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Characterization of Influenza Virus PB1 Protein Binding to Viral RNA: Two Separate Regions of the Protein Contribute to the Interaction Domain

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The interaction of the PB1 subunit of the influenza virus polymerase with the viral RNA (vRNA) template has been studied in vitro. The experimental approach included the in vitro binding of labeled model vRNA to PB1 protein immobilized as an immunoprecipitate, as well as Northwestern analyses. The binding to model vRNA was specific, and an apparent K_d of about 2×10^{-8} M was determined. Although interaction with the isolated 3′ arm of the panhandle was detectable, interaction with the 5′ arm was prominent and the binding was optimal with a panhandle analog structure (5′+3′ probe). When presented with a panhandle analog mixed probe, PB1 was able to retain the 3′ arm as efficiently as the 5′ arm. The sequences of the PB1 protein involved in vRNA binding were identified by in vitro interaction tests with PB1 deletion mutants. Two separate regions of the PB1 protein sequence proved positive for binding: the N-terminal 83 amino acids and the C-proximal sequences located downstream of position 493. All mutants able to interact with model vRNA were capable of binding the 5′ arm more efficiently than the 3′ arm of the panhandle. Taken together, these results suggest that two separate regions of the PB1 protein constitute a vRNA binding site that interacts preferentially with the 5′ arm of the panhandle structure.

The genomes of influenza A viruses, members of the *Orthomyxoviridae* family, consist of eight single-stranded RNA segments of negative polarity. They encode 10 proteins, since the two smallest RNAs have the genetic information for two different products by differential splicing (for reviews, see references 23 and 25). These RNA segments are associated with the nucleoprotein (NP) and the three P proteins (PB2, PB1, and PA) to form ribonucleoprotein (RNP) complexes (reviewed in references 23 and 25). Both transcription and replication take place in the nucleus of the infected cells (18, 20). The initiation of mRNA synthesis involves a cap-stealing mechanism by which cellular capped heterogeneous nuclear RNAs are used to generate primers that are elongated by the viral transcriptase (24). Termination and polyadenylation occur at an oligonucleotide U signal that is adjacent to the RNA panhandle structure at the 5′ terminus of the viral RNA (vRNA) template (28, 42) and may require interaction of the polymerase with the conserved 5′-terminal sequences of the template (41). vRNA replication involves the generation of a full-length RNA copy of positive polarity (cRNA) that is encapsidated with NP molecules and is used as an intermediate for the synthesis of vRNA progeny molecules (17).

The viral polymerase consists of a heterotrimer formed by the PB1, PB2, and PA proteins (7, 8, 19, 21). All three subunits are required for viral RNA replication (38). Various experiments have clarified the roles of each subunit in the transcription and replication processes. Thus, the PB2 subunit is a cap-binding protein (4, 48, 51) and may contain the cap-dependent endonuclease activity. Thus, antibodies specific for PB2 protein inhibit this activity (27) and cap primer-dependent in vitro RNA synthesis is affected by mutations in the PB2 gene (37). Nevertheless, both transcription and cap-dependent endonuclease activity require the presence of the three subunits of the polymerase and the RNA template (6, 16). Much less is known about the possible function of the PA subunit. The phenotypes of temperature-sensitive (*ts*) mutants (reviewed in reference 29) suggest its involvement in vRNA synthesis. The PA subunit is a phosphoprotein (45) whose expression by transfection leads to the degradation of coexpressed proteins (44). The regions of the PA subunit responsible for this activity map to the amino-terminal third of the protein (46), close to the nuclear localization signal (NLS) (34). The PB1 protein harbors the polymerase activity. It can be cross-linked to the triphosphate substrate (2, 5). It contains amino acid motifs present in other RNA-dependent RNA polymerases (40), and mutation of the conserved residues abolishes the transcriptional activity (3). Furthermore, extracts from baculovirus-infected cells expressing PB1 protein show some polymerase activity in vitro (22). The locations of the NLS and the putative nucleotide-binding domains have been described (1, 33), and the protein domains involved in the interaction with the other subunits of the polymerase have been mapped (15, 39, 50).

The interaction of the polymerase with vRNA template has been studied with virion cores or the enzyme complex reconstituted by coexpression of the subunits from recombinant vaccinia viruses. Both PB1 and PB2 subunits of the virion core could be cross-linked to the 3′-terminal sequence of the vRNA (12), and all three subunits were cross-linked to the vRNA 5′-terminal sequence (10). The enzyme complex bound to the 5′-terminal sequence with higher affinity than to the 3′-terminal one (49). In this report we have studied the interaction of the isolated PB1 subunit with the vRNA template. The individual PB1 protein bound specifically vRNA, with an apparent K_d of approximately 2×10^{-8} M. While PB1 binding was more efficient to the 5′ arm than to the 3′ arm of the panhandle, it was maximal when a 5′+3′ full panhandle analog structure was used. Regions of the protein corresponding to the N terminus and the C terminus appeared to be involved in binding.

