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A 48-Amino-Acid Region of Influenza A Virus PB1 Protein Is Sufficient for Complex Formation with PA†

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The concerted activity of four influenza virus proteins, PB1, PB2, PA, and NP is necessary and sufficient for transcription and replication of the viral genome in the nucleus of the cell. The three P proteins form a heterotrimeric complex in virions and the nuclei of infected cells. Biochemical analyses have shown specific interactions between PB1 and PA as well as PB1 and PB2, indicating that PB1 is the backbone of the complex. To identify domains of PB1 involved in binding PA, a two-hybrid system adapted for mammalian cells (CV-1) was implemented. First, we demonstrate the ability of PB1 and PA to interact efficiently and specifically in reciprocal combinations of two-hybrid reporter moieties, suggesting that transcription factor module fusion did not interfere sterically or allosterically with interaction between PB1 and PA. Subsequent analyses with a set of chimeric proteins with truncations of the PB1 C termini, N termini, or internal sequences led to the identification of a region at the N terminus of PB1 responsible for binding PA. Forty-eight amino acids at the N terminus of PB1 were sufficient for binding PA in vivo with the same efficiency as the complete PB1 protein. This region of PB1 responsible for binding PA does not overlap with other previously described PB1 functional domains involved in nuclear transport and RNA polymerization. We propose to name this region of interaction with PA domain α, to differentiate it from other functional domains described for PB1.

Influenza virus cell entry by endocytosis and envelope fusion releases subviral particles into the cytosol which are transported to the nucleus, the site of influenza virus replication (4, 12, 14, 16, 18). Expression and replication of the influenza virus genome relies on the activity of the viral RNA transcriptase-replicase complex (3, 7). This complex consists of three polypeptides—PB1, PB2, and PA—collectively referred to as P proteins (19, 36). Physical interactions among P proteins have been demonstrated in vitro by coprecipitation with antibodies (1, 7, 20) and by density gradient sedimentation studies (15). P proteins are found associated with each other by noncovalent interactions forming heterotrimers, inside virions and in viral replication complexes within the nucleus as well (20). The P protein complexes are normally associated with viral nucleocapsids, consisting of genomic RNA (vRNA) molecules covered with viral nucleoprotein. These ribonucleoprotein structures are transcription competent and synthesize mRNA if they are supplied appropriate substrate precursors and primers (23). Viral genomic RNA replication is primer independent and takes place only if ribonucleoprotein structures are supplemented nuclear extracts containing undefined host factors (30).

PB1 is the best characterized of the three P proteins; it contains five sequence blocks common to all RNA-dependent RNA polymerases and RNA-dependent DNA polymerases (29, 39). The importance of these motifs for viral mRNA, plus-strand copy RNA, and vRNA synthesis was demonstrated by expression of mutant PB1 proteins in a model system of influenza virus replication in mammalian cells (2). PB2 has cap-binding and endonucleolytic activities which are necessary for viral mRNA synthesis (3, 31, 42). PA is indispensable for proper plus-strand copy RNA and vRNA synthesis, but no specific function in these processes has been assigned to it (17, 37). Bipartite nuclear localization signals have been found in each of the three P proteins (27, 28, 32–34).

Expression of PB1, PB2, and PA proteins from in vitro-transcribed mRNAs microinjected in *Xenopus laevis* oocytes showed specific interaction between PB1 and PA and between PB1 and PB2. Antibodies to each of the P proteins immunoprecipitated complexes of PB1 bound to PB2 as well PB1 and PA complexes (9). Interaction between PB2 and PA was not observed by this approach. Subsequent studies with P proteins expressed by baculovirus recombinants in insect cells confirmed previous results with amphibian cells (40). Complexes of P proteins expressed in insect cells from baculovirus and reconstituted in vitro were capable of transcribing influenza virus ribonucleoprotein templates (22, 27). Furthermore, the P complex has been reconstituted from thioredoxin-renatured P proteins and displayed polymerase activity, albeit limited, when purified vRNAs and nucleoprotein were supplied (41). Thus, it appears that interaction among P proteins is an intrinsic property of these polypeptides; no additional viral proteins are necessary. P proteins are apparently transported to the nucleus after the heterotrimeric complex has formed in the cytoplasm (24). Diverse cellular hosts (mammalian, avian, amphibian, and arthropod) provide environments permissive for P-protein complex formation.

Elucidation of the molecular anatomy of the influenza virus P-protein complex is likely to provide further insight into the functions of each polypeptide in vRNA transcription and replication. Two noncovalent protein-protein interactions are considered essential for the assembly of the transcriptase-replicase, involving separate contacts of PB1 with PA and PB2. Docking of four surfaces, two of which are located in PB1, one in PA, and one in PB2, seems necessary for assembly of the P-protein complex and its subsequent structural and functional integrity. This minimalist model does not account for possible

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modulation of any bimolecular interaction among two given P proteins by attachment of the third P protein.

The purpose of the experiments described in this report was to map the PB1 region mediating association with PA in vivo. To this end, we chose a mammalian cell expression system which circumvents the potentially abnormal behavior of proteins expressed at reduced temperatures or in prokaryotic systems (40). The chosen mammalian two-hybrid strategy (43) for analysis of influenza virus protein-protein interaction in vivo has the added advantage of demonstrating physical association in the nucleoplasm, the site of viral genome replication. We demonstrate the general applicability of a modified mammalian two-hybrid system to study P protein-protein interaction and show that 48 amino acids at the N terminus of PB1 are sufficient to mediate interaction with PA in the nuclei of primate cells (CV-1) in vivo.

