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Identification of two separate domains in the influenza virus PB1 protein involved in the interaction with the PB2 and PA subunits: a model for the viral RNA polymerase structure

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ABSTRACT

The domains of the PB1 subunit of the influenza virus polymerase involved in the interaction with the PB2 and PA subunits have been defined by mutational analysis of PB1 protein. The experimental approach included *in vivo* competition of the PB1 activity, two-hybrid interaction assays and *in vitro* binding to PB1-specific matrices. Mutants of the PB1 gene including N-terminal, C-terminal and internal deletions and single amino acid insertions were constructed. They were unable to support polymerase activity in a reconstituted transcription–replication system and were tested for their competition activity when expressed in excess over wild-type PB1 protein. The pattern of competition obtained suggested that the N-terminal 78 amino acids and the sequences between positions 506 and 659 in the PB1 protein are involved in the interaction with the other components of the polymerase. We identified the N-terminal region of PB1 protein as responsible for the interaction with the PA subunit by two-hybrid assays in mammalian cells. N- and C-terminal fragments of the PB1 protein were expressed as His-tagged proteins and purified on Ni^{2+}–NTA resin. Such PB1-specific matrices were used in binding assays *in vitro* with metabolically labelled PB2 and PA proteins and mutants thereof. The results obtained indicated that the N-terminal and the C-terminal regions of PB1 are responsible for binding to PA and PB2 subunits, respectively. With this information and previously published results we propose a preliminary model for the architecture of the influenza virus RNA polymerase.

INTRODUCTION

Influenza A viruses are members of the Orthomyxoviridae family whose genome consists of eight single-stranded RNA segments of negative polarity. These RNA molecules associate with the nucleoprotein (NP) and the three P proteins (PB2, PB1 and PA) to form ribonucleoprotein (RNP) complexes [reviewed in (1,2)]. In a productive infection, the parental RNAs (vRNAs) are first transcribed into viral mRNAs by the incoming transcriptase. Cellular hnRNAs are used as cap donors to initiate transcription (3) and termination occurs at an oligo-U signal that is present adjacent to the RNA panhandle structure at the 5′ terminus of the vRNA templates (4,5). For viral RNA replication, a full-length RNA copy of positive polarity (cRNA) is used as an intermediate (6).

The influenza virus RNA polymerase is a heterocomplex composed of the three polymerase (P) proteins—PB1, PB2 and PA—present in a 1:1:1 ratio (7–9). Some information has become available in regard to the roles of each subunit in the complex. The PB1 protein is probably responsible for polymerase activity, since it shares common amino acid motifs with other RNA-dependent RNA polymerases (10). Mutation of these conserved residues abolished the transcriptional activity (11). The PB2 subunit binds to cap structures (12,13) and antibodies specific for PB2 protein inhibit the cap-dependent endonuclease activity (14). These results suggest that PB2 protein is responsible for the transcription initiation step (15). The role of PA protein is poorly understood, although the phenotypes of ts mutants [reviewed in (16)] suggest its involvement in vRNA synthesis. The only biochemical activity so far described for the PA subunit is the induction of proteolysis (17). The regions of the PA subunit responsible for this activity map to the N-terminal third of the protein (18), close to the nuclear localization signal (19).

In spite of the functions tentatively assigned to its subunits, the functionality of the reconstituted polymerase in either the CAT assay [reviewed in (20)], *in vitro* transcription and replication (B. Perales, unpublished results) and cap-snatching (21) requires the participation of every polymerase subunit and the template. Therefore, it is essential to unravel the molecular architecture of the complex(es) formed by the polymerase subunits. Polymerase complexes have been described in influenza virus infected cells (8) or from subunit expression in baculovirus vectors (22). Co-expression in frog oocytes demonstrated that both PB2 and PA subunits can form complexes with the PB1 protein but can not interact directly (23). Previous studies in our laboratory had determined the regions of the influenza virus polymerase subunits PB2 and PA involved in the interaction with the PB1 protein

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(24,25). A small portion of PB2 protein (amino acids 1–124) was sufficient for the interaction (24), but the PA sequences responsible for binding to PB1 could only be mapped to the C-terminal three-quarters of the protein, probably due to conformational constraints (25). In this report we complete the mapping of the interaction domains in the influenza virus polymerase by analyzing the competition of a collection of PB1 protein mutants with wild-type PB1 (wtPB1) *in vivo*, as well as by *in vitro* binding assays and in a two-hybrid system in mammalian cells. While this manuscript was in preparation, the report by Prez and Donis (26) was published. We confirm their conclusions indicating that the N-terminal end of the PB1 protein interacts with the PA subunit, and extend them by proposing that the region between amino acids 506 and 659 of the PB1 protein is involved in the interaction with the PB2 subunit. Taken together the results presented here and those recently published (24,25), we propose a model for the architecture of the influenza virus polymerase complex.

MATERIALS AND METHODS

Biological materials

The COS-1 cell line (27) was provided by Y. Gluzman and was cultivated as described (28). The vaccinia recombinant virus vTF7-3 (29) was a gift from B. Moss. The *Escherichia coli* strains MH1638 and HB101 were kindly provided by S.G. Sedgwick. The origin of plasmids pGPB1, pGPB2, pGPA and pGNP have been described previously (30). The pIVACAT1-S plasmid (31) and pGL-G5 plasmid, containing the luciferase gene under control of the GAL4 promoter, were provided by P. Palese and P. Stäheli, respectively. The origin of plasmids for the two-hybrid assay has been described previously (25). The pRSETA plasmid and the Ni^{2+}–NTA resin were purchased from Invitrogen. The anti-T7 tag antibody was purchased from Novagen. Cationic liposomes were prepared as described (32).