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MATERIALS AND METHODS

Biological materials. The COS-1 cell line (14) was provided by Y. Gluzman and was cultivated as described earlier (35). The recombinant vaccinia virus vTF7-3 (13) was a gift of B. Moss. Generation of the VPB1 recombinant vaccinia virus has been reported earlier (45). It contains the PB1 gene under the control of a T7 promoter, downstream of the encephalomyocarditis virus internal ribosome entry site. The origin of plasmids pGPB1, pGPB1Δ84–757, pGPB1Δ394–757, pGPB1Δ1–69, pRB1Nter (pRPB1Δ267–757), and pRB1Cter (pRPB1Δ1–493) has been described previously (15, 32). Plasmid pRPB1267–493 was constructed by cloning an *Hin*dIII fragment of the PB1 gene (positions 831 to 1516) into the *Hin*dIII site of pRSET-C. The antiserum specific for the N-terminal region of PB1 protein was prepared by immunization of rabbits with purified HisB1Nter protein (15). A general anti-PB1 protein serum was prepared by immunization of rabbits with purified PB1 protein obtained by isolation from sodium dodecyl sulfate (SDS)-polyacrylamide gels. The antiserum specific for His-NS1 protein has been described previously (30).

Transfection. Cultures of COS-1 cells growing in 35-mm dishes were infected with vTF7-3 virus at a multiplicity of infection of 5 to 10 PFU per cell. After virus adsorption for 1 h at 37°C, the cultures were washed with Dulbecco modified Eagle medium (DMEM) and transfected with 10 μg of pGPB1, with mutants thereof or pGEM3 as a control. The DNAs were diluted to 100 μl of DMEM and, in a separate tube, cationic liposomes (2 μl per μg of DNA) were diluted to 100 μl in DMEM. The contents of both tubes were mixed, kept at room temperature for 15 min, and added to the culture plates containing 1 ml of DMEM. Cationic liposomes were prepared as described previously (43).

RNA probe labeling. The synthesis of vNSZ probe, which contains a deleted version of the chloramphenicol acetyltransferase gene in negative polarity with the termini of the NS segment, was carried out as described earlier (38), with [^{32}P]GTP as a precursor. For transcription of the 5′-arm and 3′-arm probes, the strategy described by Seong and Brownlee (47) was used. Oligodeoxynucleotides 5′-CACCCTTGTTTCTACTCCTATAGTGAGTCGTATTAACC-3′ and 5′-AGCAAAAGCAGGGTGCCTATAGTGAGTCGTATTAACC-3′, which contain the T7 promoter (underlined) fused to the 5′-arm and 3′-arm template sequences, respectively, were annealed to a T7 promoter complementary oligodeoxynucleotide (5′-GGTTAATACGACTCACTATAGG-3′). Such DNA templates were transcribed with T7 RNA polymerase as described earlier (47) to yield the 18- or 17-nucleotide-long 5′-arm or 3′-arm probes. Two control probes were generated: plasmid pGEM4 was digested with *Sma*I and transcribed with T7 RNA polymerase to produce a short (26-nucleotide) unspecific probe (G4S probe). In addition, a 330-nucleotide probe (G3N probe) was synthesized by transcription with T7 polymerase of pGEM3 plasmid digested with *Nhe*I.

RNA analyses. For in vitro binding of the labeled probes to PB1 protein, cultures of COS-1 cells were transfected with pGPB1 plasmid, or pGEM3 as a control, and labeled with [^{35}S]methionine-[^{35}S]cysteine as described below. Soluble extracts were used for immunoprecipitation with 10 μl of a matrix of anti-PB1Nter immunoglobulin G (IgG) (or anti-HisNS1 IgG for His-tagged PB1 mutants) bound to protein A-Sepharose. After the immune complexes were washed three times with radioimmunoprecipitation assay buffer, the resin was washed seven times with TNE–NP-40 buffer (100 mM NaCl–1 mM EDTA–50 mM Tris-HCl–1% Nonidet P-40, pH 7.5) containing 100 μg of yeast RNA per ml. For binding, the immune complexes were incubated with about 30,000 cpm of probe (1 to 20 fmol, depending on the probe) in TNE–NP-40 buffer containing 25 ng of yeast RNA for 1 h at 4°C. After three washes with TNE–NP-40 buffer, the radioactivity retained in the resin was determined by Cerenkov counting. The resin was split into two identical portions that were used to analyze the bound RNA and the protein content. The bound RNA was isolated by boiling with TNE buffer containing 0.5% SDS and incubation with 50 μg of proteinase K per ml for 30 min at 56°C in the same buffer. After phenol extraction, the eluted RNA was precipitated with ethanol and analyzed by electrophoresis on a 4% or an 18% polyacrylamide sequencing gel, depending on the probe. The protein present in the immune complexes was extracted in Laemmli sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography. The quantitation of both RNA and protein was performed in a phosphorimager. As a standard for quantitation of PB1 concentrations, we used a total extract of cells doubly infected with vTF7-3 and VPB1 viruses and labeled with [^{35}S]methionine-[^{35}S]cysteine as indicated below. Bovine serum albumin was used as a standard for protein concentration after Coomassie blue staining.

