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Mutational Analysis Identifies Functional Domains in the Influenza A Virus PB2 Polymerase Subunit

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A collection of influenza A virus PB2 mutant genes was prepared, including N-terminal deletions, C-terminal deletions, and single-amino-acid insertions. These mutant genes, driven by a T7 promoter, were expressed by transfection into COS-1 cells infected with a vaccinia virus encoding T7 RNA polymerase. Mutant proteins accumulated to levels similar to that of wild-type PB2. Immunofluorescence analyses showed that the C-terminal region of the protein is essential for nuclear transport and that internal sequences affect nuclear localization, confirming previous results (J. Mukaijawa and D. P. Nayak, J. Virol. 65:245–253, 1991). The biological activity of these mutants was tested by determining their capacity to (i) reconstitute RNA polymerase activity in vivo by cotransfection with proteins NP, PB1, and PA and a virion-like RNA encoding the *cat* gene into vaccinia virus T7-infected COS-1 cells and (ii) compete with the wild-type PB2 protein in this reconstituted system. These experiments showed that many of the mutants behaved as dominant negative; i.e., they were unable to induce *cat* gene expression but interfered with wild-type PB2 activity. In addition, when tested at different temperatures in vivo, two mutant PB2 proteins showed a temperature-sensitive phenotype. The lack of interference shown by some N-terminal deletion mutants and the complete interference obtained with a C-terminal deletion mutant encoding only 124 amino acids indicated that this protein domain is responsible for interaction with another component of the polymerase, probably PB1. To further characterize the mutants, their ability to induce in vitro synthesis of viral cRNA or mRNA was tested by using ApG or β-globin mRNA as a primer. One of the mutants, I299, containing an isoleucine insertion at position 299, was able to induce cRNA and mRNA synthesis in ApG-primed reactions but required a higher β-globin mRNA concentration than wild-type PB2 for detection of in vitro synthesis. This result suggested that mutant I299 has diminished cap-binding activity.

The genetic information of influenza type A viruses is encoded in eight single-stranded RNA molecules with negative polarity. Together, they encode 10 different proteins, one in each RNA segment, except for the two smallest, which code for 2 proteins each (for a review, see reference 24). Expression and replication of the viral genome require, at least, the action of the three subunits of the polymerase (PB1, PB2, and PA) and the nucleoprotein (NP) (8, 18, 21, 31).

The parental viral ribonucleoproteins are transcribed in the nucleus of the infected cell by using a cap-snatching mechanism of initiation (22). Polyadenylation of the mRNAs takes place by stuttering of the transcriptase at an oligo(U) signal close to the 5′ end of the template (46), probably as a consequence of the panhandle structure of the viral ribonucleoprotein (17, 28). Replication of the viral ribonucleoproteins requires viral protein, at least NP (51), synthesis and also takes place in the cell nucleus (19, 27, 51).

The roles of the three subunits of the polymerase in these processes have been partly unraveled. Sequence comparisons have revealed that the PB1 gene contains all of the conserved protein motifs present in other RNA virus polymerases (45), and site-directed mutagenesis has indicated that these sequences are essential for polymerase activity (5). This subunit is probably responsible for nascent chain elongation (7). Although no specific role for the PA subunit has been reported, the phenotype of temperature-sensitive (TS) mutants with mutations in the gene for PA implicates it in viral RNA synthesis (reviewed in reference 29). Recently, a general proteolysis induced in cells expressing the PA protein by transfection has been described (49).

The PB2 subunit has been shown to interact with cap-1 structures (6, 55) and presumably recruits capped RNA in the nucleus of the infected cell (7). It has been suggested that the PB2 subunit acts as an endonuclease in the generation of transcription primers (26), but the enzymatic activity requires the association of all three polymerase subunits and both template RNA ends (16), supporting the notion that the active viral ribonucleoproteins are in a panhandle structure (17). A number of reports have described the isolation and characterization of TS PB2 gene mutants (23, 25, 29, 30, 34, 50, 54). These mutants showed a block in mRNA synthesis and hence in virion RNA and protein accumulation, and one of the mutants described (34) showed an altered pattern of mRNA synthesis at the permissive temperature. Moreover, a monoclonal antibody specific for the PB2 subunit inhibited capped-RNA-dependent in vitro transcription by interference at the initiation step (4). Together, these results suggest that the PB2 subunit is directly involved in viral RNA transcription. Although it has been recently reported that the PB2 subunit is not an absolute requirement for viral RNA synthesis, but rather is involved in the generation of viral capped mRNAs (36), we have recently shown that this subunit is necessary for synthesis of both mRNA and cRNA in vitro (43). On the other hand, a host range mutation has been mapped to the PB2 gene (53) and the use of the host range mutant has allowed the construction of transfectant viruses in which the PB2 gene was replaced with an in vitro-generated mutant gene (52), opening

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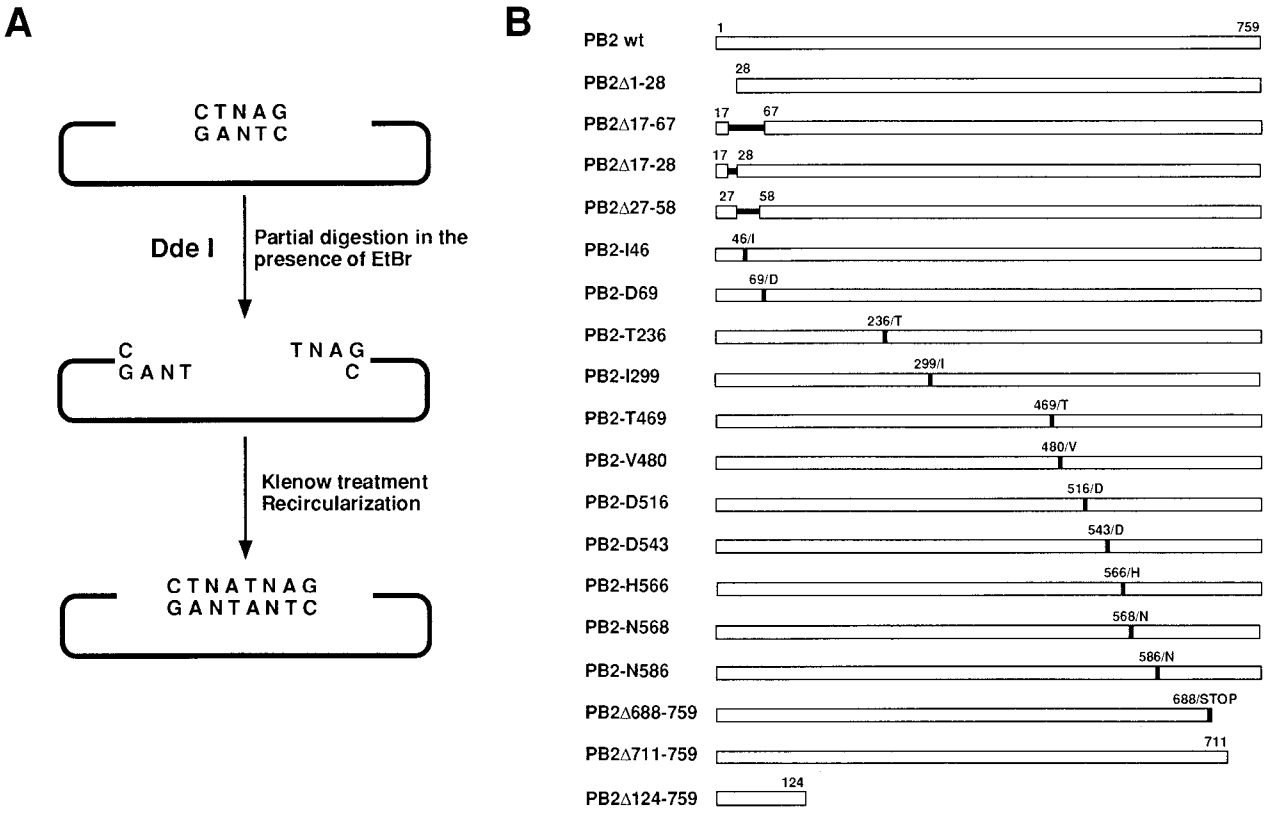