MATERIALS AND METHODS

Enzymes and reagents. DNA restriction and modification enzymes were purchased from Promega (Madison, Wis.) and New England Biolabs (Beverly, Mass.). Reagents for DNA sequencing were obtained from United States Biochemical (Cleveland, Ohio). Fetal bovine serum, antibiotic solution (containing penicillin and streptomycin), L-glutamine, sodium butyrate, and acetyl coenzyme A were from Sigma (St. Louis, Mo.). Minimum essential medium (MEM) and the Lipofectamine reagent were bought from GIBCO/BRL (Grand Island, N.Y.), and [^{14}C]chloramphenicol was bought from ICN (Costa Mesa, Calif.). Oligonucleotides were purchased from Ransom Hill (Ramona, Calif.). A polyclonal rabbit antibody to the DNA binding domain of *Saccharomyces cerevisiae* GAL4 was kindly provided by James E. Hopper, Pennsylvania State University. A similarity plot was constructed and secondary structure prediction analysis was performed with the GCG-Wisconsin package.

Cells. CV-1 African green monkey cells (American Type Culture Collection, Rockville, Md.) were maintained in MEM supplemented with 5% (vol/vol) fetal bovine serum. All cloning procedures were carried out in *Escherichia coli* MC1061.

Plasmid constructions. Plasmids pSG424, pAASVVP16, pGALVP16, and pG5EC for testing the two-hybrid system in mammalian cells were kindly provided by H. A. Vasavada and G. N. Nallur (Yale University, New Haven, Conn.) (43). Plasmid pGEM:PB1, encoding influenza virus A/WSN/33 PB1 was generously provided by D. Nayak (University of California at Los Angeles, Los Angeles, Calif.) (2). Plasmid pBSK/PA6 encoding influenza virus A/Wilson-Smith/33 (A/WSN/33) PA was a gift from T. Odagiri (Jichi Medical School) (35). DNA amplification of PB1 and PA open reading frames from pGEM:PB1 and pBSK/PA6 templates was performed by PCR using the Vent DNA polymerase according to the manufacturer's instructions. Primers for PB1 were 5′-AAAGATCT TAATGGATGTCAATCCGACTT-3′ and 5′-TTGGGGATCCAGGAGTGTG TTG-3′. Primers for PA were 5′-CTGGATCCGAATGGAAGATTTTGTGCG AC-3′ and 5′-GCGGATCCTATCTCAATGCATGTGTGAG-3′. pSGPB1t was obtained by insertion of PB1 PCR-amplified DNA digested with *Bgl*II and *Bam*HI into the unique *Bam*HI site of pSG424 (see Fig. 2A). pSGPB1t encodes the N-terminal 654 amino acids of PB1 fused to the C terminus of the GAL4 DNA binding domain (GAL4 DBD) (amino acid residues 1 to 147). A *Bam*HI-*Sal*I fragment encoding the last 103 amino acids of PB1 was obtained from pGEM:PB1 and cloned into the unique *Bam*HI-*Sal*I sites of pSGPB1t, generating plasmid pSGPB1c (Fig. 1A). pVP16PB1t was obtained from a *Bgl*II-*Bam*HI-digested PB1 PCR fragment cloned into pAASVVP16 at the unique *Eco*RI site (Fig. 1A). pVP16PB1t contains the first 654 amino acids of PB1 protein fused to the VP16 activation domain C terminus (VP16 AD) (amino acid residues 413 to 490). pSGPA was obtained by ligation of the PCR-amplified product of a PA-encoding plasmid with *Bam*HI cohesive ends, cloned into a *Bam*HI-digested pSG424. pVP16PA was obtained from a *Bam*HI-treated PA PCR product cloned into an *Eco*RI-digested pAASVVP16. pSGPA and pVP16PA encode an intact PA protein (716 amino acids) fused to the C terminus of the GAL4 DBD and VP16 AD, respectively (Fig. 1A).

PB1 C-terminal-deletion chimeric proteins. *Kpn*I-*Sal*I-digested pSGPB1t DNA was treated with exonuclease III and mung bean nuclease to generate unidirectional deletions extending from the 3′ end of the PB1 coding sequence, as described by Henikoff (13). The extent of the deletion was analyzed by DNA sequencing (38). Three mutants were selected for further analysis: pSGPB1-1201, pSGPB1-980, and pSGPB1-961 (Fig. 2A). Two other 3′ deletion mutants were produced by cleaving pSGPB1t with *Afl*II-*Sal*I or *Acc*I-*Sal*I and filling in with Klenow to generate deletion mutants pSGPB1/3′AflII and pSGPB1/3′AccI, respectively. pSGPB1/3′ScaI was obtained by subcloning a *Hin*dIII-*Sca*I fragment encoding the first 48 amino acids of PB1 (PB1CΔ48) fused to the GAL4 DBD (GAL4PB1CΔ48) into pSG424 digested with *Hin*dIII and *Sma*I (see Fig. 4A). pVP16PB1/3′ScaI was generated by subcloning an *Eco*RI fragment from pSGPB1/3′ScaI, encoding PB1CΔ48, into an *Eco*RI-digested vector, pAASVVP16.

PB1 internal deletions. pSGPB1idmSca/Afl was obtained by subcloning a *Hin*dIII-*Sca*I fragment containing GAL4PB1CΔ48 from pSGPB1 into an *Afl*II-*Hin*dIII vector fragment from pSGPB1. pSGPB1-961idmAfl/Acc was assembled by cleaving pSGPB1-993 with *Afl*II and *Acc*I, blunting the recessed 3′ ends by polymerization with Klenow enzyme, and religating. pSGPB1idmBcl/Bam was obtained by directional cloning of a *Xho*I-*Bcl*I fragment from pSGPB1-993 into the unique *Xho*I-*Bam*HI sites of pSGPB1c (Fig. 3A).