Northwestern assays were carried out with extracts of COS-1 cells doubly infected with vTF7-3 and VPB1 viruses or singly infected with vTF7-3 virus as a control, as well as with extracts from cells infected and transfected with pGPB1 plasmid or mutants thereof. These extracts were prepared in sample buffer (8% glycerol–0.1% SDS–1 mM dithiothreitol–0.1% bromophenol blue–12 mM Tris-HCl, pH 6.8, in phosphate-buffered saline) by heating for 10 min at 30°C and centrifugation for 5 min at 10,000 × *g* and 4°C. The samples were separated by SDS-PAGE and transferred to nitrocellulose filters in Tris-glycine buffer. The filters were incubated for 4 h at room temperature or overnight at 4°C in renaturation buffer (50 mM NaCl–1 mM EDTA–0.02% concentrations each of Ficoll, bovine serum albumin, and polyvinylpirrolidone–0.1% Triton X-100–10 mM Tris-HCl, pH 7.5) and further incubated in the same buffer containing labeled 5′ probe or G4S probe in the presence of 1 μg of yeast RNA per ml. After three 30-min washes at room temperature with renaturation buffer, the filters were autoradiographed. The filters were further processed by Western blotting with anti-PB1 serum as indicated below.

Protein analyses. Labeling in vivo of PB1 protein or its mutant derivatives was carried out as follows. At 6 h posttransfection or at 1 h postinfection, infected-transfected cultures or cultures infected with vTF7-3 and VPB1 viruses were washed and starved for 1 h in Met-Cys-deficient DMEM. At this point, [^{35}S]methionine-[^{35}S]cysteine was added to a final concentration of 200 μCi/ml in DMEM containing one-tenth the normal Met-Cys concentration, and the cultures were incubated for 16 to 20 h. Soluble extracts were prepared by lysis of the culture in TNE buffer containing 0.5% deoxycholate and centrifugation for 10 min at 10,000 × *g* and 4°C. Total extracts were prepared in Laemmli sample buffer.

Western blotting was done as described earlier (31). In brief, cell extracts were processed by SDS-PAGE and transferred to Immobilon filters; the membranes were then saturated with 3% bovine serum albumin for 1 h at room temperature. The filters were incubated with a 1:1,500 dilution of the anti-PB1 serum for 1 h at room temperature. The filters were washed two times for 30 min with phosphate-buffered saline containing 0.25% Tween 20 and were incubated with a 1:10,000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase. Finally, the filters were washed two times for 30 min as described above and developed by enhanced chemiluminescence.

RESULTS

The PB1 subunit of the polymerase binds specifically viral RNA. The capacity of isolated PB1 subunit to interact with model virion RNA was determined by an in vitro binding test. PB1 protein was expressed from cloned DNA by double infection with VPB1 and vTF3-7 vaccinia virus recombinants. As a control, single infection with recombinant vaccinia virus vTF3-7 was used. The proteins synthesized after infection were labeled continuously with [^{35}S]methionine-[^{35}S]cysteine. Extracts from infected cells were immunoprecipitated with an antiserum specific for the N-terminal region of PB1 protein. The immunoprecipitates were used as a solid phase for binding of radiolabeled vNSZ probe, a model vRNA with the NS segment termini and a deleted *cat* gene in negative polarity (38), or G3N probe as a control, in the presence of a 250-fold excess of total yeast RNA. After the unbound probe was washed away, the complexes were split into two identical fractions. The bound RNA was isolated from one of them and analyzed by gel electrophoresis, while the other fraction was used to analyze the protein present in the immunoprecipitate. The results are presented in Fig. 1. The PB1-specific immunoprecipitates (B1-IPP; Fig. 1A, bottom panel) were able to retain the vNSZ probe but were not capable of binding the G3N probe. In contrast, the control immunoprecipitates (CTRL-IPP; Fig. 1A, bottom panel) were unable to retain either probe (Fig. 1A, upper panels). To confirm the specificity of the binding of PB1 protein to vRNA, competition experiments were carried out. Increasing amounts of unlabeled vNSZ RNA were mixed with a constant dose of radiolabeled probe, which included excess yeast RNA as indicated above, and were incubated with PB1-specific immunoprecipitates. The results of the probe binding are presented in Fig. 1B, upper panel, and the protein present in the immunoprecipitates is shown in Fig. 1B, lower panel. The presence of increasing amounts of unlabeled vNSZ probe led to a reduction of the label retained in the complex, as expected for a specific binding, although equal amounts of PB1 protein were present in the immunoprecipitates (Fig. 1B, lower panel).