Construction of mutants

The generation of single amino acid insertion mutants was carried out exactly as described (24). The insertion at position 416 generated an ochre codon, leading to deletion mutant PB2Δ416–757. A series of mutants (mutants PB2Δ83–757, PB2Δ592–757, PB1Δ659–757 and PB2Δ711–757) were produced by random insertion of a tagged Tn1000 transposon derivative (33), which due to termination codons present close to its ends and in all frames, resulted in C-terminal deletions of the PB1 protein. Cells harbouring transposon TnXR (present in R388 in the MH1638 *E.coli* strain) were transformed with plasmid pGPB1 and selected in medium containing ampicillin and methicillin. Such a donor strain was mated with strain HB101 ($strep^R$-$recA^-$) by co-culture for 2 h at 37°C on LB-agar plates in the absence of antibiotics. Transferred cointegrates were selected in LB-agar plates containing ampicillin, methicillin and streptomycin. After characterization by restriction analysis, the plasmid was sequenced using primers specific for the TnXR termini to determine the site of the integration.

The construction of N-terminal deletion mutant PB1Δ1–179 was carried out by digestion with *Sma*I in the plasmid polylinker and *Eag*I (position 438 in the PB1 gene), filling in with Klenow enzyme and self ligation. The C-terminal deletion mutant PB1Δ393–757 was constructed by digestion of pGPB1 plasmid with *Stu*I (position 1204) and *Bgl*II (position 2259), filling in with Klenow enzyme and self ligation. Similar strategy was used to generate a number of internal deletion mutants: plasmid pGPB1 was digested with *Mun*I (position 228) and *Stu*I (mutant PB1Δ69–394), *Bsu*36I (position 246) and *Eag*I (mutant PB1Δ78–139), *Eag*I and *Stu*I (mutant PB1Δ139–394) or *Bsu*36I and *Stu*I (mutant PB1Δ78–394), the digested DNA was filled in with Klenow enzyme and self ligated. In addition, mutants PB1Δ394–506, PB1Δ722–752 and PB1Δ722–749 were generated by digestion with *Stu*I and *Xcm*I (position 1540) or *Apa*I and *Bgl*II, respectively, trimming the ends with T4 DNA polymerase and self ligation. Double-deletion mutant PB1-69-416 was constructed by digestion of pGPB2Δ416–757 plasmid with *Sma*I and *Mun*I, filling in with Klenow enzyme and self ligation.

Expression of polymerase subunits

The expression of polymerase subunits (or mutants thereof) in mammalian cells was carried out as follows: cultures of COS-1 cells in M24 wells were infected with vTF7-3 virus at a multiplicity of infection (moi) of 10 p.f.u./cell. After 1 h, the cells were transfected with 4 µg pGPB1 (or pGPB2 or pGPA) plasmid, the corresponding mutant plasmid or pGEM3 as a control, using cationic liposomes. Transfection and all steps thereafter were carried out in the presence of 50 µg/ml citosine arabinoside (AraC). After 6 h of incubation, the cultures were washed and incubated for 1 h in methionine and cysteine free medium. Finally, the cells were labelled for 15 h with [^{35}S]met–cys (0.5 mCi/ml) in a DMEM medium containing 1/10 of the normal concentrations of these amino acids.

For expression of the C-terminal portion of PB1 protein in *E.coli*, a *Hin*dIII DNA fragment (positions 1507–2341 in the PB1 gene sequence) was subcloned into the *Hin*dIII site of pRSETA plasmid. To express the N-terminal region, the full-length PB1 open reading frame, cloned into pRSETA plasmid, was digested with *Hin*dIII and self-ligated. In this way, the sequences between position 831 and 2341 in the PB1 gene were eliminated. These constructs were checked by restriction analysis and transferred to *E.coli* BL21 pLysS. The expression of the His-PB1 protein N- or C-terminal fragments was accomplished by induction with 1 mM IPTG for 2 h at 37°C. The bacterial cells were opened by sonication in a buffer containing 500 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, 100 mM imidazol and 50 mM Tris–HCl, pH 7.5 and bound to Ni^{2+}–NTA resin by incubation overnight at 4°C. The resin was washed with 50 vol of a buffer containing 1 M KCl, 5 mM $MgCl_2$, 10% glycerol, 0.1% Brij36T, 20 mM Tris–HCl, pH 7.5.