FIG. 1. Construction of PB2 gene mutants. (A) Diagram of the technique used to generate single-amino-acid insertion mutants. Although a single restriction nuclease site is shown, the target plasmid may contain many sites. See the text for details. Et Br, ethidium bromide. (B) Map of the mutants produced. The last residues present in the deletion mutants, are indicated. For the insertion mutants, the position and nature of the inserted residue are shown. In mutants PB2Δ688-759 and PB2Δ124-759, a stop codon was inserted.

the way for the development of engineered attenuated influenza virus strains.

We have described (31, 32) the in vivo reconstitution of an influenza virus transcription-replication system and its use for the synthesis of viral RNA in vitro (43). These systems are based on the expression of each polymerase subunit and the NP by plasmid transfection into cells infected with a T7 RNA polymerase-expressing vaccinia virus (14). These systems are very amenable to gene manipulation and allow the dissection of viral gene functions in relation to the influenza virus transcription and replication processes. In this report, we present the construction of a set of PB2 gene insertion and deletion mutants and their phenotypic characterization, including their intracellular localization, their activity, and their interference in the chloramphenicol acetyltransferase (CAT) assay system, as well as their capacity to estimulate the synthesis of viral mRNA and cRNA in vitro.

MATERIALS AND METHODS

Biological materials. The generation of plasmids pGPB1, pGPB2, pGPA, and pGNP from the original cDNA clones (9, 39) has been described previously (31). Plasmid pIVACAT1-S (44) was kindly provided by P. Palese. The vaccinia virus recombinant vTF7-3 (14), capable of expressing bacteriophage T7 RNA polymerase, was kindly provided by B. Moss. The preparation and characterization of rabbit serum and mouse monoclonal antibodies specific for the PB2 polymerase subunit have been described previously (4).

DNA manipulation and cloning. Except when indicated otherwise, standard conditions were used for DNA restriction, isolation, and ligation and *Escherichia coli* transformation (47). The construction of PB2 gene deletion mutants was carried out as follows: mutant PB2Δ17-67 was generated by ligation of the *Asp*700-*Sal*I fragment (positions 241 to 2367) of plasmid pGPB2 into the same vector digested with *Nru*I and *Sal*I. Mutant PB2Δ17-28 was constructed by ligation of the *Nde*I fragment (filled in with Klenow)-*Sal*I fragment (positions 121 to 2367) of plasmid pGPB2 into the same vector digested with *Nru*I and *Sal*I. Likewise, the generation of mutant PB2Δ27-58 was carried out by ligation of the *Pvu*II-*Sal*I fragment (positions 216 to 2367) into plasmid pGPB2 digested with *Nde*I (filled in with Klenow) and *Sal*I. Amino-terminal deletion mutant PB2Δ1-28 was produced by digesting plasmid pGBP2 with *Nde*I and *Asp*718, filling in both termini with Klenow, and ligating at a low DNA concentration to circularize the DNA. Carboxy-terminal deletion mutant PB2Δ124-759 was generated by site-directed mutagenesis (Clontech Transformer kit) with the oligonucleotide 5′-CCAAAGGTTCAATGTTTTCACCTTTCG-3′. This introduced in-frame termination codons at positions 125 and 128 in the PB2 protein sequence. For the generation of single-amino-acid insertion mutants, an adaptation of the procedure of Barany (3) was used, as indicated schematically in Fig. 1A. This procedure does not rely on the use of linkers and allows the insertion of single codons. Plasmid pGPB2 was digested with limiting amounts of restriction endonuclease *Dde*I or *Hin*fI for 15 min at 37°C. The precise amounts of enzyme able to produce partial digestion with a sizable fraction of linear plasmid DNA was determined after a dose-effect trial experiment. By using such a limiting amount of enzyme, a second trial experiment was performed to determine the amount of ethidium bromide in the reaction mixture sufficient to ensure that only one cut was introduced per DNA molecule (42). The plasmid DNA linearized under these conditions was isolated from an agarose gel, the ends were filled in with the Klenow fragment of DNA polymerase, and the DNA was recircularized at a low DNA concentration (approximately 2 ng/ml). The background of uncleaved plasmid DNA was controlled in a separate reaction without DNA ligase. DNA preparations from the recircularized plasmids were characterized by complete digestion with either restriction endonuclease *Dde*I or *Hin*fI, and those showing a mutant phenotype (generation of a new restriction fragment, presumably fusion of two contiguous ones) were analyzed further by sequencing. Occasionally, the ligation was not accurate and a nucleotide was deleted, leading to a frameshift. This was the case with mutant PB2Δ711-759, in which a termination codon occurred at position 711 in protein PB2 (Fig. 1B). Mutant plasmids were sequenced by the dideoxy method (48), by using ad hoc oligonucleotide primers.