PB1 protein binds preferentially to the 5′ arm of the panhandle. It has been reported that the polymerase complex interacts with virion RNA through the panhandle region (10, 12, 49). To study the contribution of isolated PB1 to such a interaction, binding assays were carried out in which short probes corresponding to the 5′ arm or the 3′ arm of the panhandle were used instead of the vNSZ model RNA. The 5′ probe was efficiently retained by the PB1 immunoprecipitate

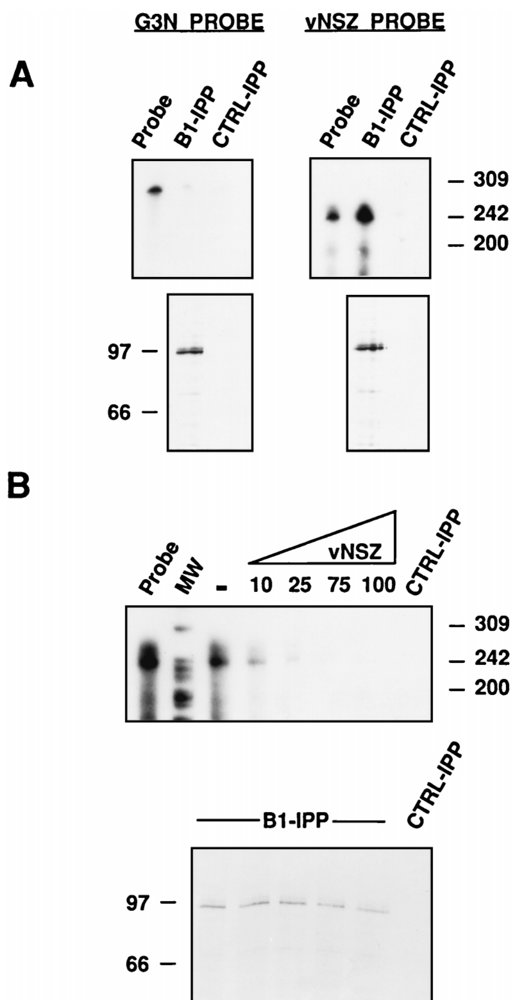

FIG. 1. Specificity of PB1 binding to vRNA. Cultures of COS-1 cells were doubly infected with vTF7-3 and VPB1 viruses or singly infected with vTF7-3 virus as a control. Newly synthesized proteins were labeled with [^{35}S]methionine-[^{35}S]cysteine, and soluble extracts were prepared as described in Materials and Methods. (A) The extracts were used for immunoprecipitation with an anti-PB1 antiserum, and the immunoprecipitates (B1-IPP or CTRL-IPP) were incubated with ^{32}P-labeled vNSZ probe or control G3N probe (specific activity of ca. 10^8 cpm/μg) in the presence of a 250-fold excess of yeast RNA. The RNA from a fraction of the complexes was isolated and analyzed by denaturing gel electrophoresis (top panels), while the protein present was isolated and visualized by SDS-PAGE and autoradiography (bottom panels). (B) The binding assay was performed as indicated for panel A except that increasing amounts of unlabeled vNSZ probe (10, 25, 75, or 100 ng; i.e., a 100- to 1,000-fold molar excess) were included in the assay. The RNA retained was isolated and analyzed as indicated above (top panel). The proteins present in the immunoprecipitates are shown in the bottom panel. Numbers to the left indicate the sizes of the protein markers in kilodaltons. Numbers to the right indicate the length of RNA markers (MW) in nucleotides. In this and in the subsequent figures, the lanes indicated as probe show one-tenth of the amount of probe that was included in the binding test.

but not by the control immunoprecipitate. The 3′ probe was only marginally retained (Fig. 2A). The binding was specific, since G4S probe, an unrelated probe of similar length, was not retained (data not shown). When a mixture of 5′ and 3′ probes was used for binding, both probes were similarly retained, and the efficiency of binding was slightly increased compared with the binding of the 5′ probe alone (compare center and right panels in Fig. 2A). These experiments indicated that the PB1-specific immunoprecipitates specifically bind to the vRNA panhandle, preferentially via its 5′ arm, but they do not certify unambiguously that PB1 protein interacts directly with the RNA probe. To study this point, Northwestern analyses were carried out in which extracts of COS-1 cells doubly infected with vTF7-3 and VPB1 vaccinia viruses or singly infected with vTF7-3 virus were used. The extracts were separated by SDS-PAGE and blotted with the 5′-arm probe or the G4S probe. The results are presented in Fig. 3. A main labeled band was detected with the specific probe and had the mobility expected for PB1 protein. Such a band was not detectable with the unrelated G4S probe. The same filter was blotted with a PB1-specific antiserum to verify the identity of the protein. As presented in Fig. 3, a band recognized by the PB1-specific antiserum was apparent, with the same mobility as the radiolabeled band, indicating that PB1 itself is responsible for the probe binding.

The affinity of PB1 protein binding to the vNSZ probe was estimated by using in vitro binding assays in which increasing amounts of PB1-containing extracts were used to prepare the immunoprecipitates. The absolute quantity of PB1 protein present in each immunoprecipitate was determined as follows. As indicated above, the expression of PB1 protein was carried out by double infection of COS-1 cells with vTF3-7 and vPB1 vaccinia viruses, and the infected cells were labeled continuously with [^{35}S]methionine-[^{35}S]cysteine. Two kinds of extracts were prepared: a soluble extract to be used for immunoprecipitation and a total cell extract to determine the specific activity of the PB1 protein. The radioactivity present in the PB1-specificc band of each immunoprecipitate was quantitated in a phosphorimager in parallel with the radioactivity associated with the PB1 protein present in the total cell extract. The determination of the absolute amount of PB1 protein in the total cell extract was done by Coomassie blue staining of the same gel with bovine serum albumin as a standard. The aggregate results obtained in three independent experiments are presented in Fig. 4. Although the concentrations of PB1 protein used were not sufficient to reach a saturation plateau, an apparent K_d of about 2×10^{-8} M was obtained.

