferase assay system (Promega, Madison, WI), and read on a BioTek Synergy 2 multidetection microplate reader (BioTek Instruments, Winooski, VT) following the manufacturer’s recommendations. All experiments were measured in duplicate and repeated in triplicate.  

RESULTS  

PA-PB1 interaction. A BiFC assay developed by Hu et al. (19) to study protein-protein interactions was used to guide our design of BiFC PA/PB1 constructs. We selected Venus, a variant of enhanced yellow fluorescent protein, as the fluorescent protein for our assay. Venus was previously demonstrated to be the most fluorescent protein for BiFC analysis of protein-protein interactions and has characteristics such as improved fluorophore maturation and reduced environmental sensitivity, making it ideal for BiFC analysis (18–20, 27, 46). Two split fragments, the N-terminal 173 residues of Venus (VN) and the C-terminal 83 residues of Venus (VC) were used to generate chimeric constructs with the target proteins (PA, PB1, and PB2). A linker sequence was inserted between the influenza virus proteins and the Venus fragments within each of the fusion constructs. This flexible linker facilitates complementation of the split fragments when they are brought into close proximity by the interacting proteins (24, 46). Figure 1 summarizes a list of BiFC constructs that were used in this study.  

To test whether BiFC reveals protein-protein interactions in the influenza A virus polymerase complex, we first studied the PA-PB1 interaction, because this interaction has been well-characterized (10, 12, 14, 35, 38, 39). In light of the fact that several lines of evidence have indicated that GFP fused to the N terminus of either PA or PB1 proteins interferes with native protein function while GFP placed at the C terminus does not do so (8, 30), we generated our BiFC PA and PB1 constructs by fusing the VC and VN fragments to the C-terminal ends of both PA and PB1, and the resulting fusion constructs were designated PA-VC, PA-VN, PB1-VC, and PB1-VN, respectively (Fig. 1). To test protein expression, we transfected COS-1 cells with equal amounts of BiFC plasmids and at 24 h posttransfection examined cell lysates by SDS-PAGE and Western blotting assays with a polyclonal anti-GFP antibody. As demonstrated in Fig. 2A, transfection with fusion constructs resulted in near-equal levels of protein expression with expected molecular weights. Next, we determined whether BiFC can efficiently detect the PA-PB1 interaction in living cells. COS-1 cells were transfected with the PA-VC/PB1-VN combination as well as with PA-VC single plasmid. The total amount of transfected DNA in each sample was held constant by using an empty vector lacking a coding region to compensate for any variations. Flow cytometry analysis was used to determine the number of Venus-expressing cells (fluorescence complementation due to PA-PB1 interaction) relative to the total number of cells, which represents the efficiency of fluorescence complementation of BiFC fusion constructs. Expression of PA-VC alone (Fig. 2B and C) or PA-VN (data not shown) gave no detectable Venus fluorescence in living cells, indicating that the Venus fragments per se have no intrinsic fluorescence. Strong Venus fluorescence was observed, however, when PA-VC and PB1-VN were coexpressed, which allowed for Venus fragment complementation triggered by PA-PB1 interaction (Fig. 2B). Moreover, coexpression of PA-VN with PB1-VC showed similar fluorescence as that with coexpression of PA-VC with PB1-VN (Fig. 2B), indicating exchangeability of the Venus fragments.  

To ascertain whether the expression of fused VN and VC fragments in the context of the BiFC PA/PB1 system showed any obvious effect on the subcellular localization and nuclear import of the PA-PB1 complex, at 24 h posttransfection COS-1 cells transfected with PA-VC and PB1-VN were fixed, stained with DAPI, and examined for the intracellular localization pattern of the PA-PB1 complex by fluorescence microscopy (Fig. 2C). We found that fluorescence, representing the PA-PB1 complex, was distributed throughout the cell but enriched in the nucleus, which is in agreement with the previous reports that the interaction between PA and PB1 proteins induces efficient nuclear accumulation of PA and PB1 proteins (4, 5, 8, 30). Furthermore, our imaging data appeared to support one of the previously proposed models for the polymerase complex assembly (4, 5, 8). This model suggested that PA and PB1 subunits of the viral polymerase interact first in the cytoplasm of infected cells and are then transported as a dimer into the nucleus (4, 5, 8).