Competition assays *in vivo*

The competition assays were carried out as described previously (24). In brief, cultures of COS-1 cells were infected with vTF7-3 virus at a moi of 10 p.f.u./cell. After virus adsorption, the cultures were co-transfected with 500 ng pGPB2, 25 ng pGPB1, 50 ng pGPA and 2 µg pGNP plasmids (20,30). A 40-fold excess of competing pGPB1 mutant plasmid (or pGEM3 as a control) was also included in the transfecting mixture. After 5 h adsorption of DNA-liposomes to the cells at 37°C, the cultures were washed with DMEM and further transfected with 100 ng NS-CAT vRNA as described (24) and incubated at 37°C. Twenty to 24 hours post-infection the cells were collected in DMEM, washed in TNE and opened by freezing and thawing three times with 0.25 M

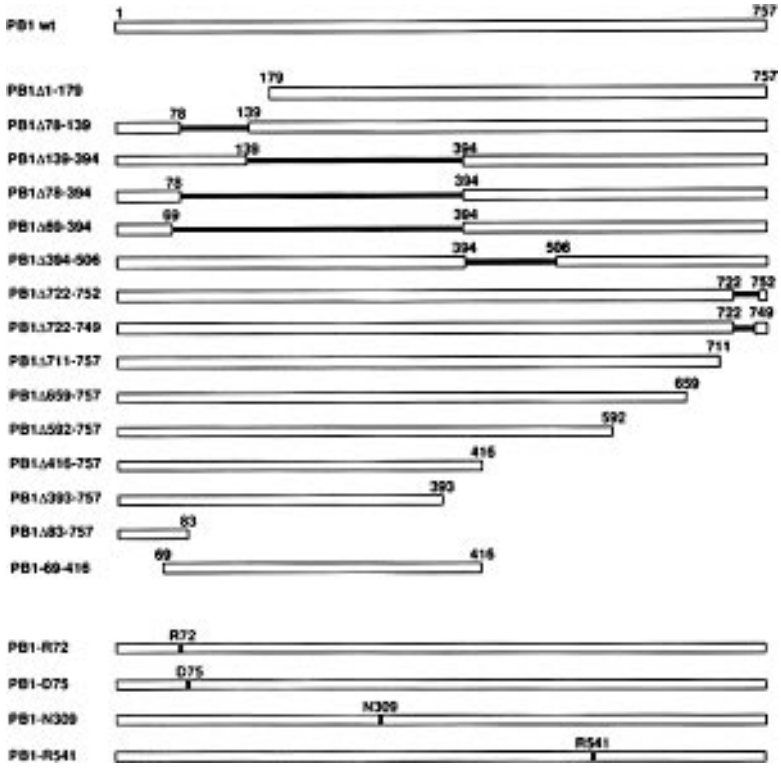

Figure 1. Structure of mutant PB1 proteins. The diagram shows the structure of the PB1 protein mutants studied. The numbers indicate the amino acid positions that limit the deletions produced. In addition to the deletion mutants, some single amino acid insertion mutants were analyzed. They are named by the nature of the amino acid inserted and the position in the PB1 protein after which the additional amino acid is present.

Tris–HCl pH 7.5. The extracts were used for CAT assays as described previously (20).

Two-hybrid assays in mammalian cells

The association of PA protein with PB1 protein or their mutants was tested in a two-hybrid system developed for animal cells (34 and references therein). COS-1 cells were co-transfected with 1 µg each of pGL-G5 plasmid, pVP-PA plasmid and pGALPB1 plasmid (or mutants thereof), using cationic liposomes. The mutations present in plasmids pGPB1Δ394–506, pGPB1Δ722–749, pGPB1Δ711–757, pGPB1Δ659–757, pGPB1Δ592–757, pGPB1Δ416–757 and pGPB1R641 were transferred to plasmid pGALPB1 by swapping the *Bsu*36I–*Xba*I fragment including most of the PB1 gene (positions 246–2341). The cultures were harvested 40–48 h post transfection and extracts were prepared in a buffer containing 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT, 1% Triton X-100, 25 mM glycyl-glycine, pH 7.8. The extracts were assayed for luciferase activity as described (35).

Binding assays *in vitro*

The preparation of labelled extracts containing the polymerase subunits PB1, PB2 or PA or mutants thereof was carried out as described above for the expression of PB1 mutant proteins, except that 35 mm dishes were used and the labelling was for 1 h. The labelled cultures were washed with PBS, scraped off the plates and lysed in 100 µl buffer containing 7.5 mM ammonium sulphate, 1 mM EDTA, 1 mM DTT, 0.025% NP-40 and 10 mM Tris–HCl, pH 7.9. After vortexing, the extract was centrifuged for 3 min at 3000 r.p.m. and 4°C and the supernatant was further centrifuged for 15 min at 10 000 r.p.m. and 4°C. For the binding reaction, 20 µl of extracts were diluted to 500 µl of 100 mM NaCl, 1 mM $MgCl_2$, 1% NP-40, 10 mM Tris–HCl, pH 7.5 and incubated with 20 µl of the PB1-specific or control matrices for 2 h at 4°C. The resin was washed 10 times with 1 ml of the same buffer and the retained protein was eluted by boiling in gel-loading buffer.

Western blotting

For western blot assays, the protein extracts were separated in SDS–polyacrylamide gels, transferred to nitrocellulose membranes (Inmobilon) and saturated with 3% bovine serum albumin for 1 h at room temperature. The membranes were incubated with either anti-PB2 serum (at 1:100 dilution), anti-PA monoclonal antibodies (at 1:10 dilution) or with the anti T7-G10 tag monoclonal antibody (at a 1:3000 dilution) for 1 h at room temperature. After washing two times for 30 min with PBS containing 0.05% Tween 20, the filters were incubated with a 1/3000 dilution of protein A–peroxidase or with a dilution of 1/3000 of goat anti-mouse IgG conjugated to horseradish peroxidase, respectively. Finally, the filters were washed two times for 30 min as above and developed by enhanced chemiluminescence (ECL, Amersham).

