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Functional domains of the influenza A virus PB2 protein: identification of NP- and PB1-binding sites

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Abstract

Influenza virus genomic RNA segments are packaged into ribonucleoprotein (RNP) structures by the PB1, PB2, and PA subunits of an RNA polymerase and a single-strand RNA-binding nucleoprotein (NP). Assembly and function of these ribonucleoproteins depend on a complex set of protein–protein and protein–RNA interactions. Here, we identify new functional domains of PB2. We show that PB2 contains two regions that bind NP and also identify a novel PB1 binding site. The regions of PB2 responsible for binding NP and PB1 show considerable overlap, and binding of NP to the PB2 fragments could be outcompeted by PB1. The binding domains of PB2 acted as trans-dominant inhibitors of viral gene expression, and consistent with the *in vitro* binding data, their inhibitory activity depended on the concentration of wild-type PB2, NP, and PB1. This provides evidence for functionally significant and potentially regulatory interactions between PB2 and NP.

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Keywords: RNA dependent; RNA polymerase; Ribonucleoprotein

Introduction

Influenza A virus possesses a single-strand RNA genome comprised of eight individual segments. These vRNA segments are negative polarity and are neither capped nor polyadenylated. They are always found in the form of ribonucleoprotein (RNP) particles with four core viral polypeptides. These are the PB1, PB2, and PA subunits of an RNA-dependent RNA polymerase, and a single-strand RNA binding nucleoprotein (NP). These four polypeptides are necessary and sufficient to catalyse transcription of the vRNA segments into capped and polyadenylated mRNA and to replicate them via an uncapped and non-polyadenylated cRNA replicative intermediate (Huang et al., 1990). The process of mRNA transcription is understood in some detail. A capped RNA fragment is generated from a host cell mRNA through an RNA endonuclease activity present in

the viral polymerase and then used to prime viral mRNA synthesis. Polyadenylation is achieved by repetitive transcription of a poly(U) stretch just adjacent to the 5'-end of the vRNA segments, most likely because of a self-imposed steric block arising from the polymerase remaining bound to the 5'-end of the genomic template (Fodor et al., 1994; Poon et al., 1998; Pritlove et al., 1999; Tiley et al., 1994). Genome replication is less well understood, but requires viral protein synthesis and unprimed transcription initiation (recently reviewed by Elton et al., 2002).

In many cases, individual functions have been ascribed to specific RNP polypeptides. PB1 is the nucleotide polymerase and probably the RNA endonuclease (Li et al., 2001; Ulmanen et al., 1981), although PB2 recognizes the cap structures on host cell mRNAs (Blaas et al., 1982; Ulmanen et al., 1981). Mutations in the PA subunit of the polymerase affect both genome replication and mRNA transcription (Fodor et al., 2002; Mahy, 1983), and although the protein does not have a specifically identified role during transcription, there is evidence that it stimulates binding of PB1 to 5'-promoter sequences (Lee et al., 2002). PA is also associated with a protease activity of uncertain function (Hara et al., 2001; Sanz-Ezquerro et al., 1995). NP primarily has a structural role in maintaining the organization of the RNP,

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but is also an essential cofactor and possible regulator of genome replication (reviewed by Portela and Digard, 2002). However, all four polypeptides are required for the transcriptional life-cycle of the virus (Huang et al., 1990), and their function is rendered mutually interdependent through a network of protein–protein interactions. The polymerase functions as a trimer containing one copy of each of the three P proteins (Detjen et al., 1987; Honda et al., 1990), with PB1 acting as the backbone of the complex (Digard et al., 1989). NP forms homo-oligomers (Elton et al., 1999a; Ruigrok and Baudin, 1995) and also interacts with two of the three polymerase (P) proteins (Biswas et al., 1998; Medcalf et al., 1999). These protein–protein interactions are likely to be functionally significant. Genetic analysis indicates that the PB1–PA interaction is essential for virus replication (Perez and Donis, 2001). Moreover, there is evidence for allosteric interactions between the P proteins, as binding of PB1 and PB2 to viral RNA and cellular mRNAs, respectively, modulates the activities of the other protein (Cianci et al., 1995; Lee et al., 2003; Li et al., 1998; Penn and Mahy, 1984; Rao et al., 2003).