To investigate the specificity of BiFC, we studied whether the deletion of the N-terminal 100 amino acids of PB1, encompassing the PA-binding domain, could disrupt the PA-PB1 interaction. The deletion of this region disrupted binding to PA in a yeast two-hybrid system (10, 38, 39), so we sought to compare results obtained using BiFC. For this study, PB1

N

100-VN was generated, in which the N-terminal 100 residues of PB1 were deleted and fused to VN through a linker at its C terminus in an identical manner to wild-type (WT) PB1-VN (Fig. 1). Flow cytometry analysis showed that the deletion of the PA-binding domain specifically inhibited PB1 from binding to PA, as the BiFC PB1

N

100-VN/PA-VC com-
Combination resulted in only about 5% Venus-expressing cells, whereas wild-type PB1-VN pairing with PA-VC gave rise to approximately 25% Venus-expressing cells (Fig. 3A). To rule out possibilities such as aberrant targeting, instability, or misfolding of the PB1/H9004N100-VN construct, which could account for the lack of PA-PB1 interaction, we also tested PB1/H9004N100-VN for its interaction with PB2-VC. To this end, the PB2-VC plasmid was generated by the same strategy as described above (Fig. 1). Because PB1 utilizes its C-terminal region to interact with PB2 (3, 10), previous studies had shown that PB1 mutants with a deletion of the N-terminal PA-binding domain, still efficiently bind to PB2 (3, 10, 36). As expected, interaction between the PB1 and PB2 proteins was not affected by the deletion, because BiFC analysis of the PB2-VC/PB1/H9004N100-VN pair produced about 18% Venus-expressing cells, which was very similar to that observed with the WT PB2-VC/PB1-VN combination (i.e., 21% Venus-expressing cells) (Fig. 3A). In addition, the expression levels of PB1/H9004N100-VN and PB2-VC fusion proteins were similar to that of WT PB1-VN (Fig. 3B) and those observed in Fig. 2A, further confirming the above results. We also found that luciferase activity (from the internal reference plasmid) was highly comparable among samples (Fig. 3B), indicating similar levels of transfection efficiency. The results of our experiments demonstrated the specificity of BiFC to detect the PA-PB1 interaction, supporting the feasibility of the BiFC approach in studying protein-protein interactions involved in the assembly of the influenza A virus polymerase complex.

**PA-PB2 interaction.** So far, we have provided a proof of concept for the utility of the BiFC approach in detecting the PA-PB1 interaction, which had previously been established using alternative techniques (4, 5, 10, 30, 35, 38, 39, 45, 48). As previously discussed, the binding of PB1 to both PB2 and PA forms the functional RNA polymerase complex. In a three-dimensional structural model, extensive contact among the three subunits, including PA and PB2, was revealed (1, 44). However, previous attempts to demonstrate a direct interaction between PA and PB2 by biochemical approaches were not productive (36). Because BiFC is based on the association between fluorescent protein fragments, this association is likely to stabilize interactions between many proteins (24). Thus, we reasoned that BiFC may detect transient and weak interactions.
protein-protein interactions that are not efficiently captured by other assays. Therefore, we applied BiFC to examine whether there is an interaction between PA and PB2 proteins in living cells.