RESULTS

Construction and characterization of PB1 mutants

To study the regions of the PB1 protein involved in polymerase complex formation, a collection of PB1 mutants were constructed whose description is summarized in Figure 1. It included

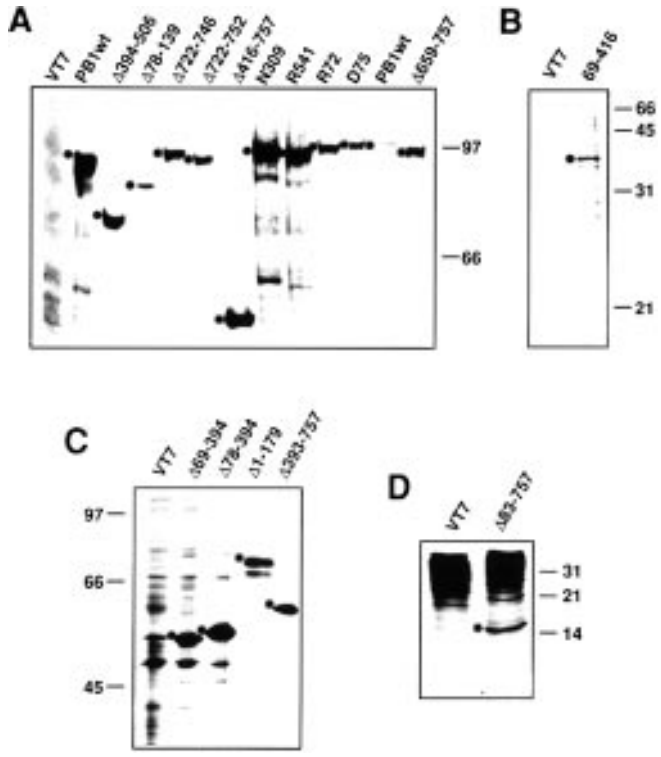

Figure 2. Expression of PB1 mutants. Cultures of COS-1 cells were infected with vTF7-3 vaccinia virus and transfected with either pGEM3 plasmid (lanes VT7), pGPB1 plasmid or its mutant derivatives, as indicated in the Figure. The infected-transfected cultures were labelled continuously for 15 h with [^{35}S]met–cys and total cell extracts were prepared. The labelled proteins were analyzed by electrophoresis in polyacrylamide gels and autoradiography. The numbers to the left of each panel indicate the mobility of molecular weight markers.

C-terminal, N-terminal and internal deletion mutants as well as single-amino acid insertion mutants (see Materials and Methods for details). All mutants were generated in the background of the pGEM vector, under the control of the T7 promoter, and hence they could be expressed by transfection into mammalian cells previously infected with a vaccinia recombinant virus capable of expressing the T7 RNA polymerase (vTF7-3). When COS-1 cell cultures were infected-transfected by such an expression strategy and labelled continuously with [^{35}S]met–cys to ascertain the synthesis and stability of the mutant proteins, the results presented in Figure 2 were obtained. The levels of label obtained for the insertion mutants (Fig. 2A), the mutants containing small deletions (Fig. 2A), those containing intermediate deletions (Fig. 2B and C) as well as the smallest mutant protein PB1Δ83–757 (Fig. 2D) were somewhat variable, but within the values obtained for wtPB1 protein (see the two separate samples of wtPB1 shown in Fig. 2A). In any case, the expression levels obtained exceeded those required for competition of activity. Thus, even mutant PB1Δ78–130, the one expressed to the lowest level, could effectively compete for wtPB1 activity (see Fig. 3). The mutant proteins were characterized in regard to their ability to support CAT activity in the transcription–replication system reconstituted *in vivo* (24,30). All mutants were completely negative (data not shown). This result is not surprising, since relatively large portions of the protein were absent in the various deletion mutants, and allowed us to carry out competition experiments *in vivo* to determine the functional interactions of these mutants with the rest of the polymerase components.

Competition of PB1 mutants for the activity of PB1 *in vivo*

As described previously for the competition experiments regarding PB2 mutants (24), the first step carried out was to determine the minimal amount of PB1-expressing plasmid to be transfected in order to obtain maximal CAT activity in the reconstituted system. Such previous experiments indicated that 25 ng of pGPB1 plasmid were sufficient to yield 100% of the CAT activity in the assay (data not shown) and such a dose was therefore used in the competition experiments carried out subsequently. They involved the transfection of a 40-fold excess of mutant, competing plasmid, in addition to pGPB1 plasmid and the rest of the components required for the reconstitution *in vivo* (24,30). The results of several such competition experiments are shown in Figure 3. Since PB1 protein interacts with both the PB2 and PA subunits of the polymerase (23), the most informative mutants were those able to compete fully for the PB1 wt activity, i.e. those not affected in the interaction domains. All insertion mutants tested, used as positive control for the competition experiments, were able to compete almost completely for the activity of the PB1 protein (Fig. 3; mutants PB1R72, PB1D75, PB1N309 and PB1R541). Using the results obtained with these mutants, a value of CAT activity of 20% was established as cut-off limit to identify fully competing mutants (Fig. 3). Two regions of the PB1 protein appeared as non-essential for the interaction with the other polymerase components: the C-terminal part of the protein (amino acids 659–757) (Fig. 3; see mutants PB1Δ659–757, PB1Δ722–749 and PB1Δ722–752) and an internal portion comprising amino acids 78–506 (Fig. 3; see mutants PB1Δ78–139, PB1Δ139–394 and PB1Δ394–506). In agreement with these results, it was not possible to compete the activity of wtPB1 by overexpression of mutant PB1-69-416, that only encodes the internal protein region (Fig. 3). The lack of competition observed with other deletion mutants might reflect alterations in the interacting sequences that reduce the binding affinity or might simply represent global misfolding of the resulting proteins. In summary, the results of competition experiments suggest that interacting regions exist at the very N-terminal end of PB1 protein and at its C-terminal third, excluding the C-terminus. However, these experiments do not allow the identification of the protein partner of the interaction at each of these separate ends of the PB1 subunit.