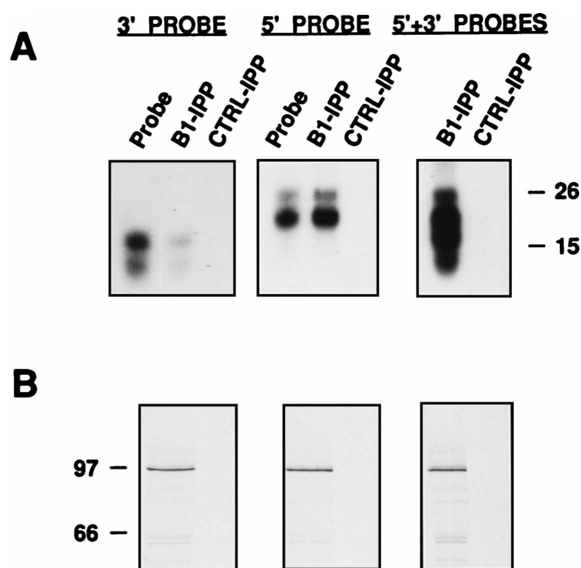

FIG. 2. Interaction of PB1 protein with the 5′ and the 3′ arms of the panhandle. In vitro RNA-binding assays were carried out as described in Materials and Methods and in the legend to Fig. 1 except that 3′ probe, 5′ probe, or a mixture of both was used. (A) Analysis of the RNA retained. (B) Analysis of the proteins present in the immunoprecipitates. Numbers to the left indicate the size of the protein markers in kilodaltons. Numbers to the right indicate the length of the RNA markers in nucleotides.

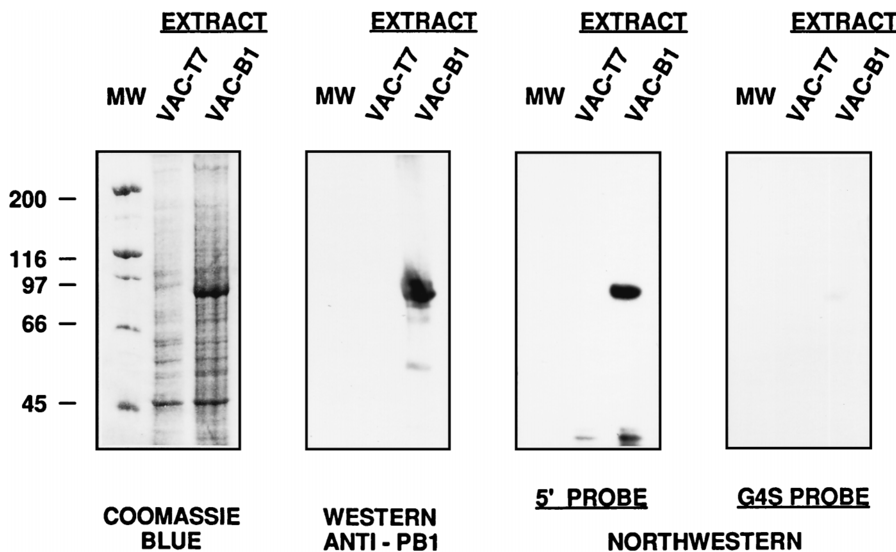

FIG. 3. Northwestern assays. Cultures of COS-1 cells were doubly infected with vTF7-3 and VPB1 viruses (VAC-B1) or singly infected with vTF7-3 virus (VAC-T7) as a control. Extracts prepared as indicated in Materials and Methods were separated by SDS-PAGE and transferred to nitrocellulose filters. After renaturation, the filters were incubated with 5′ probe or with the control G4S probe. After autoradiography, the filters were developed by Western blotting with an anti-PB1 serum and, finally, the filters were stained with Coomassie blue. Numbers to the left indicate the sizes of the protein markers (MW) in kilodaltons.