In addition to the PA-VC, PA-VN, and PB2-VC constructs used in the above experiments (Fig. 1), PB2-VN was generated by the same method and used in this study. As demonstrated in Fig. 4A, all constructs were expressed with the anticipated molecular weights. Next, we asked whether BiFC can detect an interaction, if any, between PA and PB2 in living cells. Strikingly, when PA fusion constructs were coexpressed with their corresponding PB2 constructs, we were able to detect a BiFC signal that indicated an interaction between PA and PB2 (Fig. 4B). Interestingly, the PA-VC/PB2-VN pair produced slightly higher BiFC fluorescence than PA-VN/PB2-VC. The discrepancy in fluorescence could not be explained by variability in transfection efficiency, as the levels of luciferase activity among samples were highly comparable (Fig. 4C). Previous studies of protein interactions using BiFC have reported that the orientation of fluorescent fragments in the fusion constructs can affect spatial arrangements of the VN and VC fragments as a result of protein-protein interactions, thereby affecting fluorescence complementation in BiFC (19, 20, 24, 46). Regardless of the mechanisms leading to different complementation efficiencies observed between these two pairs of BiFC PA/PB2 plasmids, we concluded that PA can interact with PB2 in living cells. Considering that the PA-VC/PB2-VN pair constantly produces good fluorescence complementation with high reproducibility, we selected the PA-VC/PB2-VN pair for further analysis. It should be noted that despite PA interacting with PB2, this interaction is generally weak, being about two- to threefold less intense than that observed with PA-PB1 (Fig. 2B) or with PB1-PB2 (Fig. 3A).

The N-terminal domain of PA is responsible for binding of PA to PB2. To investigate the specificity of the interaction and map the domain of the PA protein that mediates the PA-PB2 interaction, we generated two groups of PA-VN deletion mutants (Fig. 1). One group involved truncations from PA’s N termini toward its center, with each containing fragments of PA after the progressive deletion of 100 amino acid residues from the previous construct, which were designated PA\textsuperscript{SN100-VN}, PA\textsuperscript{SN200-VN}, and PA\textsuperscript{SN300-VN}. Another group harbored the truncations from PA’s C termini by using the same deletion strategy and consisted of four deletion mutants: PA\textsuperscript{AC100-VN}, PA\textsuperscript{AC200-VN}, PA\textsuperscript{AC300-VN}, and PA\textsuperscript{AC400-VN}. These deletions from both the N terminus and C terminus encompassed the entire PA protein and allowed us to sufficiently identify the region of PA responsible for the BiFC PA-PB2 interaction. COS-1 cells transfected with deletion mutants expressed similar amounts of fusion proteins relative to the parental PA-VC, and electrophoretic mobilities of these proteins were consistent with the expected sizes of the fusion proteins with the exception of PA\textsuperscript{SN200-VN} (Fig. 5A). Two very close bands of the PA\textsuperscript{SN100-VN} fusion protein were observed, for an unknown reason. We speculated that the lower band may correspond to an in vitro cleavage product derived from the full-length fusion protein, although further study is needed to define the exact mechanism of this cleavage or the presence of two close fusion proteins of PA\textsuperscript{SN100-VN}. For PA\textsuperscript{SN200-VN}, this mutant constantly expressed a
vivo protein-protein interaction assay, such as BiFC, reassay and the potential translation of this phenotype to an in vitro cleavage by the unknown cellular protease (Fig. 5A). Considering that the protein cleavage associated with PA-PB2 or single-plasmid (PA-VC) control (mock). At 24 h posttransfection, cells were harvested and analyzed by flow cytometry to determine the percentage of cells expressing fluorescence above background (mock). Results represent the means of three independent experiments plus standard deviations. (C) Luciferase assay. A Renilla luciferase reporter vector (0.25 µg) was cotransfected into COS-1 cells expressing pairs of BiFC plasmids or a single BiFC plasmid (PA-VC) as indicated. At 24 h posttransfection, cells were lysed to characterize Renilla luciferase reporter activity with a luciferase assay system (Promega) which measured relative light units. Results (in panels B and C) represent the means of three independent experiments plus standard deviations.

Subcellular localization of the BiFC PA-PB2 binary complex in living cells. Finally, we studied the subcellular localization pattern of the PA-PB2 complex that was demonstrated by BiFC. Previous studies showed that PB2 alone localized to the nucleus, while PA alone was observed in both the cytoplasm and the nucleus (4, 5, 8, 30). To determine in detail the subcellular localization pattern of the PA-PB2 complex, we randomly counted 105 Venus-positive cells expressing PA-VC/PB2-VC at 18 h posttransfection and 100 Venus-positive cells each for 24 h and 48 h posttransfection, respectively. Localization patterns (cytoplasm, cytoplasm plus nucleus, and nucleus) were determined and compared among three time points. The number of Venus-positive cells showing each localization pattern was expressed as a percentage of the total counted Venus-positive cells (Fig. 6A). Examples of localization patterns (cytoplasm, cytoplasm plus nucleus, and nucleus) demonstrated by the BiFC PA-PB2 complex are shown in Fig. 6B. Analysis of PA-PB2 complex localization clearly indicated a temporal trend where the PA-PB2 dimer gradually migrated to the nucleus following the formation of the PA-PB2 complex in the

our further studies, but we have interpreted our data involving this mutant with caution.