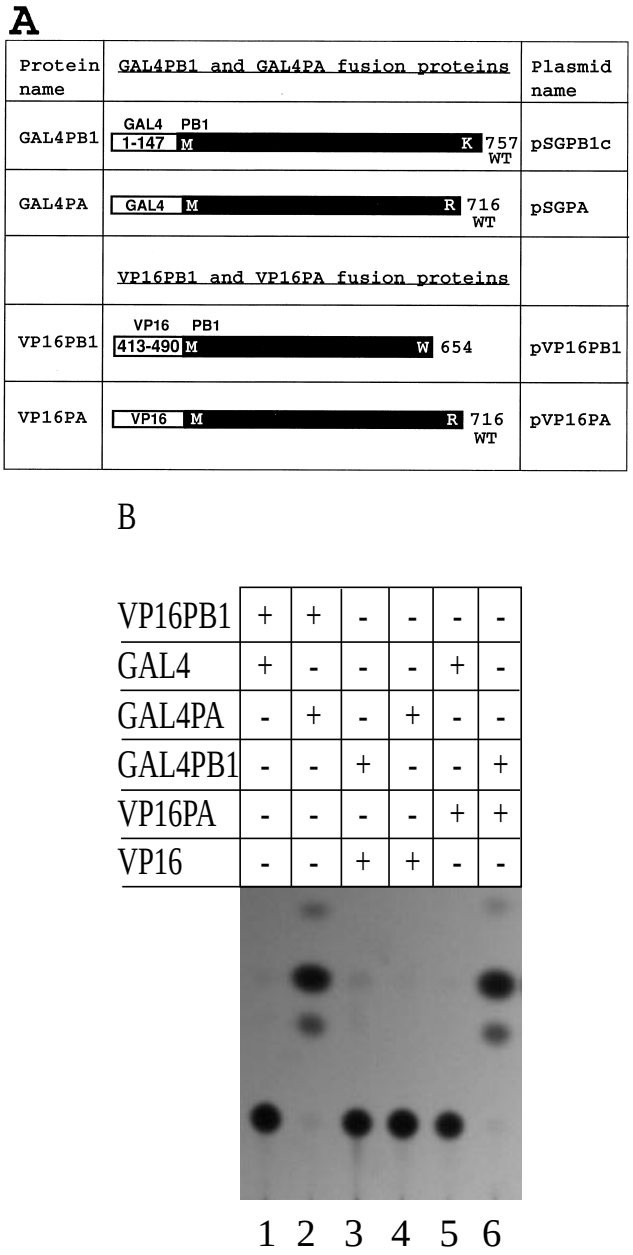
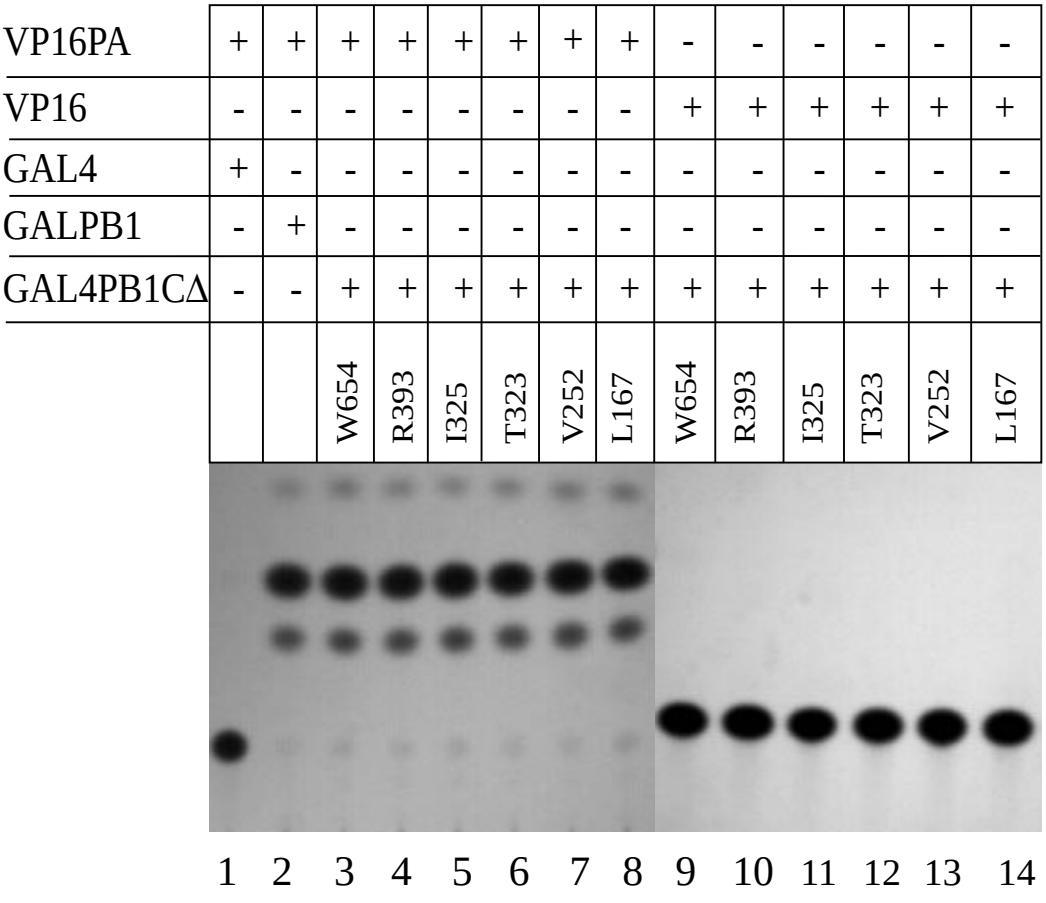