Mapping the PB1 protein sequences required for viral RNA binding. Next, we tried to identify the regions of the PB1 protein involved in binding to the template vRNA. To do this, in vitro interaction tests were carried out in which we used extracts from COS-1 cells transfected with either pGPB1 plasmid, a series of plasmids encoding deleted PB1 mutants (15), or pGEM plasmid as a control. The PB1 subunit or its mutant derivatives were immunoprecipitated with a PB1-specific antiserum or with a His-NS1 antiserum (His-tagged PB1 mutants PB1Δ267–757, PB1Δ1–493, and PB1-267–493), and the immunoprecipitates were used as a solid phase for binding to the vNSZ probe as indicated above. The results are presented in Fig. 5. Mutants PB1Δ267–757 and PB1Δ1–493 proved positive for probe retention, while mutant PB1-267–493 was unable to bind vNSZ probe. The binding capacity of the N-terminal region of PB1 protein was confirmed by the results obtained for mutant PB1Δ394–757, which showed a retention similar to that obtained with wild-type PB1 protein. Furthermore, mutant PB1Δ84–757, encoding only the first 83 amino acids of the protein, was almost as active as wild-type PB1 in binding to the vNSZ probe. These results indicate that the N terminus of the protein contains a strong determinant for template binding and that the C-terminal third of the protein also participates in the interaction. This latter conclusion is supported by the fact that mutant PB1Δ1–69, which lacks most of the N-terminal sequences involved in RNA binding, was able to retain vNSZ probe (data not shown).

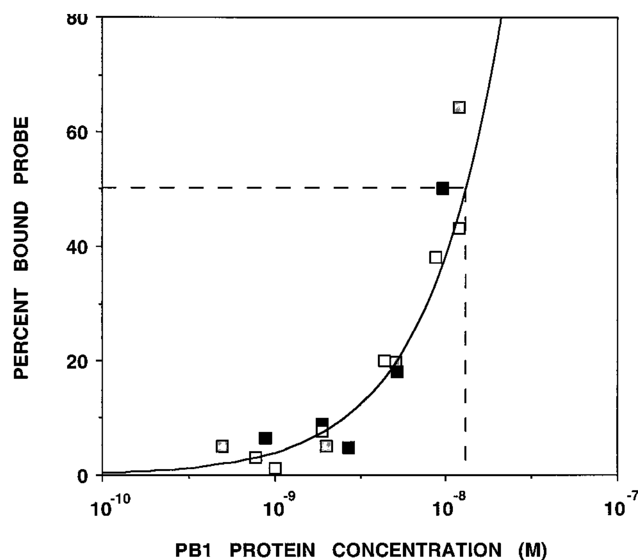

FIG. 4. Affinity of binding of PB1 to vRNA. In vitro RNA-binding assays were carried out as described in the legend to Fig. 1 with vNSZ as a probe. Increasing amounts of VAC-B1 extracts were used, and the actual concentration of PB1 protein present in each assay was determined as indicated in the text. The percentage of probe retained after each washing was determined by Cerenkov counting. Different symbols represent the results of three independent experiments. The curve is the best exponential fit of the data.

To test whether each RNA-binding region of PB1 protein interacts with both arms of the panhandle, the mutant versions of PB1 protein able to bind vNSZ probe were assayed for interaction in vitro with either the 5′ probe, the 3′ probe, or the mixture of both probes. The results are presented in Fig. 6. Every one of the mutants tested for vNSZ RNA binding was able to interact with the 5′ probe (Fig. 6A), as well as the 3′ probe to a much lower extent (Fig. 6B), and induced the increase in the retention of the 3′ probe by the presence of the 5′ probe (Fig. 6C). These results suggest that both RNA-binding regions of the protein interact predominantly with the 5′ arm of the panhandle.

DISCUSSION

The promoter region of vRNA was first located at the 3′-terminal sequences of the molecule (36, 47), but it was later shown that both 5′- and 3′-terminal sequences were required for efficient transcription of vRNA (10, 16). Mutational analyses have suggested a number of models for the promoter structure (9–11, 26). In agreement with these studies, the influenza virus polymerase interacts with its template vRNA at