As demonstrated in Fig. 5B, all four PA-VC mutants with C-terminal deletions maintained substantial interactions with PB2-VN, displaying approximately about 80 to 90% Venus-positive cells produced by WT PA-VC pairing with PB2-VN. In contrast, mutants with N-terminal deletions showed a significant reduction in interaction with PB2-VN. For example, cell cultures transfected with PA-VCN100/PB2-VN only produced about 16% Venus-positive cells relative to the WT PA-VC/PB2-VN combination. The reduction of BiFC fluorescence in all N-terminal deletion constructs was statistically significant, with P values of <0.05, as determined by two-tailed t tests. The amount of luciferase activity was highly comparable among samples (data not shown), indicating that the different levels of BiFC fluorescence observed were not caused by discrepancies in transfection efficiencies. We conclude that the deletion of 100 amino acids at the N terminus of PA is sufficient to disrupt binding of PA to PB2, suggesting that a pocket in PA for interaction with PB2 might be located within the N-terminal 100-amino-acid region of PA.

The PA protein consists of two discrete domains: the N domain (amino acid residues 1 to 256) and the C domain (residues 257 to 716), and it has been shown that the C domain of PA mediates the PA-PB1 interaction (12, 14, 35). To further validate the PA-PB2 interaction that is mediated through the N domain of PA, two approaches were used. The first approach involved testing whether PA with the deletion of the N-terminal 100 amino acids, which is unable to bind to PB2, could retain its ability to interact with PB1. The second approach was to examine whether the N domain of PA could interact with PB2 in the BiFC system. As expected, like WT PA-VC, the PAAN100,VC mutant efficiently interacted with PB1-VN (Fig. 5C). Furthermore, the N-terminal domain of PA (PAN-VC) could interact with PB2 with even a slightly higher efficiency than the full-length PA (Fig. 5D). Taken together, these observations provide further support for our conclusion that PA interacts with PB2 in the BiFC system and this interaction is likely mediated through the N-terminal 100-amino-acid region of PA protein.
cytoplasm. For example, 72% and 53% of Venus-positive cells counted exhibited the cytoplasmic localization of BiFC fluorescence at 18 h and 24 h posttransfection, respectively, whereas after 48 h, only 13% of Venus-positive cells counted expressed the same pattern (Fig. 6A). Conversely, cells exhibiting exclusively nuclear fluorescence became clearly evident only later after transfection (Fig. 6A). At 48 h posttransfection, 35% of Venus-positive cells counted showed exclusive nuclear fluorescence, while 1 to 4% of Venus-positive cells with nuclear accumulation of BiFC signals were present at early time points posttransfection. The subcellular localization pattern of PA-PB2 demonstrated here was similar to what we have observed for BiFC PA-PB1 (Fig. 2 and data now shown) and PB1-PB2 complex (Fig. 7) as well as to the localization patterns of these binary complexes in living cells reported previously (4, 5, 8, 30).