Mapping of the PB1–PA binding region by two-hybrid assays

To identify which of the PB1 interacting domains is the responsible for the binding of the PA subunit we made use of a two-hybrid assay based on the expression of PB1 and PA proteins fused to the GAL4 DNA binding domain (GAL) or the VP16 activation domain (VP) in mammalian cells. This assay was used previously to investigate the regions of PA protein important for its interaction with PB1 (25). Co-transfection of wt alleles of GAL–PB1 and VP–PA led to the expression of cotransfected luciferase marker (up to 10% of the levels obtained by transfection of intact GAL4–VP16 transactivator; data not shown). In contrast, the luciferase activity obtained in the assay when only GAL–PB1 was transfected or when GAL–PB1 was co-transfected with a mutant allele of VP–PA (25) was greatly

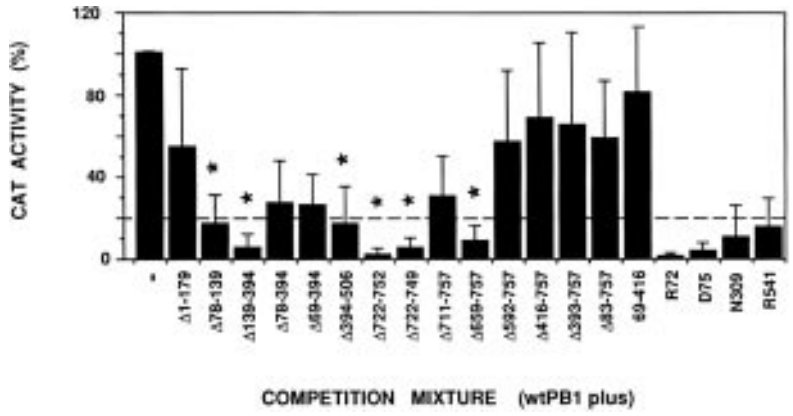

Figure 3. Competition of PB1 polymerase mutants for PB1 activity *in vivo*. The diagram shows the average values and standard deviation of CAT activity in competition experiments with the different PB1 mutant proteins indicated. Cultures of COS-1 cells were infected with vTF7-3 vaccinia virus and transfected with a mixture of plasmids pGPB1, pGPB2, pGPA, pGNP and excess mutant pGPB1. At 5 h post infection, the cells were further transfected with NSCAT vRNA. Total cell extracts were prepared at 20–24 h post infection and the CAT activity was determined (see Materials and Methods for details). The stars indicate the deletion mutants whose competition leads to CAT values <20% of control (indicated by dashed line), a value comparable with those obtained with single amino acid-insertion mutants.

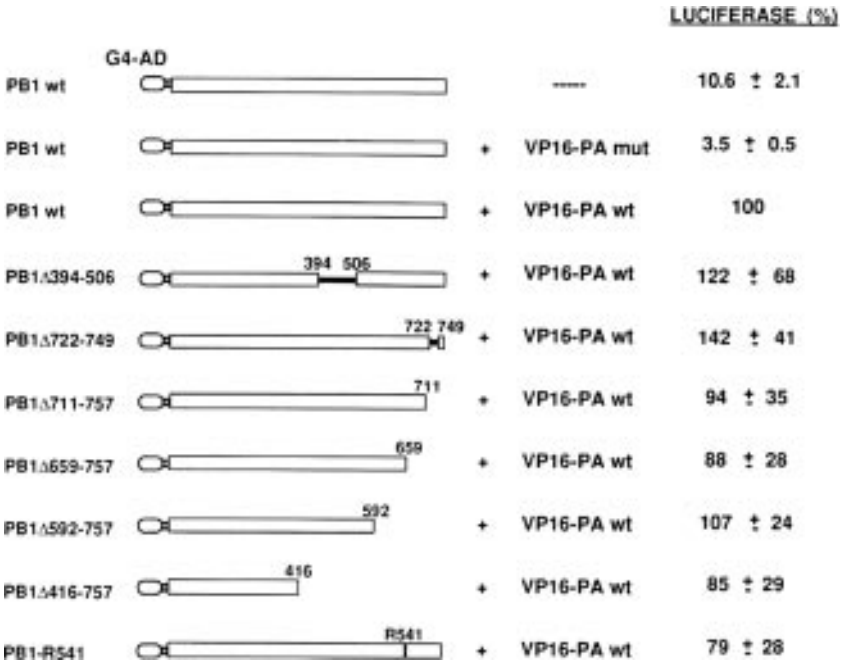

Figure 4. Interaction of PA protein with PB1 mutant proteins *in vivo*. Cultures of COS-1 cells were co-transfected with plasmids pGL-G5, pVP-PA and pGAL-PB1 or the mutants indicated in the Figure. The cultures were harvested and extracts were prepared and assayed for luciferase activity as described in Materials and Methods. The values shown are the averages and standard deviations of three independent experiments.