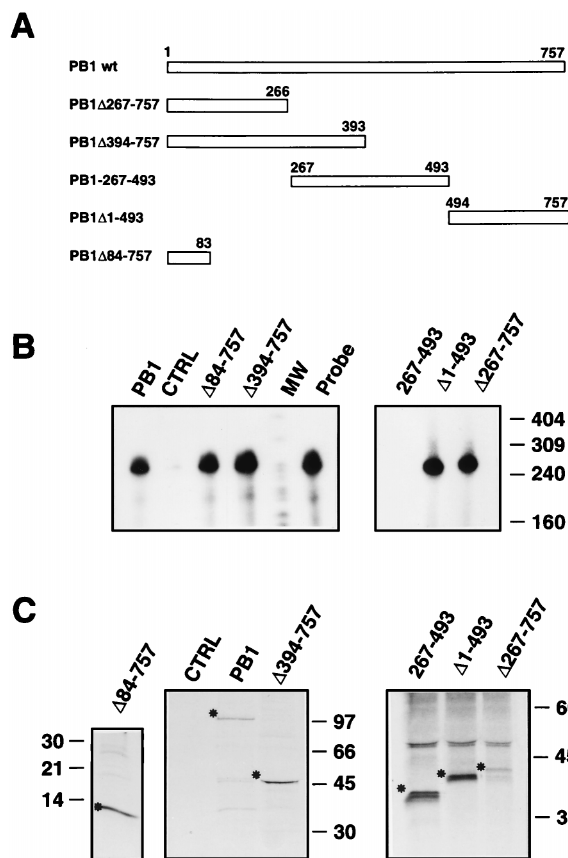

FIG. 5. Binding of mutant PB1 proteins to vRNA. Cultures of COS-1 cells were infected with vTF7-3 vaccinia virus and transfected with pGPB1 plasmid (PB1), the mutant plasmids indicated in panel A, or pGEM3 plasmid as a control (CTRL). The RNA binding assay was carried out as indicated in Materials and Methods and in the legend to Fig. 1. Mutants PB1Δ267–757, PB1Δ1–493, and PB1-267–493 were expressed as His-tagged proteins and immunoprecipitated with anti-HisNS1 serum. (B) Analysis of the RNA retained. Numbers to the right indicate the length of the RNA markers (MW) in nucleotides. (C) Analysis of the proteins present in the immunoprecipitates. Numbers indicate the sizes of the protein markers in kilodaltons.

the panhandle region. Thus, cross-linking studies have shown that the enzyme present in virion RNPs is able to interact with exogenous RNA probes corresponding to the 3′ terminus (12) and the 5′ terminus (10) of vRNA. Moreover, mutational analyses indicated that nucleotides at positions 1 to 3 and positions 8 to 10 from the 5′ terminus are important for polymerase binding (10). In addition, the polymerase expressed from vaccinia virus recombinant was shown to contact the panhandle preferentially at its 5′ terminus, and the contact sites were located at the predicted loop residues in the panhandle model (49). The results presented here deal with the interaction of the isolated PB1 subunit with the template vRNA. We show that there is a specific interaction of PB1 protein with model vRNA probes, since in vitro binding was not competed with an excess of unrelated RNA present in the assay, binding was not observed with an unspecific probe, and it was competed with an excess of unlabeled, specific probe (Fig. 1). The apparent K_d for the interaction of PB1 protein with its template was estimated to be ca. 2×10^{-8} M (Fig. 4), indicating a substantial affinity. It is likely that other subunits of the polymerase might also add on to the affinity of binding of the polymerase complex to the vRNA template, but our data indicates that PB1 alone makes an important contribution to the decrease in free energy due to the interaction.

The interaction of PB1 protein with its template was mapped to the 5′ arm of the panhandle (Fig. 2), a finding in agreement with the results obtained with the complete polymerase complex by gel shift experiments (49) or by cross-linking (10), although a measurable interaction was also observed for the 3′ arm. Our results indicate that the main determinant of the interaction of the 3′ arm with the PB1 subunit of the polymerase is its binding to the 5′ arm, since retention of the 3′ probe was much more efficient when presented to PB1 as a panhandle analog (a 5′+3′ hybrid probe). Moreover, retention of the 5′+3′ hybrid probe was more efficient than that observed for the single-stranded 5′ probe (Fig. 2), suggesting that a more restricted conformation of the 5′ arm in the panhandle analog is better suited for interaction with PB1 protein. Northwestern experiments showed that PB1 protein itself bound directly the 5′ probe. This result rules out the possibility that a cellular contaminant present in the PB1-specific immunoprecipitates is responsible for the binding of vRNA in the in vitro assay (Fig. 1). Moreover, the specific binding in the Northwestern assay indicates that the interaction takes place with a monomeric form of PB1.

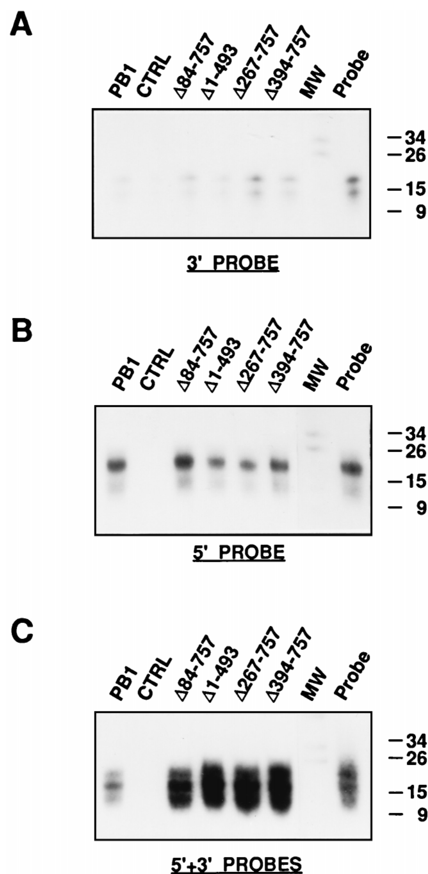

FIG. 6. Binding of mutant PB1 proteins to the 5′ and the 3′ arms of the panhandle. Cultures of COS-1 cells were infected with vTF7-3 vaccinia virus and transfected with pGPB1 plasmid (PB1), the mutant plasmids indicated in Fig. 5A, or the pGEM3 plasmid as a control (CTRL). The RNA-binding assay was carried out as indicated in Materials and Methods and in the legend to Fig. 1. (A) Analysis of the RNA retained with the 3′ probe. (B) Analysis of the RNA retained with the 5′ probe. (C) Analysis of the RNA retained with a mixture of the 3′ and 5′ probes. Numbers to the right indicate the length of RNA markers (MW) in nucleotides.