**FIG. 5.** The N-terminal domain of PA is responsible for binding of PA to PB2. (A) Protein expression by COS-1 cells transfected with mutant PA-VC plasmids with deletions in PA, as indicated. Cell lysates were analyzed by Western blotting with anti-GFP antibody. Numbers on the right indicate the migration positions of molecular mass markers (in kilodaltons). An arrowhead in the PA<sub>N200</sub>-VC lane indicates the doublet protein band, while an arrow in the PA<sub>N100</sub>-VC lane indicates a cleavage product of PA<sub>N200</sub>-VC. (B) Flow cytometry analysis of Venus fragment complementation in COS-1 cells transiently transfected with WT PB2-VN pairing with each of the PA-VC deletion mutants or the WT PA-VC plasmid. Cells transfected with the single-plasmid control (PA-VC) and with WT PA-VC/PB2-VN served as negative and positive controls, respectively. At 24 h posttransfection, cells were harvested and analyzed by flow cytometry to determine the percentage of cells expressing fluorescence above background (mock). The number of Venus-positive cells for the WT PA-VC and PB2-VN combination was set at 100%. BiFC fluorescence for each of the PA-VC deletion mutants paired with PB2-VN was normalized by comparison with WT PA-VC/PB2-VN. Two-tailed t tests were used for statistical comparisons of differences among groups. These data represent an average of at least three independent experiments. Experimental variability is indicated by the standard deviation bars. (C and D) Validation of the BiFC PA-PB2 interaction. COS-1 cells were transfected with WT PB1-VN (0.5 μg) together with either WT PA-VC (0.5 μg) or PA<sub>N100</sub>-VC (0.5 μg) (C), or COS-1 cells were transfected with WT PB2-VN (0.5 μg) together with either WT PA-VC (0.5 μg) or PA<sub>N200</sub>-VC (0.5 μg) (D). Transfection of COS-1 cells with PA-VC plasmid (1.0 μg) alone was used as a negative control (mock). At 24 h posttransfection, cells were harvested and analyzed by flow cytometry to determine the percentage of cells expressing higher than background fluorescence (PA-VC). The number of Venus-positive cells by the combination of BiFC WT PA/PB1 (C) or WT PA/PB2 (D) was set at 100%, respectively. BiFC fluorescence for each of the mutant BiFC pairs was normalized by comparison with the corresponding WT BiFC combination.
DISCUSSION

Studies of polymerase complex assembly have greatly contributed to our current understanding of influenza virus genome transcription and replication. However, measuring the dynamics of spatial and temporal macromolecular interactions involving virus and host proteins during the formation of the polymerase complex (PA-PB1-PB2) presents many challenges. A particular difficulty has been the detection of transient pro-

FIG. 6. Subcellular localization of the BiFC PA-PB2 binary complex in living cells. (A) Subcellular localization pattern of the PA-PB2 complex. At 18 h, 24 h, and 48 h posttransfection, cells expressing PA-VC and PB2-VN proteins were fixed, stained with DAPI (blue), counted, and examined for the intracellular localization pattern of the PA-PB2 complex by fluorescence microscopy. The total number of cells expressing Venus was 105 for 18 h, 100 for 24 h, and 100 for 48 h posttransfection. The number of cells showing each localization pattern (cytoplasm, cytoplasm plus nucleus, and nucleus) was expressed as a percentage of the counted Venus-positive cells. (B) Localization of the PA-PB2 complex in COS-1 cells expressing BiFC PA-VC and PB2-VN proteins. COS-1 cells grown on glass coverslips were transfected with a pair of BiFC PA-VC/PB2-VN plasmids or PA-VC plasmid alone. At 24 h posttransfection, cells were fixed, stained with DAPI, and imaged with a confocal microscope at 60× magnification. A representative localization pattern (cytoplasm, cytoplasm plus nucleus, and nucleus) of the PA-PB2 complex is shown.
tein-protein interactions with low affinity that may exist in the assembly of a functional polymerase complex but are not amenable to traditional approaches, such as pull down or GFP-tagged-based imaging experimental systems (4, 5, 8, 23, 30, 36). The advantage of BiFC is its unique capacity to precisely detect weak protein-protein interactions while emitting strong fluorescence, allowing for high spatial and temporal resolution (24, 46). Though only recently introduced, this assay has been used to visualize more than 200 protein interactions in many different cell types and organisms (24, 46). Given the rapid pace of newly emerging fluorophores with improved biophysical properties, BiFC is likely to become even more valuable and commonplace for future studies of protein-protein interactions. Multicolor BiFC analysis and BiFC-based fluorescent resonance energy transfer (FRET) have been recently developed (20, 42, 43), opening more possibilities for monitoring interactions involving multiple proteins within the same cell. As a starting point toward better understanding of influenza virus A polymerase complex assembly, we developed and validated a BiFC approach by first directly visualizing interactions between PA and PB1. The BiFC PA/PB1 combination readily resulted in the emission of fluorescence, whereas a mutant PB1 containing the deletion of the PA-binding domain produced only about 20% of WT BiFC fluorescence, testifying to the assay's specificity. Detection of the PA-PB1 interaction by BiFC is consistent with coimmunoprecipitation and TAP-based affinity purification approaches (4, 5, 36). Furthermore, we noted that the BiFC PA-PB1 binary complex was distributed throughout