In the absence of atomic-resolution structures for any of the protein components of the RNP, several studies have employed genetic and biochemical means to identify the regions of the polypeptides that are responsible for recognizing their partners. In the polymerase complex, sequences have been identified at the N- and C-termini of PB1 that bind to the C- and N-termini of PA and PB2, respectively (Gonzalez et al., 1996; Perez and Donis, 1995; Toyoda et al., 1996). Within NP, two regions have been implicated in multimerization (Elton et al., 1999a), although multiple sequences have been identified as capable of binding to PB2 (Biswas et al., 1998). In this study, we set out to identify the PB2 sequences responsible for interacting with NP. We show that two separate regions of PB2 function as NP-binding sites. Furthermore, one of these sequences closely overlaps a previously identified PB1-binding site while the other contains a hitherto unrecognized PB1-interacting domain. Consistent with the sequence overlap of the binding sites, both PB2 domains could bind either NP or PB1, but not both simultaneously. The PB2 fragments acted as trans-dominant inhibitors of influenza virus gene expression in cells, and their inhibitory activity was out-competed by an excess of wild-type PB2. The inhibitory activity of the N-terminal fragment also depended on the concentration of NP and PB1. This provides evidence for functionally important protein–protein interactions among PB2, NP, and PB1.

Results

Identification of the NP-binding regions of PB2

We and others have identified a protein–protein interaction between the influenza virus NP and PB2 polypeptides

(Biswas et al., 1998; Elton et al., 1999b; Medcalf et al., 1999). The functional significance of this interaction is not yet known, although it has been suggested to be important in the switch between mRNA and cRNA synthesis (Biswas et al., 1998; Mena et al., 1999), or as a structural element during the formation or transcription of RNP structures (Medcalf et al., 1999). To further delineate the interaction, we determined the regions of PB2 that bind to NP, by examining the ability of PB2 fragments to bind to full-length NP. To provide a source of radiolabeled PB2, *Xenopus* oocytes were microinjected with synthetic mRNAs derived from cDNA copies of influenza A segment 1 and incubated in the presence of ^{35}S -methionine. Cell extracts were subsequently prepared and tested for their ability to bind to glutathione transferase (GST) or a GST–NP fusion protein immobilized on glutathione sepharose. As a positive control for expression of the desired PB2 fragment, aliquots of the extract were also immunoprecipitated with antisera directed against PB2, or in certain cases, precipitated using a GST–PB1 fusion protein (see later). As we have previously shown (Medcalf et al., 1999), full-length PB2 was precipitated by GST–NP but not by GST (Fig. 1a, lanes 2 and 3). In addition, GST–NP precipitated as much PB2 as anti-PB2 sera but as few cellular polypeptides (compare lanes 1 and 3 and see Fig. 4a for an example of a total cell extract), indicating that the GST–NP/PB2 interaction is efficient and

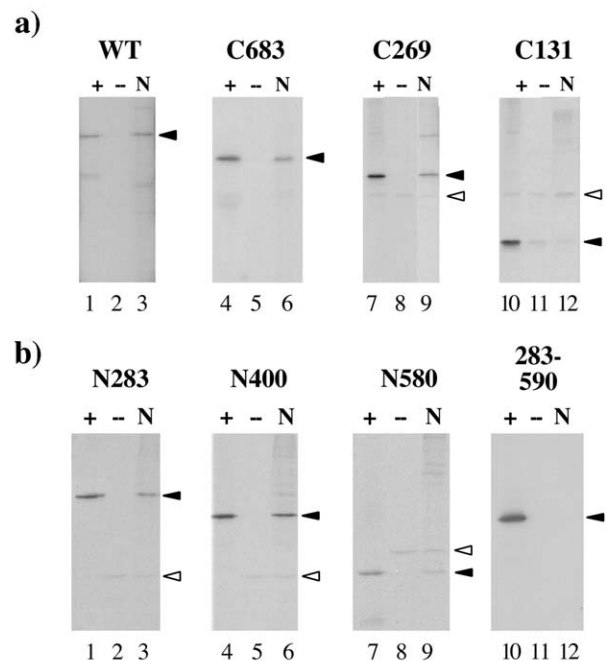


Fig. 1. NP-binding activity of PB2 deletion mutants. Radiolabeled cell lysates from *Xenopus* oocytes microinjected with mRNAs encoding WT, or deleted PB2 polypeptides were analyzed by SDS-PAGE and autoradiography after precipitation with GST (–), GST–NP (N), or anti-PB2 sera (+, except panel (a), lanes 7 and 10, where GST–IN683 was used). Black arrows indicate PB2 polypeptides, white arrows a *Xenopus* glutathione–sepharose binding protein. (a) WT and C-terminally deleted PB2 molecules. (b) N-terminal and internal PB2 fragments.