Infection and transfection. The conditions used for cell culture have been described previously (41). Cultures of COS-1 cells in 35-mm-diameter dishes were infected with vTF7-3 virus at a multiplicity of infection of 5 to 10 PFU per cell. Adsorption was carried out for 1 h at 37°C. After virus adsorption, the cultures were washed with Dulbecco modified Eagle medium (DMEM) and transfected with a mixture of plasmids encoding the virus polymerase subunits and the NP as previously described (31, 32). In brief, 500 ng each of plasmids pGPB1 and pGPB2 (or mutants thereof) plus 50 ng of pGPA and 2 μg of pGNP were diluted to 100 μl with DMEM. In a separate tube, cationic liposomes (1 to 3 μl/μg of DNA) were diluted to 100 μl with DMEM. The contents of both tubes were mixed, kept at room temperature for 15 min, and added to culture plates containing 1 ml of DMEM. After 5 h of adsorption of DNA-liposomes to the cells at 37°C, the cultures were washed again with DMEM and further transfected with 100 ng of NS-CAT RNA (negative polarity) as described above and incubated at 37°C. For in vitro RNA synthesis assays, NSZ RNA (negative polarity) was used (43). These RNAs were synthesized by in vitro transcription of plasmid pIVACAT1-S or pNSZ that had been previously digested with endonuclease *Hga*I. The transcription conditions were those described for the Ambion Megascript kit. Quantification of the RNA was done by including [^{3}H]CTP in the reaction mixture, and the quality of the RNA product was checked by denaturing agarose gel electrophoresis. Twenty to 24 h postinfection, the cells transfected with NS-CAT viral RNA were collected in DMEM, washed in TNE (100 mM NaCl, 50 mM Tris-HCl, and 1 mM EDTA (pH 7.5), and separated into two portions. One of them was resuspended in gel electrophoresis sample buffer, and the other was frozen and thawed three times in 0.25 M Tris · HCl (pH 7.5). They were used for Western blot (immunoblot) analysis and CAT assays, respectively, as described below.

For immunofluorescence analysis, transfections were carried out as described above, except that only 50 ng of plasmid pGPB2 (or mutants thereof), diluted to 500 ng with plasmid pGEM3, was used and the cultures were fixed at 7 h postinfection.

Protein analyses. Total cell extracts were separated by electrophoresis in 7.5% polyacrylamide gels and transferred to Immobilon membranes as previously described (11). The filters were saturated with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and incubated with a 1:200 dilution of rabbit anti-PB2 serum in PBS containing 1% BSA for 1 h at room temperature. After being washed in PBS containing 0.25% Tween 20, the filter was incubated with a 1:3,000 dilution of goat anti-rabbit immunoglobulin G serum labeled with peroxidase. The filter was further washed with PBS–0.25% Tween 20 and developed by enhanced chemiluminiscence (Amersham).

For immunofluorescence, infected-transfected cultures were fixed with methanol for 15 min at −20°C and rehydrated in PBS. Fixed samples were incubated with a 1:1 dilution of mouse anti-PB2 monoclonal antibody (culture supernatants) PB2-20, PB2-22, PB2-28, or PB2-8 for 1 h at room temperature, washed with PBS, and further incubated for 1 h with a 1:200 dilution of fluorescein isothiocyanate-labeled rabbit anti-mouse immunoglobulin G serum and a Hoechst 33258 dye at a final concentration of 0.5 μg/ml. After further washing in PBS, the samples were mounted in Mowiol. The samples were observed and photographed in a Zeiss Axiophot fluorescence microscope.

Two types of CAT assays were used. The thin-layer chromatography assay was carried out as previously described (15). The phase extraction method used was previously described (8). Briefly, cell protein extracts (up to 10 μg of protein) were incubated in a reaction mixture containing 0.05 M Tris · HCl (pH 7.5), 0.2 mM ^{3}H-labeled acetyl coenzyme A (2 to 10 cpm/pmol) and 0.2 mM chloramphenicol in a final volume of 50 μl for 1 h at 37°C. The reaction was stopped by adding 250 μl of 0.1 M sodium borate–5 M NaCl (pH 9.0), the acetylated chloramphenicol was extracted into 1.5 ml of scintillation cocktail, and the radiation was counted. The reaction conditions were chosen such that measurements were made in the linear range regarding both enzyme concentration and incubation time. Protein determinations were made by the bicinchoninic acid method (Bio-Rad) with BSA as the standard.

In vitro RNA synthesis. Fractionation of cells for in vitro transcription was done by a modification of a previously described method (27) as detailed in reference 43. In brief, infected-transfected cells were washed, scraped off the plates, and resuspended in 7.5 mM ammonium sulfate–1 mM EDTA–1 mM dithiothreitol–0.025% Nonidet P-40–10 mM Tris · HCl (pH 8.0). After being vortexed the suspension was centrifuged, the pellet was washed with 100 mM KCl–5 mM $MgCl_2$–0.5 mM CaCl–10 mM Tris · HCl (pH 7.5), and the resulting pellet was resuspended in 100 mM KCl–0.2 mM EDTA–20% glycerol–0.5 mM dithiothreitol–1 mM phenylmethylsulfonyl fluoride–20 mM HEPES (*N*-2-hydroxyethylpiperazin-*N'*-2-ethanesulfonic acid; pH 7.9). In vitro synthesis was carried out in 50 mM Tris · HCl (pH 8.0)–2 mM $MgCl_2$–100 mM KCl–1 mM dithiothreitol–1 mM each ATP, CTP, and UTP–10 μM [α-^{32}P]GTP-10 μg of actinomycin D per ml–1 U of human placental RNase inhibitor per μl with ApG or β-globin mRNA as the primer. Unless otherwise indicated, incubation was for 1 h at 30°C. The reaction product was treated with 50 μg of proteinase K per ml in 0.5% sodium dodecyl sulfate for 30 min at 37°C and extracted with a phenol-chloroform mixture. The aqueous phase was excluded in a Sephadex G50-80 spin column, ethanol precipitated, and treated with RNase-free DNase. After further extraction with a phenol-chloroform mixture, the product was precipitated with ethanol. The poly(A)$^+$ and poly(A)$^-$ RNA fractions were isolated by two rounds of oligo(dT) cellulose chromatography (2) as described previously (43, 56). Retained and non retained RNAs were ethanol precipitated. For size analysis, RNAs were diluted in gel loading buffer, boiled for 3 min, and run on 4% polyacrylamide-urea sequencing gels. The gels were dried, and the signals were detected by autoradiography.