![FIG. 7. Subcellular localization of the BiFC PB1-PB2 binary complex in living cells. COS-1 cells grown on glass coverslips were transfected with a pair of BiFC PB1-VN/PB2-VC plasmids or PB1-VN plasmid alone. At 24 h posttransfection, cells were fixed, stained with DAPI, and imaged with a confocal microscope at 60× magnification. A representative localization pattern (cytoplasm, cytoplasm plus nucleus, and nucleus) of the PB1-PB2 complex is shown.](http://jvi.asm.org/)
the cells but mainly located to the nucleus, which was also consistent with previous reports (5, 8). Taken together, these data provide proof of concept that BiFC can be utilized in studying protein interactions in polymerase complex assembly.

It has been demonstrated that individually expressed PB1, PB2, and PA can independently perform nuclear import (22, 29, 32, 34); however, several lines of evidence have indicated that polymerase subunits more likely enter the nucleus either through a PA-PB1 or PB1-PB2 dimeric complex (4, 5, 8, 30). The general consensus is that the PA-PB1 complex is formed in the cytoplasm prior to being imported into the nucleus (4, 5, 8, 30). The location of the initial interaction between PB1 and PB2 in the cell is still controversial (5, 30). By using BiFC, we visualized both PA-PB1 and PB1-PB2 complexes (Fig. 2 and 7). Their trafficking patterns appear to suggest that both complexes are formed first in the cytoplasm and then imported into the nucleus. These data are in agreement with a previous report on the assembly and trafficking of the influenza A virus RNA polymerase complex (30), which suggested that the PB1-PB2 interaction, like PA-PB1, formed a binary complex in the cytoplasm prior to nuclear import.

Most notably, by using BiFC, we observed an interaction between PA and PB2 proteins that has not been reported previously, although we cannot rule out that the interaction of PA with PB2 is indirect and mediated by the cellular factor(s) that binds both PA and PB2 proteins. The subcellular localization pattern and nuclear import of the BiFC PA-PB2 complex is similar to those of PA-PB1 (Fig. 2) and PB1-PB2 (Fig. 7). Because all three pairs of polymerase subunits appeared to interact with each other in the cytoplasm and also followed the similar trend in the nuclear import, we favored the polymerase assembly model originally proposed by Fodor and colleagues, in that PB1, PB2, and PA interact first in the cytoplasm and are then transported into the nucleus as a trimeric complex (8). With multicolor BiFC analysis or a BiFC-based FRET approach, we can test and verify this model in our future studies and address the sequential nature and dynamics of nuclear import in terms of the protein-protein interactions in the polymerase assembly. We view this model as more appealing because it can take into account the following observations: first, after virus infection the incoming RNPs appear to enter the nucleus as a complex rather than each protein isolated (3); second, there is growing evidence that influenza virus polymerase subunits can form a heterotrimer in vivo (23); third, PA-PB1 or PB1-PB2 or PA-PB2 interactions described in this report all occur initially in the cytoplasm prior to their nuclear import; finally, this model could also reconcile the discrepancy presented by two current assembly models (5, 30).