FIG. 1. (A) Details of PB1 and PA fusion proteins used. Filled boxes, PB1 and PA; open boxes, GAL4 DBD and VP16 AD. PB1 and PA were fused to the C terminus of the GAL4 DBD (GAL4PB1 and GAL4PA) and to the C terminus of the VP16 AD (VP16PB1 and VP16PA). Plasmids were transfected as described in Materials and Methods. (B) CAT activity assays of CV-1 cell lysates harvested at 60 h after transfection with the reporter plasmid DNA encoding CAT and plasmid DNAs for VP16PB1 and GAL4 (lane 1), VP16PB1 and GAL4PA (lane 2), GAL4PB1 and VP16 (lane 3), GAL4PA and VP16 (lane 4), GAL4 and VP16PA (lane 5), and GAL4PB1 and VP16PA (lane 6). CAT assay images were captured with a video camera and Image 1.49 software. Files were edited with Aldus Freehand on a Macintosh IIfx computer.

A

Protein name	GAL4PB1 carboxy-terminal deletion mutants	Plasmid name	PA Binding
GAL4PB1	GAL4 PB1 1-147 M ... K 757 WT	pSGPB1c	+
PB1CΔW654	GAL4 M ... W 654	pSGPB1t	+
PB1CΔR393	GAL4 M ... R 393	pSGPB1 1201	+
PB1CΔI325	GAL4 M ... I 325	pSGPB1 980	+
PB1CΔT323	GAL4 M ... T 323	pSGPB1 961	+
PB1CΔV252	GAL4 M ... V 252	pSGPB1/3′ AccI	+
PB1CΔL167	GAL4 M ... L 167	pSGPB1/3′ AflII	+

B



PB1 amino-terminal deletions. 5′ deletion mutants were constructed by cleavage with appropriate restriction enzymes and, depending upon the construct, filled in with Klenow fragment. The correct frame at the junctions of 5′ and internal PB1 deletion mutants was verified by DNA sequencing as described elsewhere (38). Plasmids obtained were pSGPB1/5′EcoRI, pSGPB1/5′AccI, pSGPB1/5′AflII, and pSGPB1/5′NcoI. A *Xho*I-*Bam*HI fragment from pSGPB1/5′NcoI was cloned into a *Xho*I-*Bam*HI-digested pSGPB1c to obtain pSGPB1/5′NcoIc (Fig. 3A).

Transfection procedures. CV-1 cells (5×10^5 cells per well) were plated on 35-mm dishes (Costar, Cambridge, Mass.) 2 to 4 h before transfection. Optimal transfection efficiency was achieved with 2 μg of total DNA and 24 μg of the Lipofectamine reagent per well in 1.2 ml of MEM. DNA-Lipofectamine complexes were left in contact with cells for 6 h at 37°C according to the manufacturer's directions (GIBCO/BRL). After 6 h of transfection, cells were supplemented with 5% fetal bovine serum–MEM and 5 mg of sodium butyrate per ml for 12 h. Eighteen hours posttransfection, DNA-Lipofectamine- and sodium butyrate-containing medium was removed and replaced with fresh 5% fetal bovine serum–MEM.

CAT assay. Cells were harvested 60 h after the start of transfection, and chloramphenicol acetyltransferase (CAT) assays were performed as described elsewhere (11). Approximately 100 μg of total protein was assayed for CAT activity.

RESULTS

In vivo interaction between PB1 and PA proteins fused to reporter moieties. A two-hybrid system developed by Vasavada et al. (43) to study protein-protein interaction in mammalian cell nuclei was used to dissect part of the molecular anatomy of the influenza virus P-protein complex. This mammalian system is analogous to the yeast system described by Fields and Song and relies on the modular nature of transcriptional activators GAL4 and VP16 (10). PB1 and PA subunits, known to form heterodimers, were fused to two-hybrid transcription factor modules to determine if they yield chimeric polypeptides which interact and transactivate a reporter gene under the control of the cognate promoter, GAL4. Influenza A/WSN/33 virus genes encoding PB1 and PA were cloned in plasmids as GAL4 and VP16 fusion proteins. Two plasmids were obtained, with the complete open reading frames of PB1 (757 codons) and PA (716 codons) fused to the 3′ end of the gene encoding the yeast transcription factor GAL4 DBD (codons 1 to 147; Fig. 1A). PA (716 codons) and 3′-truncated-PB1 (codons 1 to 654) genes were also fused 3′ of the gene encoding the herpes simplex virus VP16 AD (codons 413 to 490; Fig. 1A).

Plasmids encoding DBD and AD domains fused to P proteins were cotransfected into CV-1 cells with a reporting plasmid featuring a CAT transcription unit controlled by five tandem GAL4 promoter elements. Expression and nuclear translocation of two interacting hybrid proteins are expected to transactivate the GAL4 promoter and produce CAT. Transfected CV-1 monkey cells harvested at 60 h posttransfection were examined for CAT expression. Presence of CAT activity in lysates indicated that PB1 and PA interacted efficiently in this two-hybrid system (Fig. 1B, lanes 2 and 6). CV-1 cells cotransfected with GAL4PB1 and VP16PA expressed CAT activity levels equivalent to those of the reciprocal pair VP16PB1 and GAL4PA, demonstrating that interaction between PB1 and PA was independent of the fused reporting polypeptide module (VP16 AD or GAL4 DBD).