RESULTS AND DISCUSSION

Generation of a collection of PB2 gene deletion and insertion mutants. A comparison of the available sequences of influenza type A PB2 genes shows a high degree of homology. However, the similarity among type A, B, and C genes is very limited and reveals no obvious indication of the possible biological roles of the different regions of the PB2 molecule. In view of the lack of predictive information in the protein sequence, we used a random-mutagenesis approach to investigate the protein regions involved in the function of PB2 in viral RNA transcription. Two types of mutants were constructed: terminal deletion mutants and single-amino-acid insertion mutants. The first type of mutant was constructed by conventional recombination techniques, except for mutants PB2Δ688-759 and PB2Δ711-759, which were the result of the introduction of premature termination codons, and mutant PB2Δ124-759, which was generated by site-directed mutagenesis (Fig. 1B). The insertion mutants were constructed by a modification of the procedure of Barany (3), as described schematically in Fig. 1A and detailed in Materials and Methods. By filling in the overhangs of restriction cuts generated with endonuclease *Dde*I or *Hin*fI, three nucleotide insertions were produced at various sites in the PB2 gene. In this way, a collection of 11 mutants covering the PB2 gene essentially at random was generated (Fig. 1A and B). The mutants were characterized by sequencing the appropriate sections of the gene to verify the insertion of the predicted amino acid. In every case, the mutations obtained were those expected from the restriction site modified. Their nature and location are indicated in Fig. 1B.

Expression and intracellular localization of PB2 mutant proteins. To analyze the expression and stability of the mutant proteins, the mutant genes, cloned under the control of the T7 promoter of plasmid pGEM3, were expressed by transfection into COS-1 cells previously infected with a recombinant vaccinia virus able to express the T7 RNA polymerase (14). Total cell extracts of cultures, mock transfected or transfected with a mixture of plasmids pGPB1, pGPB2, pGPA, and pGNP or with mixtures including the mutant plasmids indicated in Fig. 1B, were analyzed by Western blotting with a PB2-specific polyclonal serum. Every mutant tested showed the presence of PB2-specific signals with intensity comparable to that of the wild-type gene (Fig. 2 and data not shown). The sizes of the deletion mutant proteins were compatible with those predicted by the sequence analysis, and that of the insertion mutants was indistinguishable from that of the wild type (Fig. 2 and data not shown). These results indicate that every mutant protein was expressed and accumulated to levels similar to that of the wild-type PB2 protein and, hence, that the analysis of their phenotype was feasible.

To start, we studied the consequences of the mutations introduced for the nuclear transport of the PB2 protein. This subunit of the polymerase, as well as PB1 and PA, is transported to the nucleus both in influenza virus-infected cells and in cells expressing each subunit as the sole viral protein (1, 20, 35, 37, 38). The signal for PB2 subunit transport into the nucleus has been mapped to two separate regions of the protein (35). By immunofluorescence studies with the mutants indicated in Fig. 1B, we confirmed that the main determinant in the PB2 protein nuclear localization signal is placed at the carboxy-terminal end of the molecule. Thus, deletion mutants PB2Δ688-759 and PB2Δ711-759 showed a clear cytoplasmic

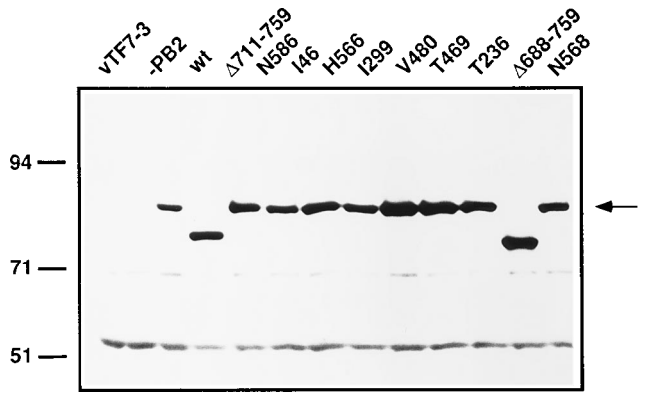

FIG. 2. Expression of mutant PB2 proteins. Extracts from COS-1 cultures infected with vTF7-3 vaccinia virus and transfected with plasmids pGPB1, pGPA, pGNP, and pGPB2 (or mutants thereof) were separated by polyacrylamide gel electrophoresis, transferred to filters, and analyzed by Western blotting with anti-PB2 polyclonal serum. See the text for experimental details. The arrow points to the position of the PB2-specific band, and the numbers to the left are molecular sizes in kilodaltons. wt, wild type.

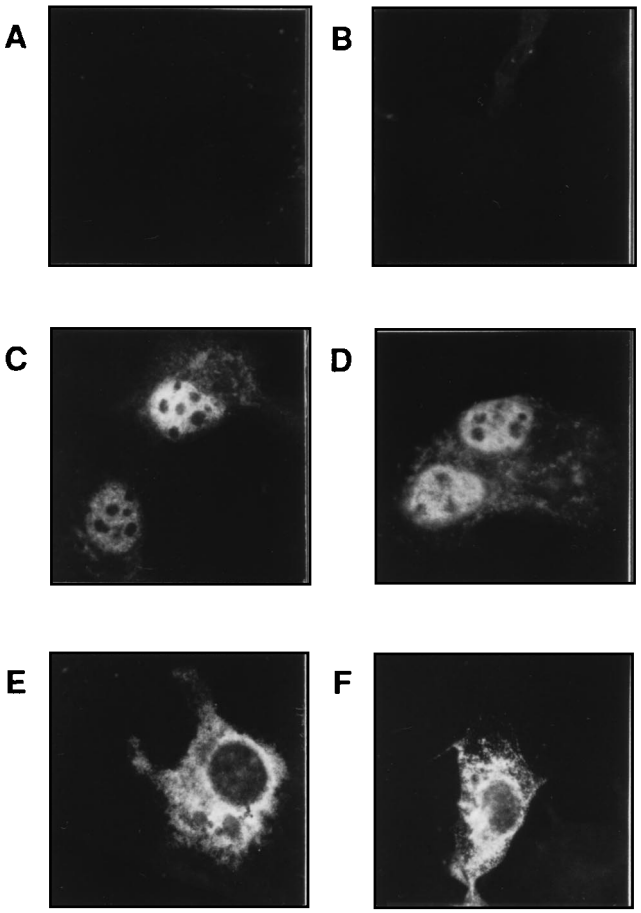

FIG. 3. Intracellular localization of PB2 protein mutants. Cultures of COS-1 cells were infected-transfected with 50 ng of plasmid pGPB2 and 450 ng of pGEM3 DNA or just pGEM3 DNA. The cells were fixed and processed for immunofluorescence as described in Materials and Methods. (A) Cells transfected with pGEM3 DNA. (B and C) Cells transfected with plasmid pGPB2. (D) Cells transfected with mutant plasmid pGPB2I299. (E) Cells transfected with mutant plasmid pGPB2Δ688-759. (F) Cells transfected with mutant plasmid pGPB2V480. (B) Result of staining with a control monoclonal antibody. The rest of the panels were stained with the PB2-specific antibodies indicated in Materials and Methods. The exposure time used for panels A and B was equal to the average of the exposure times needed for the stained cells (C to F).