The PA-PB2 interaction we detected by BiFC is relatively weak, showing about two- to threefold less intensity than those observed in the PA-PB1 and PB1-PB2 interactions. A weak interaction between the PA and PB2 protein cannot be fully explained by the expression levels of BiFC PA/PB2 fusion proteins, as they are highly comparable with the BiFC proteins utilized for PA-PB1 interaction studies (Fig. 2, 4, and 5). It is worthwhile to mention that the weak PA-PB2 interaction we observed in BiFC may not truly reflect its native interaction in the context of a trimeric PA-PB1-PB2 complex. It could be possible that interactions between PA-PB1 and PB1-PB2 trigger conformational changes, either in PA or PB2 or both proteins, that in turn promote PA-PB2 interaction. Alternatively, a weak PA-PB2 interaction might be interpreted as needed for ready modulation of structural changes of the PA-PB1-PB2 trimeric complex that accommodate its various functions in virus genome transcription and replication. The low-affinity interaction between PA and PB2 observed in this study may help to explain why various attempts in the past to elucidate this interaction using biochemical approaches were not productive (36), as upstream assay processing may interfere with the native PA-PB2 complex, preventing subsequent detection of the weak interaction. It should be noted that using GFP-tagged PA coupled with nontagged PB2, a live cell imaging study similar to the BiFC assay in concept, Fodor and colleagues found that PB2 had no effect on the nuclear localization of PA-GFP, concluding there was no obvious interaction between these two proteins (8). However, by carefully comparing fluorescence imaging data of cells transfected with PA-GFP/PB2 to those of cells transfected with PA-GFP alone, as shown in that earlier report (8), we noticed that the expression of PB2 triggered some nuclear localization of PA protein, indicating that PB2 may interact with PA, probably at a low level in that study. We do not fully understand the reason for this difference in detecting the PA-PB2 interaction between GFP-tagged imaging and BiFC assays, but we propose that it could be due to the sensitive nature of BiFC in detecting weak protein-protein interactions, as the reconstituted Venus can stabilize low-affinity interactions, such as the PA-PB2 complex.

Alternatively, it is possible that PA-GFP displayed no obvious interaction with PB2 because the GFP tag potentially imposed some steric hindrance on the PA-PB2 interaction, which has been clearly demonstrated in detecting the PA-PB2 interaction by BiFC, even involving fragments rather than the whole molecule of a fluorescent protein (Fig. 4). The observation that PA interacts with PB2 in the absence of PB1 raises an interesting question of whether the PA-PB2 dimer could perform any polymerase activity independent of the intact heterotrimeric complex. Several studies have shown that the PB1-PA binary complex can efficiently synthesize cRNA in the absence of PB2 protein (17, 31). Future studies are needed to address the role the PA-PB2 dimer can play in virus polymerase activity.

In this study, we have identified the N-terminal 100-amino-acid region within the PA protein as a determinant of interaction with PB2. We noticed that this defined region does not overlap with other regions of PA required for interaction with PB1 (3). Interestingly, a large-scale sequence comparison among the PA genes of influenza A virus subtypes (15) recently identify a number of highly conserved amino acids (residues 29 to 54) within the N-terminal 100-amino-acid region of PA that could be implicated in binding the PB2 protein. It will be important to determine in the future whether these conserved residues represent contact sites of PA interaction with PB2. The precise function of the N-terminal 100-amino-acid region of PA is not well-established, although limited mutagenesis studies have indicated that this region may play a role in viral RNA synthesis and viral assembly (13, 40). The results of our current study suggest a previously unrecognized role for PA, particularly this region, in the assembly of viral polymerase complex. While it is conceivable that the PA-PB2 interaction may allow for the efficient assembly of a heterotrimer PA-PB1-PB2 complex, future studies are needed to establish its biolog-
ical relevance in the polymerase activity and viral replication. Further investigation of the PA-PB2 interaction may also aid in the elucidation of a novel therapeutic target that could be explored in the design of a new generation of anti-influenza drugs that could inhibit polymerase assembly and activity.

ACKNOWLEDGMENTS

We thank Philip Hardwidge and members of the Li lab for helpful discussions and critical reviews of the manuscript. We acknowledge use of the SDSU-Functional Genomics Core Facility, supported in part by NSF/EPSCoR grant no. 0901948, the Center of Excellence in Drought Tolerance through the South Dakota 2010 Initiative, and the South Dakota Agricultural Experimental Station. We also thank Michael Hildreth for his expertise in confocal microscopy.

This work was supported by the South Dakota 2010 Initiative through the Center for Infectious Disease Research and Vaccinology and Public Health Service grant AI078177 (F.L.) from NIAID.

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