Interactions between the two chimeric P proteins not resulting from direct binding of PA to PB1 could lead to false interpretation of GAL4 transactivation. Thus, it is paramountly important to demonstrate absence of spurious interactions between influenza virus P proteins and one of the transcription reporter modules as well as transactivation functions by the influenza virus proteins themselves. To this end, each of the four chimeric proteins was cotransfected with the complementary module of the two-hybrid system lacking a fused influenza virus P-protein domain. CAT activity was absent when the following pairs were expressed: GAL4PB1 and VP16 AD, GAL4PA and VP16 AD (Fig. 1B, lanes 3 and 4), VP16PB1 and GAL4, and VP16PA and GAL4 (Fig. 1B, lanes 1 and 5).

In vivo interaction between PB1 and PA is in agreement with previous biochemical evidence demonstrating the intimate association between PB1 and PA in the polymerase complex of influenza virus and validates the two-hybrid system for investigating this particular interaction in more detail (9). PB1 and PA were very robust as chimeras in the two-hybrid system inasmuch as heterodimer interaction interfaces were preserved in fusions to either GAL4 DBD or VP16 AD, substantiating the flexibility of this mammalian two-hybrid system for studying PB1-PA interactions in vivo.

The N terminus of PB1 interacts with PA. To identify the boundaries of the PB1 domain(s) interacting with PA, we tailored plasmids with three types of GAL4PB1 deletion mutants: plasmids encoding PB1 proteins with C-terminal deletions (CΔ), N-terminal deletions (NΔ), and internal deletions (iΔ) as described in Materials and Methods. Six different PB1 mutants with C-terminal deletions were examined: PB1CΔW654, PB1CΔR393, PB1CΔI325, PB1CΔT323, PB1CΔV252, and PB1CΔL167 (Fig. 2A). Surprisingly, all PB1 constructs with C-terminal deletions were equally effective in binding VP16PA (Fig. 2B, lanes 3 to 8). These results suggested that 167 amino acids at the N terminus of PB1 suffice for binding PA. The specificities of these interactions were subsequently tested with pairwise combinations of plasmids containing PB1CΔ and a plasmid encoding VP16 AD alone (Fig. 2B, lanes 9 to 14). CAT activity was not detected in cells cotransfected with PB1CΔ mutants and VP16 AD.

Amino-terminal deletions of PB1 protein abrogated binding to PA. To delimit the N-terminal region of PB1 involved in binding PA, five plasmids encoding PB1 mutant proteins with N-terminal deletions were constructed. Deletion mutants PB1NΔ M40c, PB1NΔ M40, PB1NΔ K168, PB1NΔ Y253, and PB1NΔ E479 (Fig. 3A) showed no interaction with PA (Fig. 3B, lanes 3 to 6 and 11). Interestingly, deletion of 40 amino acids at the amino terminus of PB1 was sufficient to abolish binding of PB1 to PA (Fig. 3B, lanes 6 and 11), suggesting that a pocket for interaction with PA might be located within the N-terminal end of PB1. However, aberrant targeting, instability, or misfolding of PB1 N-terminal deletion chimeras could also account for lack of transactivation of the CAT reporter and needed to be investigated. Steady-state nuclear levels of chimeric PB1 proteins with a truncated N terminus (PB1NΔ) in transfected cells were analyzed by Western blots (immunoblots) probed with an antibody to GAL4 DBD. Nuclear fractions of transfected CV-1 cells lysed at 60 h posttransfection contained PB1NΔ chimeric proteins in concentrations comparable to those of intact or PB1CΔ chimeras (data not shown).

FIG. 2. (A) GAL4PB1 chimeric proteins with C-terminal deletions (CΔ). Six different PB1 chimeric proteins were tested. PB1CΔ chimeric proteins were W654, R393, I325, T323, V252, and L167 (letters indicate the last PB1 amino acid encoded in the fusion protein, and numbers mark the position of each amino acid in the intact PB1 protein sequence). Binding to PA is indicated on the right. WT, wild type. (B) CAT assays from extracts of cells transfected with pairwise combinations of plasmids encoding GAL4 and VP16PA (lane 1; negative control), GAL4PB1 and VP16PA (lane 2; positive control), and VP16PA cotransfected with GAL4PB1CΔ W654 (lane 3), R393 (lane 4), I325 (lane 5), T323 (lane 6), V252 (lane 7), or L167 (lane 8). The specificity of the interaction was investigated by cotransfection of plasmids encoding VP16 AD and GAL4PB1CΔ mutants (lanes 9 to 14).