phenotype when infected-transfected cells were stained with PB2-specific monoclonal antibodies (Fig. 3E and Table 1), in contrast to the nuclear phenotype observed for the wild-type protein (Fig. 3C) and most of the mutants (Fig. 3D and Table 1). In addition, some mutants, like PB2V480, PB2T469, and PB2T236, presented a mixed cytoplasmic-nuclear phenotype (Fig. 3F and Table 1). Two of them (PB2T469 and PB2V480) are located within the region of the protein previously defined as necessary for efficient nuclear transport (35). Therefore, these results confirm the previous mapping of the main determinant of the nuclear localization signal of protein PB2 at its carboxy-terminal end and the requirement of other internal sequences for efficient transport to occur.

Biological activity of PB2 mutant proteins. The effects of the mutations introduced in the PB2 gene on its biological activity was studied by reconstitution of an active transcriptase-replicase in vivo. The system used (31) is experimentally convenient, since mutant plasmids can be tested directly, and has been optimized to yield maximal CAT activity (32). It is noteworthy that the activity obtained is essentially independent of the dosage of the PB2-expressing plasmid used (32; see below). This characteristic of the system is important for comparison of the activities of different plasmids and for competition experiments. The results obtained by the thin-layer chromatographic assay with some of the mutants are presented in Fig. 4, and the average values of three independent experiments are summarized in Table 1. None of the deletion mutants showed any CAT activity. This was expected for mutants PB2Δ688-759 and PB2Δ711-759, which are affected in nuclear transport, and mutant PB2Δ124-759, which lacks most of the PB2 protein sequence (Fig. 3 and Table 1). Many of the single-codon insertion mutants were also inactive, but four mutants showed CAT activity, three of them at levels comparable to that of wild-type PB2 (PB2I299, PB2H566, and PB2N568) and the fourth (PB2D69) at a residual level (Fig. 4 and Table 1). These results indicate that most of the PB2 protein is involved in biological activity and are not surprising, since the CAT assay used is a rather demanding one. Thus, activity of a mutant requires formation of an accurate complex with the other subunits of the polymerase and the template (16), recognition of the capped RNA primer, and possibly endonuclease activity (26).

Some insertion mutants show a TS phenotype. It is a common finding reported in the literature and also in relation to protein PB2 (25) that TS alleles of a gene are the result of missense mutations. To learn whether any of the deletion or insertion mutations introduced into the PB2-encoding gene (Fig. 1) could show a TS phenotype, cultures of COS-1 cells were infected-transfected to reconstitute the influenza virus transcription-replication system as described above and incubated at 37°C (restrictive temperature) or 33°C (permissive temperature). Total protein extracts were prepared and assayed for CAT activity. The results for wild-type PB2 and some of the mutants are presented in Fig. 5. Wild-type PB2 showed a ratio of activity at the permissive versus the restrictive temperature of close to 1, but the active mutants PB2I299, PB2H566, and PB2N568 gave ratios of about 2 to 3 (Fig. 5 and Table 1). Most of the mutants inactive at 37°C remained as such at 33°C; i.e., they did not present a TS phenotype. However, one mutant (PB2T469) showed activity at the permissive temperature in contrast to the restrictive temperature. In addition, another active mutant (PB2D69) gave enhanced CAT

TABLE 1. Phenotypic analyses of mutant PB2 proteins

Allele	Intracellular localization[a]	CAT activity[b]	Relative CAT activity[c] ratio (33/32°C)	In vivo competition[d]	In vitro transcription[e]	
					ApG	β-Globin
Wild-type PB2	N	100	1.2	ND	++++	++++
PB2Δ1-28	N	<0.1		−	−	−
PB2Δ17-67	N	<0.1		−	−	−
PB2Δ17-28	N	<0.1		+	−	−
PB2Δ27-58	N	<0.1		−	−	−
PB2-146	N	<0.1		+	−	−
PB2-D69	N	1	7.7	+	−	−
PB2-T236	N/C	<0.1		+	−	−
PB2-I299	N	49	2.7	ND	+++	+/−
PB2-T469	N/C	<0.1	>36	+	+/−	+/−
PB2-V480	N/C	<0.1		+	−	−
PB2-D516	N	<0.1		+	−	−
PB2-D543	N	<0.1		+	−	−
PB2-H566	N	45	2.9	ND	++	++
PB2-N568	N	42	1.9	ND	++	++
PB2-N586	N	<0.1		+	−	−
PB2Δ688-759	C	<0.1		+	−	−
PB2Δ711-759	C	<0.1		+	−	−
PB2Δ124-759	C	<0.1	NT	+	−	−

[a] N, nuclear localization; C, cytoplasmic localization.
[b] Standardized to the value obtained for wild-type PB2.
[c] Average of two to four determinations. Dashes indicate no significant effect of incubation temperature. NT, not tested.
[d] See the text and the legend to Fig. 6 for details. ND, not detectable.
[e] ++++, 100%; +++, >50%; ++, 25 to 50%; +/−, 5 to 10%; −, not detectable.

activity at the permissive temperature (Fig. 5 and Table 1). Quantification of the CAT activities indicated that the TS mutants were between 7.7-fold and more than 36-fold more active at the permissive temperature than at the restrictive one, although their activities at the permissive temperature were only a few percent of that obtained with wild-type PB2 (about 7 and 3% of wild-type activity; Table 1). These TS insertion mutations are located at sites in the PB2 protein where no sequence homology is observed when the PB2 proteins of influenza virus types A, B, and C are compared (data not shown), and the D69 insertion, in particular, is very close to a previously described TS mutation (position 65) (25).

To the best of our knowledge, these are the first influenza virus mutant genes for which insertion mutations, which are expected to be genetically more stable than missense mutations, are responsible for a TS phenotype. It remains to be determined whether phenotype reversion can be induced by compensatory mutations intra- or extragenically, as has been reported before for other influenza virus mutants (30, 33, 54). These potential problems notwithstanding, the TS mutants described might be of use for construction of influenza viruses with reduced pathogenicity and enhanced genetic stability by following the rescue procedures previously described (12, 13, 52).