reduced (Fig. 4) allowing an experimental window to measure the interaction of mutant versions of GAL–PB1 with wt VP–PA. The results of a series of co-transfection experiments are shown in Fig. 4. All mutants tested, that covered serial deletions from the PB1 protein C-terminus, led to luciferase values similar to that of wt GAL–PB1, indicating that the interaction with the PA subunit is mediated by the N-terminal domain of PB1 protein. Since deletion of the C-terminal end of PB1 did not affect binding to PA protein, it is tempting to speculate that this region could be involved in PB1 binding to PB2. Unfortunately, the attempts to detect PB1–PB2 interaction in the two-hybrid system were unsuccessful and hence this prediction could not be tested using such an experimental approach.

Mapping of the PB1–PA and PB1–PB2 binding regions *in vitro*

To establish whether the PB2 binding region of the PB1 subunit maps to the C-terminal third of the protein and to confirm that the PA binding region is located at its N-terminal end, fragments of the PB1 gene, encoding amino acids 1–266 or 494–757, were fused to a histidine tag and an antigenic T7 tag in the pRSET

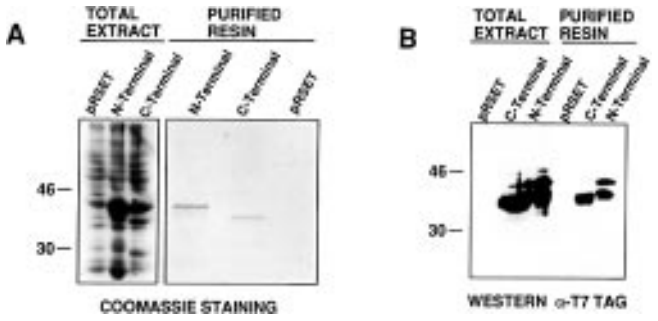

Figure 5. Characterization of PB1-specific affinity matrices. Cultures of *E.coli* BL21pLysS transformed with either pRSETA vector, the construct containing the PB1 C-terminal or the construct containing the PB1 N-terminal region were induced with IPTG. The corresponding protein extracts were analyzed directly or after purification on Ni^{2+}–NTA resin as described in Materials and Methods. (**A**) Analysis of the extracts or the material in the resin by staining with Coomassie Blue. (**B**) Analysis of the same samples by western blot using a T7 tag-specific antiserum. The numbers to the left indicate the positions of molecular weight markers.

vector and expressed in *E.coli*. The tagged PB1 fragments were bound to and purified on a Ni^{2+}–NTA resin as indicated in Materials and Methods. The characterization of such affinity matrices is shown in Figure 5. The his-PB1 fragments were essentially the only proteins retained in the resin after extensive washing, as detected by Coomassie blue staining (Fig. 5A). Their identification as the recombinant protein fragments was carried out by western blotting using anti-T7 tag antibodies (Fig. 5B). As a control matrix, Ni^{2+}–NTA resin was incubated with extracts of *E.coli* transformed by empty pRSETA vector and washed in parallel to the his-PB1 resins. Such a control matrix was essentially devoid of protein, as detected by Coomassie blue staining and shown in Figure 5A. Although detection by Coomassie blue staining does not allow us to reach any strong conclusion on protein purity, the use of the control resin in binding experiments (see below) indicated that the possible low level of contaminants present did not contribute to the binding activities detected by the PB1-specific resins.

The his-PB1 affinity matrices, as well as the control resin, were incubated with extracts of COS-1 cells infected with vaccinia vTF7-3 virus and transfected with either pGPB1, pGPA, pGPA-Δ155 (25), pGPB2, or a series of its mutant derivatives (24). These infected-transfected cells were labelled *in vivo* with [^{35}S]met–cys to allow the detection of the proteins bound to the affinity matrix. The presence of the PB1, PA, PB2 and mutant proteins in total extracts of infected-transfected cells can be seen in Figure 6A, when compared with the extract prepared from untransfected cells. These labelled extracts were incubated with the PB1-specific matrices or the control matrix. The polymerase subunits that were specifically bound to the matrices were completely depleted from the extracts and, after the extensive washings, 30–80% of the input specific protein was recovered in the matrix. No protein was bound to the control matrix (data not shown). The specific retention of PB2 protein on the his-PB1 C-terminal matrix is shown in Figure 6. A labelled band of electrophoretic mobility identical to that of PB2 protein was present in the solid phase of the PB1 C-terminal matrix (Fig. 6B) but not in the PB1 N-terminal matrix (Fig. 6C). The specificity of the binding was checked by using extracts of cells expressing either PB1 protein or PAΔ155 protein. This PA deletion mutant was used because, in contrast to wtPA protein, it does not induce proteolysis in the expressing cells (18) and hence it accumulates to higher levels. Neither labelled PB1 nor PAΔ155 proteins were retained by the PB1 C-terminal matrix (Fig. 6B). To further control the specificity of the binding, a series of PB2 mutant proteins were included. Some of them, like PB2I299 (Fig. 6B) or PB2H566 (data not shown) were retained on the his-PB1 C-terminal matrix as wtPB2, in accordance to their capacity to compete with the activity of wtPB2 protein in the reconstituted transcription–replication system *in vivo* (24). Other mutants, like PB2Δ1–28 or PB2Δ17–67, did not bind at all, as expected from their lack of competition in the same assay (24) (Fig. 6B). On the other hand, PA and PAΔ155 proteins were specifically retained by the his-PB1 N-terminal matrix, in contrast to PB1 or PB2 proteins and in agreement with the results obtained by two-hybrid assays (25) (Fig. 6C). Western blot analysis using anti-T7 tag antibodies verified the presence of the his-PB1 C- or N-terminal fragment in the specific matrices (Fig. 6B and C).