A

Protein name	GAL4PB1 amino-terminal and internal deletion mutants	Plasmid name	PA Binding
PB1NΔM40	GAL4 – PB1 M40 … W 654	pSGPB1 /5′NcoI	-
PB1NΔK168	GAL4 – K168 … W 654	pSGPB1 /5′AflII	-
PB1NΔY253	GAL4 – Y253 … W 654	pSGPB1 /5′AccI	-
PB1NΔE479	GAL4 – E479 W 654	pSGPB1 /5′EcoRI	-
PB1iΔ167/252	GAL4 M L 167 V252 T 323	pSGPB1-961 idmAfl/Acc	+
PB1iΔ48/168	GAL4 M Q48 K168 … W 654	pSGPB1idm Sca/Afl	+
PB1NΔM40c	GAL4 – M40 … K 757	pSGPB1 /5′NcoIc	-
PB1iΔ324/655	GAL4 M 324 I655 K 757	pSGPB1idm Bcl/Bam	+

B

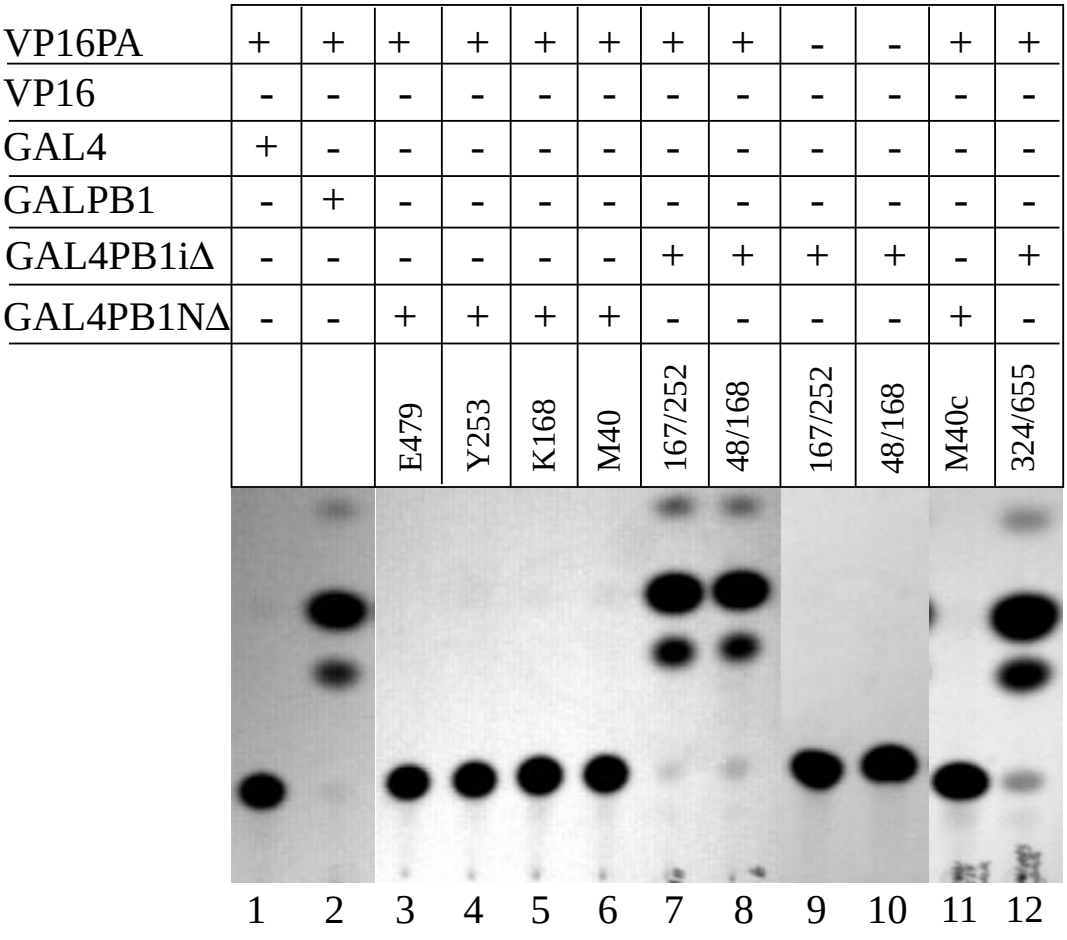


FIG. 3. (A) Plasmids encoding GAL4PB1 proteins with N-terminal (NΔ) and internal deletions (iΔ). Binding to PA (+) and absence of binding to PA (−) are indicated on the right. (B) Five N-terminal-deletion (GAL4PB1NΔ M40c [lane 11], M40 [lane 6], K168 [lane 5], Y253 [lane 4], and E479 [lane 3]) and three internal-deletion (GAL4PB1iΔ167/252 [lane 7], GAL4PB1iΔ48/168 [lane 8], and GAL4PB1iΔ324/655 [lane 12]) chimeric proteins were cotransfected with VP16PA. The specificity of the interaction for GAL4PB1iΔ167/252, GAL4PB1iΔ48/168, and GAL4PB1iΔ324/655 was tested by cotransfection with VP16 AD (lanes 9 and 10 and data not shown).

Thus, failure of truncated PB1NΔ chimeric polypeptides to interact with PA is not a consequence of nuclear transport defects, reduced expression, or accelerated degradation. Evidence indicating that the PB1 N terminus was necessary for interaction led us to engineer short deletions C terminal to this region to minimize distortion of possible higher-order structures of unknown importance. PB1iΔ167/252, PB1iΔ48/168, and PB1iΔ324/655 (Fig. 3A) showed levels of interaction similar to that for the complete PB1 protein (Fig. 3B, lanes 7, 8, and 12). Deletion mutant PB1iΔ48/168 contains the first 48 amino acids of PB1 followed by an internal deletion of 120 amino acids and shows efficient interaction with PA. In contrast, PB1NΔ M40c (Fig. 3B, lane 11) with a deletion of its N-terminal 40 amino acids is unable to bind PA. Thus, a 48-amino-acid region at the amino terminus of PB1 is predicted to be sufficient for PA binding. The data for the PB1 internal-deletion mutants also suggested relative independence of the N-terminal region of PB1 from adjacent sequences for interaction with PA.