Most of the PB2 mutants act as *trans*-dominant repressors. The CAT activity detected with mutants PB2H566, PB2I299, PB2N568, and PB2D69 necessarily implies that these PB2 pro-

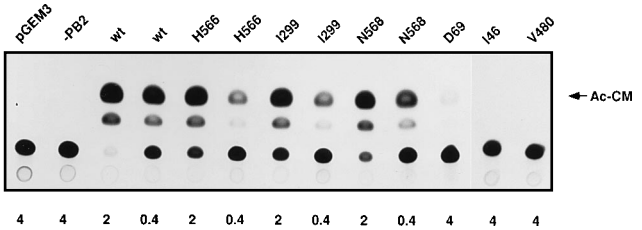

FIG. 4. Transcriptase-replicase activity of PB2 mutants. Cell cultures were infected-transfected as described in Materials and Methods. Extracts were prepared and analyzed for CAT activity by thin-layer chromatography assay with ^{14}C-chloramphenicol as the tracer. The reaction products were separated in a thin-layer chromatography plate and visualized by autoradiography. Lane −PB2 shows the results obtained after transfection with a mixture of every DNA but pGPB2, followed by transfection with NS-CAT virion-like RNA. The arrow shows the position of acetyl-chloramphenicol (Ac-CM). The number below each lane indicates the amount of cell extract (in micrograms) included in each reaction. wt, wild type.

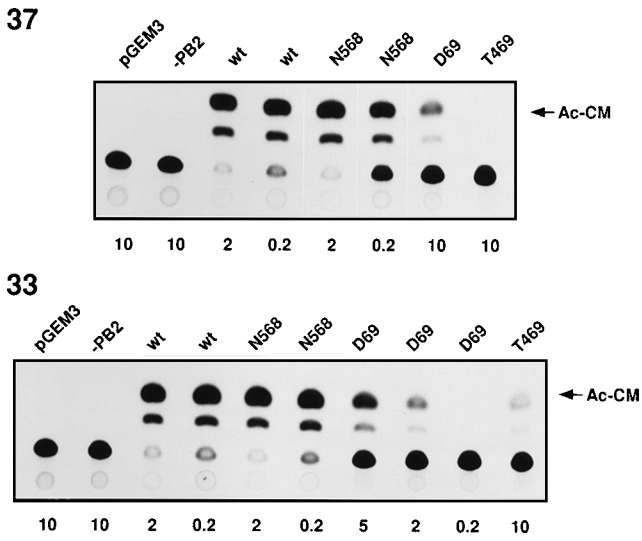

FIG. 5. TS phenotype of PB2 protein mutants. Cell cultures were infected-transfected as indicated in Materials and Methods. Parallel cultures were incubated at the permissive (33°C) or restrictive (37°C) temperature, and the CAT activities of their extracts were assayed as indicated in the legend to Fig. 4. The arrow shows the position of acetyl-chloramphenicol (Ac-CM). The number below each lane indicates the amount of cell extract (in micrograms) included in each reaction. wt, wild type.

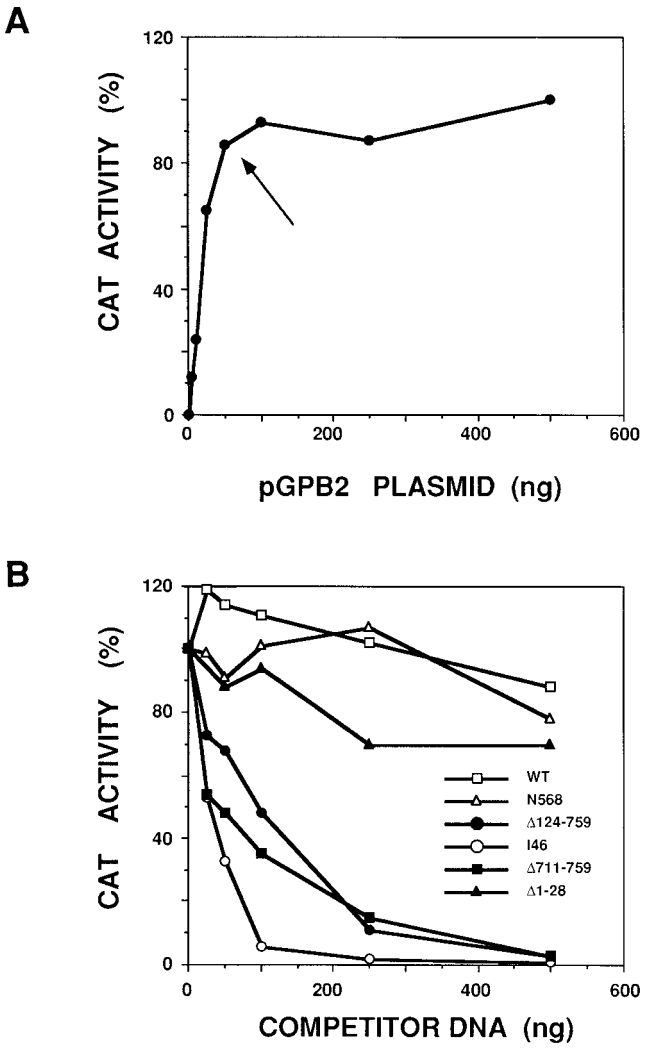

FIG. 6. Competition of mutant proteins with wild-type (WT) PB2. (A) Dose-effect curve of plasmid pGPB2. Cell cultures were infected-transfected as indicated in Materials and Methods, except that progressively smaller amounts of plasmid pGPB2 were included. The CAT activity of each extract was assayed and is indicated as a percentage of the maximal value obtained. The arrow indicates the minimal plasmid pGPB2 dosage that allowed quasimaximal CAT activity. (B) Competition between plasmid pGPB2 and mutant PB2 plasmids. Cell cultures were infected-transfected as described in Materials and Methods, except that 50 ng of plasmid pGPB2 and progressively larger amounts of pGPB2 competitor plasmids were included. The CAT activity of each extract was assayed and is indicated as a percentage of the maximal value obtained.