The identity of the labelled proteins retained by the his-PB1 matrices was verified by western blot analysis using anti-PB2 serum or anti-PA monoclonal antibodies (36) (Fig. 7). As a whole, these results indicate that the binding test used is specific and that the PB1 protein domains responsible for the interaction with the PA and PB2 subunits are located upstream of position 266 and downstream of position 494 in the PB1 sequence, respectively.

DISCUSSION

In the present report, the regions of the PB1 protein involved in the interaction with the other polymerase subunits have been studied by means of a series of PB1 mutants, most of which contained deletions and some had single amino acid insertions (Fig. 1). When these mutant genes were expressed in the vaccinia–T7 infection–transfection system (30) they showed accumulation levels comparable with that of wtPB1 (Fig. 2). The interaction with the PB2 and PA subunits was studied by a set of experimental approaches including *in vitro* binding to PB1-specific matrices, two-hybrid assays in mammalian cells and competition for the activity of wtPB1 in a reconstituted transcription–replication system *in vivo*. Taken together, the results obtained by the three experimental approaches used indicate that the PB1 protein contains two separate domains involved in the interaction with the other polymerase subunits. These functional domains can be unambiguously located at the N- and C-terminal regions of the protein and assigned to the interaction with PA and PB2, respectively, by the pull-down experiments *in vitro* (Figs 5–7). These results confirm *in vitro* the binding phenotypes of PB2 and PA mutant proteins whose interaction with PB1 had been studied *in vivo* (24,25). Furthermore, the interaction of PA protein to the N-terminal domain of PB1 was confirmed by two-hybrid assays (Fig. 4). These results are in agreement with those presented by Prez and Donis who used a similar two-hybrid system to map the PA-binding site of PB1 protein to the N-terminal 48 amino acids (26).

The fact that all mutants used were inactive in the transcription–replication system reconstituted *in vivo* allowed us to check the relevance of the interactions detected for the biological activity of the viral polymerase by competition assays. The results obtained are fully compatible with those obtained by the other approaches

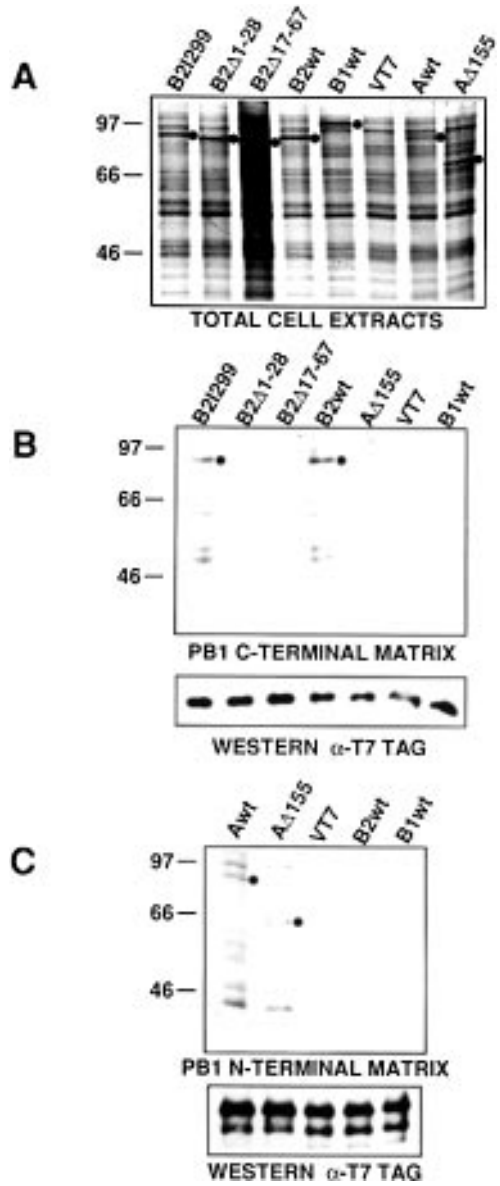

Figure 6. Binding of polymerase subunits to PB1-specific affinity matrices *in vitro*. Cultures of COS-1 cells were infected with vaccinia vTF7-3 virus and transfected with either pGEM vector (lanes VT7) or pGEM derivatives expressing the various polymerase subunits. After labelling *in vivo*, extracts were prepared and analyzed by polyacrylamide gel electrophoresis (**A**). The various extracts were incubated with the PB1 C-terminal matrix (**B**) or the PB1 N-terminal matrix (**C**) and the retained proteins were analyzed by polyacrylamide gel electrophoresis. Aliquots of the same samples were analyzed by western blotting using a T7 tag-specific antiserum. The numbers to the left indicate the positions of molecular weight markers. The stars indicate the influenza polymerase-specific bands.