A short domain at the N terminus of PB1 is sufficient for binding PA. To test the hypothesis that a 48-amino-acid peptide sequence at the amino terminus of PB1 was sufficient to bind PA, we constructed plasmids expressing GAL4PB1CΔ48 and VP16PB1CΔ48 chimeric proteins (see Fig. 4A). These chimeras consist of the first 48 amino acids of PB1 (from 1 to Q-48) fused to the GAL4 DBD or VP16 AD, respectively. Cotransfection of pairwise combinations of plasmids encoding GAL4PB1CΔ48 and VP16PA or VP16PB1CΔ48 and GAL4PA showed that the N terminus of PB1 was able to bind PA, as revealed by transactivation of CAT expression (Fig. 4B, lanes 1 and 3). The nature of the reporter moiety linked to the N-terminal 48-amino-acid domain of PB1 did not affect PA binding. GAL4PB1CΔ48 or VP16PB1CΔ48 fusions could reconstitute the transactivator and produce levels of CAT activity similar to that for the whole PB1 protein chimera, suggesting a remarkable structural and functional independence for this 48-amino-acids fragment of PB1 (data not shown). To facilitate discussion of different PB1 functional regions, we propose to name the region responsible for interaction with PA "domain α."

DISCUSSION

PB1 and PA interact with each other to form the influenza virus transcription complex, but the domain of PB1 responsible for this interaction was unknown. As shown above, 48 amino acids at the N terminus of PB1 were sufficient for binding PA in vivo (Fig. 4) with the same efficiency as the entire PB1 protein. Efficient interactions between PB1 and PA in the two-hybrid system whether fused to GAL4 or VP16 suggest an absence of spurious interactions arising fortuitously as a consequence of the chimeric nature of the proteins. Our results, however, do not preclude a role for other domains of PB1 in modulating interaction with PA in influenza virus-infected cells. In addition, the influence of PB2 in the PB1-PA interaction process remains to be studied.

The three P proteins remain stably associated with template RNA as the complex moves from the 3′ end towards the 5′ end during transcription and replication (3). The PB1 subunit is the

A

Protein name	PB1CΔQ48 mutant	Plasmid name
GAL4PB1CΔ48	PB1: GAL4 — M Q48	pSGPB1/3'ScaI
VP16PB1CΔ48	PB1: VP16 — M Q48	pVP16PB1/3'ScaI

B

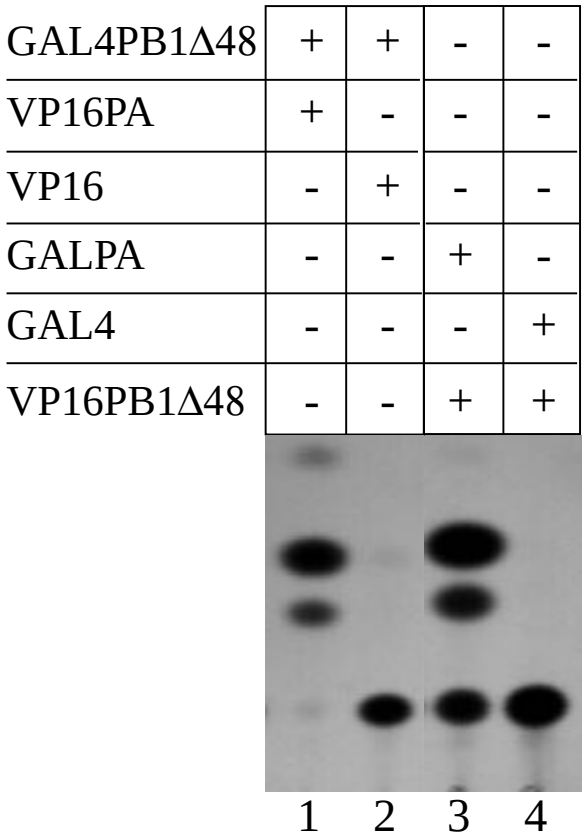


FIG. 4. (A) Plasmid constructions for expression of PB1 protein fusions. The N-terminal 48 amino acids (M-1 to Q-48) of PB1 were fused to the C terminus of GAL4 DBD (GAL4PB1CΔ48) and to the C terminus of VP16 AD (VP16PB1CΔ48). (B) Lysates of CV-1 cells transfected with pG5EC and pairwise combinations of the following plasmids were assayed for CAT activity: GAL4PB1CΔ48 and VP16PA (lane 1), GAL4PB1CΔ48 and VP16 AD (lane 2), GAL4PA and VP16PB1CΔ48 (lane 3), and GAL4 and VP16PB1CΔ48 (lane 4).