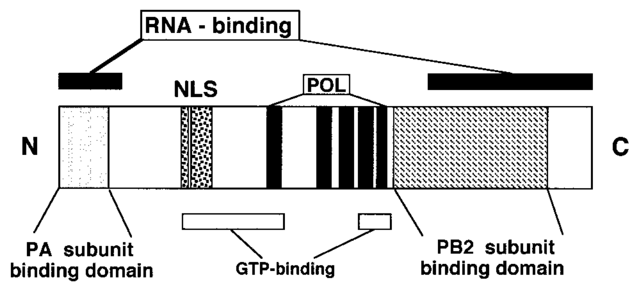

FIG. 7. Diagram of the PB1 protein indicating the vRNA binding site, as well as a number of other domains reported in the literature. See the text for details.

The results of in vitro binding of the vRNA model RNA to a variety of PB1 mutant proteins (Fig. 5), as well as the results of the Northwestern assays (data not shown), indicated that the protein sequences responsible for the interaction with the template are not contiguous in the PB1 protein primary sequence. Deletion from the C terminus did not abolish the binding activity in vitro, up to the point that a PB1 deletion mutant encoding the first 83 N-terminal amino acids was still active in vRNA binding (mutant PB1Δ84–757; Fig. 5). However, a mutant protein encoding the last 263 C-terminal amino acids was also active in vRNA retention (mutant PB1Δ1–493; Fig. 5), and deletion of the first N-terminal 69 amino acids did not abolish the activity (data not shown). Therefore, we propose that both N-terminal (positions 1 to 83) and C-terminal (positions 494 to 757) sequences contribute to vRNA binding. In agreement with this proposal, the internal region of PB1 protein (mutant PB1267–493) was unable to bind the vNSZ probe (Fig. 5). The contributions of the N-terminal and the C-terminal regions of PB1 to vRNA binding do not seem to be equal. Retention of vRNA was more efficient when N-terminal sequences were present (Fig. 5).

The results presented in Fig. 5, together with those of Fig. 2, opened the possibility that each of the PB1 regions involved in template binding would interact with one of the arms of the panhandle structure. Such a simplistic model was ruled out by the results presented in Fig. 6. Both RNA-binding determinants in the PB1 protein sequence interacted preferentially with the 5′ probe, suggesting that they cooperate in the recognition of this side of the panhandle. Recognition of the 3′ probe was poor, and its retention in the complex seemed very dependent on the interaction with the 5′ arm of the panhandle (Fig. 6). As a whole, the results presented suggest that the N-terminal and the C-terminal sequences of PB1 protein fold together to build up the recognition site of vRNA, mainly via the 5′ arm of the panhandle. Protein contacts with the 3′ arm do not appear to be important, since the binding of the 3′ probe is very dependent on the 5′ probe, although we cannot rule out the possibility that entry of the 5′ arm in the complex would open a secondary RNA-binding site that recognizes the 3′ arm. This is a minimal model in which the role of the other subunits of the polymerase complex have not been considered. Since PB2 protein can be cross-linked to the 3′ sequences of the panhandle (12), it is possible that it contributes to the polymerase-vRNA binding capacity by recognizing the 3′ arm of the panhandle, i.e., by complementing the PB1 RNA-binding site. However, this possibility is unlikely, since the contact sites of the polymerase complex onto model vRNA molecules were mapped to the 5′ arm of the panhandle (49). It is worth mentioning that the position of the main RNA-binding determinant to the N-terminal region of PB1 determined in this report overlaps with the mapped PA-binding domain of the PB1 subunit (15, 39, 50). A similar situation might occur with the PB2 binding domain and the C-terminal RNA-binding sequences (Fig. 7). Whether the PA or PB2 subunits could modify the vRNA-binding activity of PB1 protein in the polymerase complex remains to be tested. A recent report has shown that in vitro polyadenylation of influenza virus model transcripts can be inhibited by mutations at positions close to the 5′ end of vRNA (41), mutations that affect the binding of the polymerase to the 5′ arm of the panhandle (10). These results support the notion that polymerase binding to the 5′ terminus is an essential element that, coupled to cap-dependent initiation, determines the synthesis of mRNA rather than full copies of cRNA. Since we show that PB1 protein on its own binds preferentially the 5′-terminal sequence of vRNA, it could be concluded that such a default situation has to be avoided in the event of cap-independent initiations, which should not lead to polyadenylation. The mechanism by which de novo initiation is coupled to the release of the 5′ terminus of vRNA from PB1 protein is unknown at present.

The information regarding vRNA binding reported here is included in the diagram shown in Fig. 7, together with the mapping of other domains or active sites identified in the PB1 subunit. These include the polymerase motifs (3, 40), the nucleoside triphosphate-binding regions (1), the NLSs (33), and the domains responsible for interaction with the PB2 and PA subunits (15, 39, 50). Understanding of the interrelationships of these sites would require structural information at the three-dimensional level.

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