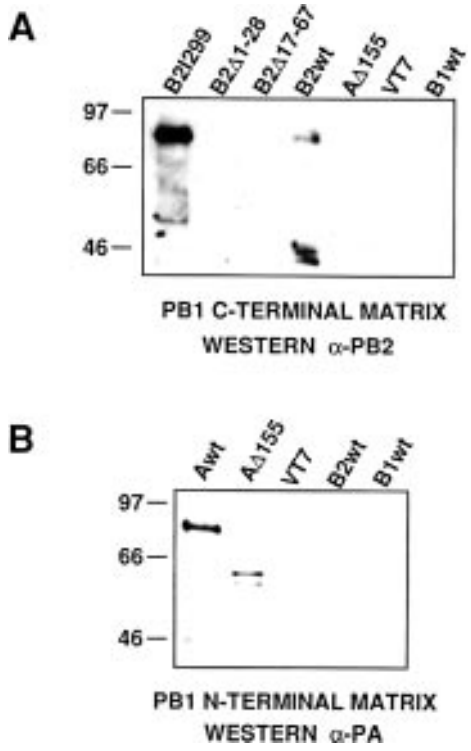

Figure 7. Western blot analysis of the proteins bound to PB1-specific affinity matrices. The proteins retained by the PB1-specific affinity matrices (see Fig. 6 legend for details) were separated by polyacrylamide gel electrophoresis, transferred to Immobilon filters and probed with anti-PB2 (**A**) or anti-PA antibodies (**B**), as indicated in the Figure and described in Materials and Methods.

and further define the interacting domains to the N-terminal 78 amino acids and to the region included within positions 506 and 659 in the protein sequence (Fig. 3).

The body of evidence presented here, together with the results reported earlier (24–26), allow us to propose a preliminary model for the architecture of the influenza virus polymerase, as shown in Figure 8. The N-terminal 78 amino acids of PB1 would be responsible of the binding to PA protein through the C-terminal three quarters of the latter (25), while the PB1 sequences comprised within positions 506–659 would mediate interaction with the first 124 amino acids of PB2 protein (24). A sequence comparison among the PB1 genes of the influenza types A, B and C, as well as the related Dhori and Thogoto viruses, show a number of conserved amino acids within the N-terminal region responsible for the binding of PA protein. The conservation in the 506–659 region of the PB1 protein is less apparent, although some conserved amino acids can be identified scattered throughout. Some of the conserved amino acids have a high propensity to be at the protein surface. Whether they represent contact sites of PB1 and the other polymerase subunits must await further experimentation. It is interesting to note that no overlap is apparent between the different functional regions so far defined in the PB1 subunit, as the nuclear localization signal (NLS) (37), the polymerase motifs (10,11) and the interaction domains responsible for PB2 and PA binding (26 and this report) (Fig. 8). Likewise, there is no overlap between the NLS, the PB1 interacting domain and a presumptive cap-binding region (24) in the PB2 protein. The situation is less clear for the PA subunit of the polymerase, because the majority of the protein seems to be required for interaction with PB1 (25) (Fig. 8).

The structural model presented can be used as a first step in the definition of the contact regions, and eventually the amino acid residues involved in the interaction, as well as an important base line towards the determination of the structure of the complex. However, this model must be considered as preliminary in several regards: (i) it only identifies rough regions of the proteins

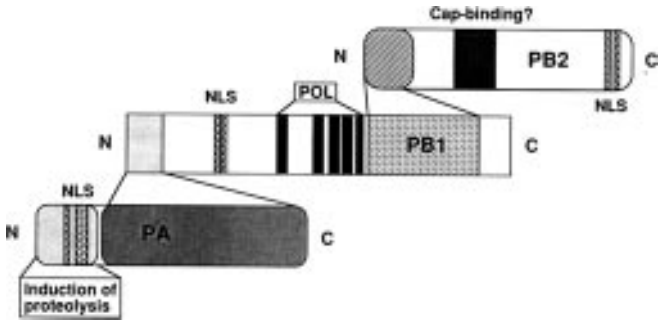

Figure 8. Model for the influenza virus RNA polymerase. The interacting domains of the three polymerase subunits are indicated, together with a series of functional regions of the proteins. See text for details.

involved in the complex formation (specially in relation to the PA subunit). (ii) the experimental evidence used do not allow to establish which sequences in the regions defined participate in interactions and (iii) it only considers interactions of pairs of polymerase subunits, since no testing has been carried out as to the modulation of the pairwise interactions by the third component of the complex. In addition, the model presented is so far a stationary model, since it does not include the possible modulations that could take place at the different steps in which the polymerase is involved, i.e. primary versus secondary transcription, transcription versus replication. To determine whether variations of the architecture presented occur in the course of the virus infection cycle will be a challenge ahead. Furthermore, the proposed model is a minimal one, because it does not consider the possible influences of other viral or cellular factors. Very specially, the influence of the template RNA has not been considered so far. Template RNA is obviously an element required for transcription and replication, that has been reported as essential for cap-snatching (21,38,39). Some cellular factors seem to be involved in the virus-specific RNA synthesis (40). Whether these or other cellular components, as for instance capped-RNAs, alter the structure of the polymerase is not known at present and will constitute future avenues of research.

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