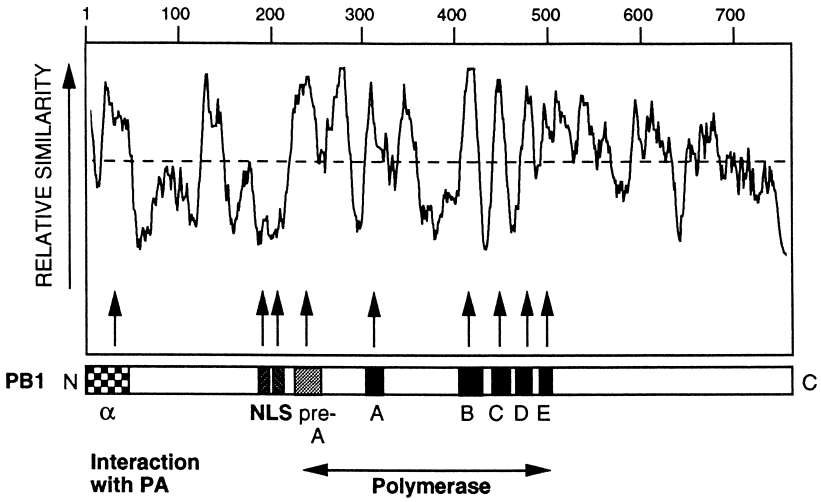


FIG. 5. Amino acid sequence similarity plot of influenza A, B, and C virus PB1 proteins. A/WSN/33, B/Ann Arbor/1/66, and C/JJ/50 PB1 sequences were aligned, and the similarity was calculated and plotted for a window of 10 amino acids with programs in the GCG software package as described elsewhere (5, 8, 38, 43). Dashed line, average level of overall similarity in the plot. The following functional domains and sequence motifs are shown below as boxes within a linear representation of the PB1 protein: α region, nuclear localization signal (NLS), and RNA-dependent RNA polymerase conserved motifs pre-A through E (2, 31). NLSs are not well conserved while the α region and polymerase motifs are highly conserved among influenza virus types A, B, and C (vertical arrows).

core of the polymerase complex, being responsible for RNA chain elongation. Several putative functional domains have been identified in PB1. Influenza A virus PB1 expressed in the absence of other viral products accumulates in the nucleus of the cell. A bipartite nuclear localization signal extending from residue 187 to 196 and from residue 203 to 216 is responsible for nuclear translocation (Fig. 5) (32). Five domains in PB1, designated A to E, are common to all RNA-dependent RNA polymerases and RNA-dependent DNA polymerases (Fig. 5) (29). Conserved residues within motifs A (residue 303) through D (residue 490) are thought to be involved in synthesis of mRNA, plus-strand copy RNA, and vRNA segments and include the canonical SDD motif (2). Single amino acid substitutions within motifs A, B, and D also render the protein inactive in transcription. An additional motif in RNA-dependent polymerases of retroviruses and positive-strand RNA viruses, called premotif A, has been described recently for negative-strand RNA viruses (29). Premotif A would be located between residues 230 and 260 in PB1 of influenza A viruses and may be required for proper positioning of vRNA molecules during transcription and replication. The N-terminal region of PB1 described herein, responsible for binding PA, does not overlap with previously described PB1 domains. We propose to name this region of interaction with PA "domain α," to differentiate it from other functional domains described for PB1.

Although further refinement of α-domain boundaries within the first 48 amino acids of PB1 is possible, several important concepts emerge from the data presented. PB1 exhibits the highest level of amino acid sequence conservation among influenza A, B, and C viruses. Influenza A/WSN/33 and B/Lee/40 virus PB1 proteins are 61% identical (21). The PB1 protein of influenza C/JJ/50 virus nears 40% sequence identity with the corresponding proteins of influenza A and B viruses (44). Three-way alignment of amino acid sequences among influenza A, B, and C viruses reveals blocks of 10 to 30 amino acids which are virtually identical in the three influenza virus types (not shown). A similarity plot of the comparison of these viruses reveals that polymerase-related motifs map to six areas of conserved sequences (Fig. 5). Six additional areas of high conservation lacking assigned functions can be identified in the plot. Among these, one of the three largest peaks maps to a region between PB1 residues 17 and 47 (Fig. 5), which is located within region α (Fig. 5). Compared with influenza B and C virus PB1 amino acid sequences, the influenza A virus PB1 is 75 and 57% identical within region α, respectively. P-protein interactions of influenza virus types B and C remain to be elucidated; it will be of interest to determine if they are analogous to those of influenza virus A. Secondary structure analysis of the PB1 α domain predicts the presence of a β-pleated sheet towards the amino terminus coincident with the presence of a hydrophobic amino acid-rich region. The carboxy-terminal region of domain α is a proline- and glycine-rich hydrophilic region where several turns are predicted and surface exposure is likely. In the PB1 proteins of the three influenza virus types, this small region is characterized by a high degree of amino acid sequence conservation, especially for amino acids with bulky side chains. Consequently, the PB1 region α may possess conserved structural features allowing for complex formation with PA proteins from orthomyxoviruses of different types. PB1 from a distant orthomyxovirus of insects, Dhori virus, has 37% identity and 67% similarity to the influenza A virus in the C-terminal 24-amino-acid portion of the PB1 α region (residues 24 to 48) (25). Clustering of conservation towards the C-terminal end of the α region suggests that the core of the interaction may reside between residues 17 and 47 of PB1. Such a remarkable level of conservation in the α domain is comparable only to RNA polymerase-related motifs and two other regions centered around residues 135 and 275 of unknown function. Some of these regions of unassigned function could participate in interactions leading to complex formation with PB2. Conservation of primary structure in the α region argues for important functional constraints limiting divergence in this region of influenza virus PB1 polymerase proteins. It is conceivable that this constraint is imposed by the need to recruit PA to assemble functional polymerase-replicase complexes. PA is thought to be required for adequate plus-strand copy RNA and vRNA synthesis. The specific role(s) of PA may include either primer-independent transcription initiation or helicase functions (6, 31).

A pharmacological strategy aimed at blocking PB1-PA in-

teraction would prevent P-protein complex formation and viral replication. Using a peptidomimetic drug, Liuzzi et al. could inhibit ribonucleotide reductase homodimer formation and, in consequence, herpes simplex virus growth in cultured cells (26). Similar approaches directed at inhibiting PB1-PA heterodimer formation may lead to identification of candidate agents for controlling influenza virus replication and disease.

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