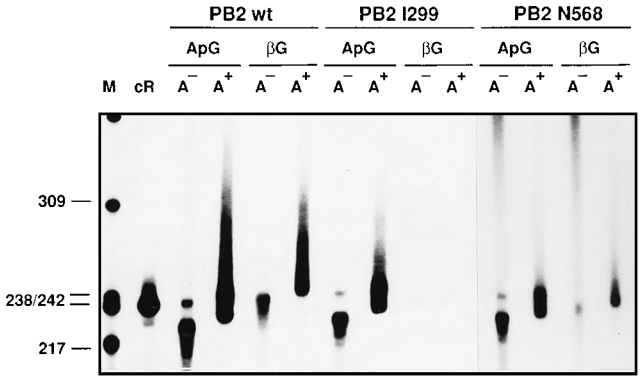

FIG. 7. In vitro RNA synthesis mediated by mutant PB2 proteins. Synthesis reactions were carried out in vitro with nuclear extracts from cells transfected with pGPB1, pGPA, pGNP, either pGPB2 or mutants thereof, and vNSZ RNA, as indicated in Materials and Methods. The RNA products were purified, separated by oligo(dT) chromatography, and analyzed in sequencing gels. The results are shown for wild-type (wt) PB2 and PB2 mutants I299 and N568. Reactions were primed with either ApG or β-globin mRNA (βG). The positions of size markers in lane M are indicated (their lengths in nucleotides are shown to the left). Lane cR shows an NSZ cRNA synthesized with T7 RNA polymerase. A^+ and A^- indicate RNA retained or not retained in oligo(dT) columns, respectively.

teins are able to form functional complexes with the rest of the components of the viral RNA polymerase. However, the negative results obtained with some of the mutants described in Fig. 1 might be the consequence of a lack of proper interactions with the other elements of the transcription-replication machinery or, alternatively, might reflect defects in the biochemical activity of the mutant PB2 protein. To differentiate among these alternatives, we performed in vivo competition experiments. First, the minimal dosage of plasmid pGPB2 able to produce maximal CAT activity in the reconstituted system was determined by diluting this component out of the transfection mixture. The results are presented in Fig. 6A and indicate that under the conditions used, 50 ng of plasmid pGPB2 is sufficient to saturate the system. Therefore, competition experiments were carried out in which a constant amount (50 ng) of wild-type plasmid pGPB2 was included and increasing amounts of competitor plasmids were added to the transfection mixture. The results obtained for representative mutants are shown in Fig. 6B, and a summary of the phenotypes is presented in Table 1. As expected, when wild-type or active mutant plasmids were used as competitors, no competition was observed (Fig. 6B, WT and N568). Most of the mutants were able to compete with wild-type PB2; i.e., they acted as *trans*-dominant repressors of activity (Table 1). Some of them, like mutant PB2I46, showed very efficient competition, while others, like mutant PB2Δ711-759, presented a more protracted competition curve (Fig. 6B). This could be interpreted as the consequence of the cytoplasmic localization of mutant PB2Δ711-759, but the other cytosolic mutant (PB2Δ688-759) competed efficiently. The ability of these cytosolic mutants to compete with the activity of wild-type PB2 might suggest that polymerase complex formation takes place in the cytoplasm, at least in the reconstituted system used.

The competition results, as a whole (Table 1), indicate that only mutants with deletions at the amino-terminal end of the protein were not able to compete, as exemplified by mutant PB2Δ1-28 in Fig. 6B. This fact suggests that a small region localized to the amino-terminal end of the PB2 molecule might be required for the interaction of protein PB2 with the rest of the viral transcription-replication components. To ascertain whether such a protein domain is sufficient for PB2 interaction, mutant PB2Δ124-759 was tested. The results obtained indicate that this is the case; i.e., full CAT activity competition was obtained with competitor concentrations similar to those of other dominant negative mutants (Fig. 6B and Table 1). Since no direct PB2-PA interaction has been detected in vitro (10), it is tempting to speculate that the N-terminal region of protein PB2 is the interaction domain with the PB1 subunit. In this context, it is worth mentioning that a PB2-specific monoclonal antibody (PB2-28), able to inhibit the initiation step in the transcription reaction in vitro (4), interacts with the amino-terminal region of the PB2 protein (40). Nevertheless, other alternatives have not been excluded. For instance, this region of the PB2 protein might be involved in interactions with a presumptive cellular component, essential for the viral RNA

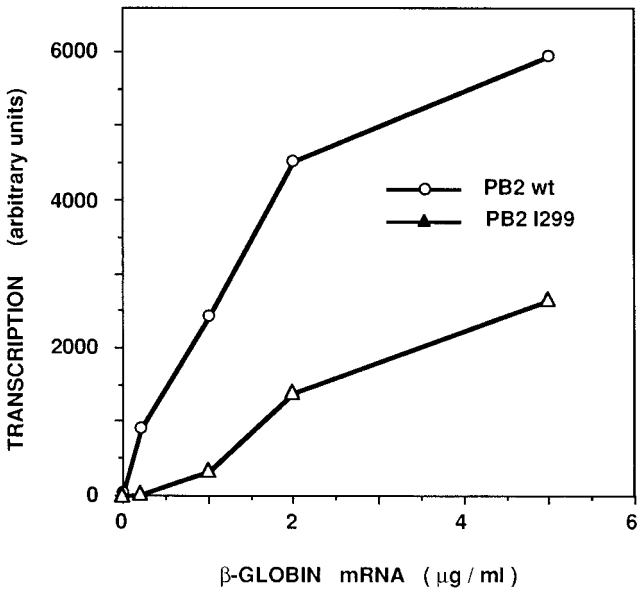

FIG. 8. Cap primer concentration dependence of in vitro transcription. Synthesis in vitro was carried out with in vivo-reconstituted RNA polymerase by using either wild-type (wt) PB2 or mutant I299, as indicated in Materials and Methods, and several concentrations of β-globin mRNA as the primer, as indicated. The RNA products were purified, separated in sequencing gels, and quantified by microdensitometry.

polymerase activity and present in limiting amounts. However, no such cellular factor has been described to date.

Identification of PB2 protein sequences involved in cap-dependent transcription. The reduced CAT activities induced by several PB2 protein mutants, which are able to interact with the other components of the polymerase, indicate that they are unable to carry out properly the transcription reaction but do not provide an explanation of these defects in molecular terms. To get further insight into the possible mechanisms affected by these mutations, we analyzed the capacity of the mutants to induce synthesis of viral RNA in vitro, by using a reconstituted system recently described, which is able to generate both mRNA and cRNA (43). Thus, wild-type and mutant polymerases were reconstituted by cotransfection of plasmids encoding each of the polymerase subunits and the NP, as well as a model viral RNA 240 nucleotides long containing the NS terminal sequences (43). Nuclear extracts of these cells were used for in vitro RNA synthesis primed with either ApG or β-globin mRNA, and the labeled products were analyzed by oligo(dT) chromatography and electrophoresis in sequencing gels. Representative phenotypes are presented in Fig. 7, and the results are summarized in Table 1. None of the CAT-negative mutants showed a capacity to generate either mRNA or cRNA in vitro after priming with ApG or β-globin mRNA. These results indicate that these mutants give rise to polymerase complexes affected at a general step in the polymerization reaction. It could be speculated that after interaction with protein PB1, a mutant PB2 interferes with proper binding with protein PA or, alternatively, its interaction with PB1 does not allow the necessary conformation for proper recognition of the ribonucleoprotein template or the triphosphate. Further experiments are required to elucidate whether any of these

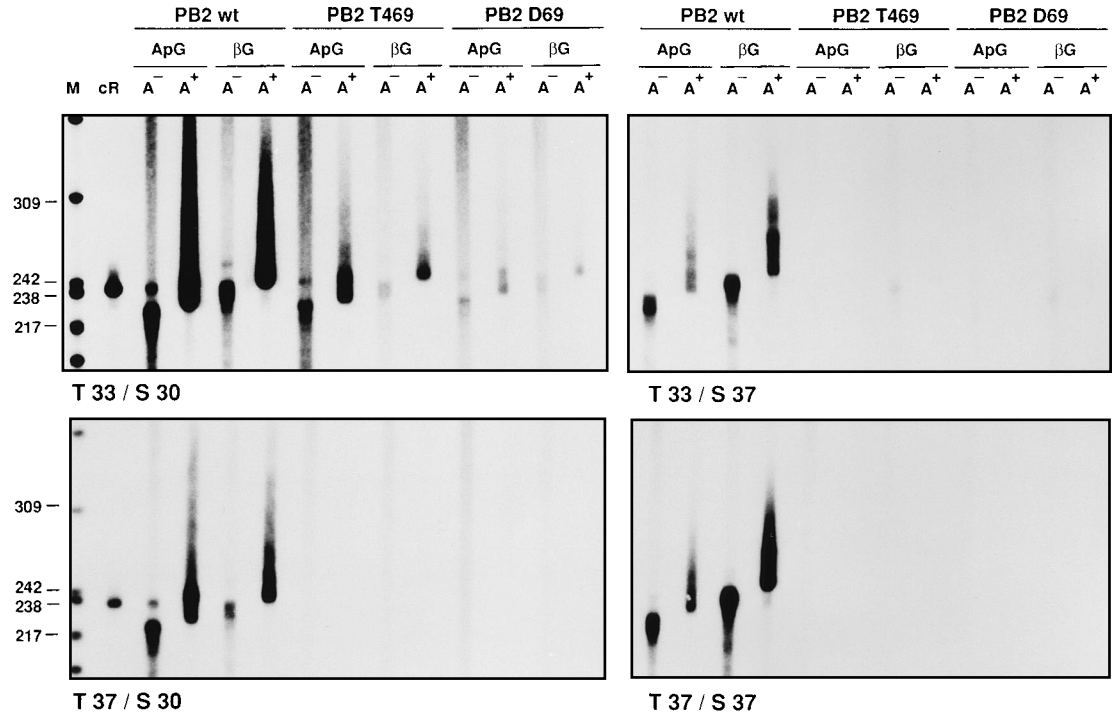

FIG. 9. PB2 mutant proteins induce TS synthesis in vitro. Viral RNA was synthesized in vitro at the permissive temperature (30°C [S 30]) or the restrictive temperature (37°C [S37]) after reconstitution of polymerase in vivo at the permissive temperature (33°C [T 33]) or the restrictive temperature (37°C [T 37]) by using either wild-type protein PB2 or PB2 mutant D69 or T469 and the conditions indicated in Materials and Methods and in the legend to Fig. 7. Reactions were primed with either ApG or β-globin mRNA (βG). The positions of size markers in lane M are indicated (their lengths in nucleotides are shown to the left). Lane cR shows an NSZ cRNA synthesized with T7 RNA polymerase. A^+ and A^- indicate RNA retained or not retained in oligo(dT) columns, respectively.

explanations, among others, account for the defective phenotype of these mutants. Among CAT-positive mutants, two phenotypes were observed: mutants H566 and N568 were active in vitro, albeit at a reduced level, and showed a pattern of RNA products indistinguishable from that of the wild type (Fig. 7 and data not shown). In addition, mutant I299 showed normal activity after ApG priming but much-reduced activity when β-globin mRNA was used as a primer (Fig. 7). This result suggests that insertion I299 affects either cap binding or cap-dependent endonuclease activity. To ascertain which of these possibilities could hold, the in vitro transcription mediated by either wild-type PB2 or mutant I299 was measured by using several concentrations of β-globin mRNA. As shown in Fig. 8, at lower β-globin mRNA doses, the activity induced by mutant I299 was much less than that of wild-type PB2. This result suggests that mutant I299 has reduced affinity for the cap primer. In this regard, it may be speculated that this mutation and mutations T236, T469, and V480 define a region of protein PB2 involved in cap binding. Thus, the partial cytoplasmic localization of the latter mutants (Table 1) might be explained by a failure in nuclear retention due to defective binding to cap structures in the nucleus.

In vitro activity of TS mutants. The activity of the PB2 TS mutants in vitro was tested by carrying out transfections at the permissive (33°C) or restrictive (37°C) temperature and in vitro synthesis at the permissive (30°C) and restrictive (37°C) temperatures. In every case, either ApG or β-globin mRNA was used as the primer and the products were analyzed by oligo(dT) chromatography and electrophoresis in sequencing gels. The results obtained are shown in Fig. 9 and indicate that the TS CAT phenotype of PB2 mutants (Fig. 5 and Table 1) is the consequence of both TS reconstitution in vivo (compare upper and lower panels in Fig. 9) and TS activity in vitro (compare upper left and right panels in Fig. 9). It is not clear why the relative activities of mutants T469 and D69 in the CAT assay and in the in vitro transcription are reversed (Fig. 5 and 9), but their TS phenotype is clear in both assays.

Conclusions. The results presented in this report indicate that the N-terminal region of the PB2 protein is necessary and sufficient for interaction with the influenza virus transcription complex, most probably with the PB1 protein (10). Mutations at a number of sites in the molecule lead to its inactivation in vivo and in vitro. Some of these mutations affect the proper nuclear localization of the protein, others alter its interaction with the transcription complex, and still others inhibit its biological activity. Of special interest are insertion mutations that show a TS phenotype or are defective in cap-binding activity.

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