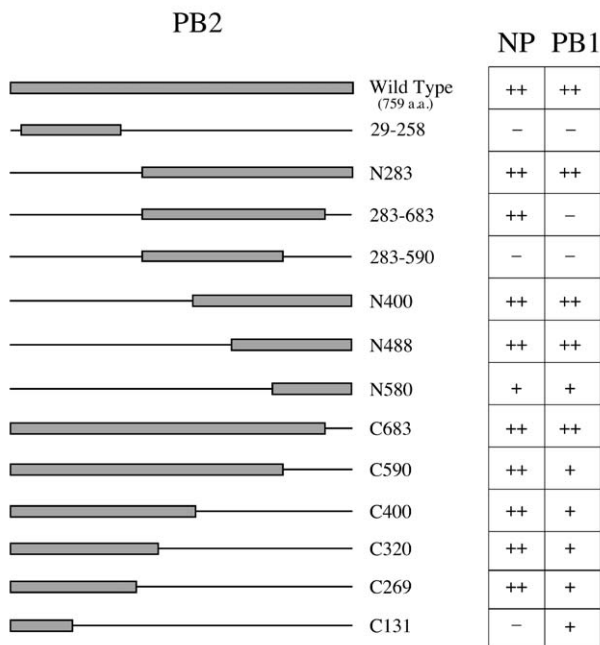


Fig. 2. Summary of the ability of PB2 deletion mutants to bind PB1 and NP. The coding sequences of the indicated mutants are depicted by boxes relative to the WT protein. Binding activity is summarized as essentially WT (+), clearly above background but less than WT (++) and no significant binding activity (-).

specific. Next, we examined the ability of C-terminally truncated PB2 mutants to bind NP. Removal of the C-terminal 76 residues had little apparent effect on the ability of PB2 to bind NP (Fig. 1a, C683, lanes 4–6). Increasing removal of C-terminal sequences, ranging from 169 to 490 amino acids, also caused little decrease in binding activity (C590, 400, 320; data not shown but summarized in Fig. 2, C269, Fig. 1a, lanes 7–9). However, further truncation of the polypeptide to leave only the N-terminal 131 residues reduced binding to background levels (Fig. 1a, C131, lanes 10–12). In the converse experiment, we tested the effects of progressive N-terminal truncation of PB2. Removal of the first 282 residues to generate PB2 N283 had little effect on NP binding (Fig. 1b, lanes 1–3). Similarly, polypeptides with N-termini beginning at PB2 codons 400 or 488 also bound well to GST–NP (N400, Fig. 1b, lanes 4–6; N488, summarized in Fig. 2). Further truncation to leave just the C-terminal 170 residues of PB2 reduced but did not abolish binding (N580, Fig. 1b, lanes 7–9). When the effects of simultaneous N- and C-terminal truncations of PB2 were tested, a fragment of PB2 comprising residues 283–683 still bound efficiently to NP (Fig. 2). However, removal of further C-terminal sequences to generate the polypeptide 283–590 abolished binding activity (Fig. 1b, lanes 10–12). Similarly, an N-terminal internal fragment of PB2 containing amino acids 29–258 showed no NP-binding activity (Fig. 2). Overall, these data suggest that PB2 contains independent N- and C-terminal regions capable of binding NP, located between residues 1–269 and 580–683, respec-

tively (Fig. 2). In confirmation of this, identical results were obtained when the mutant PB2 polypeptides were expressed in rabbit reticulocyte lysate (data not shown), indicating that the pattern of binding specificities obtained was not peculiar to *Xenopus* oocyte expressed material.

To facilitate further analysis of the PB2 NP-binding regions, we next expressed and affinity purified the appropriate sequences from *Escherichia coli* as gene fusions with either GST or maltose-binding protein (MBP). C-terminal PB2 sequences were successfully obtained with the last 180 amino acids of PB2 fused to MBP or GST (MBP-PB2-C, Fig. 3b, lane 9; GST-PB2-C; data not shown), or as the last 271 residues of PB2 fused to GST (GST-PB2-CL; data not shown). Expression of the N-terminus of PB2 was achieved by fusing the first 258 residues of the polypeptide to GST (GST-PB2-N), although the protein co-purified with substantial quantities of a presumed proteolytic product corresponding closely in size to native GST (Fig. 3b, compare lanes 3 and 8). Next, we tested the abilities of these polypeptides to bind to NP translated in vitro in rabbit reticulocyte lysate. Aliquots of the radiolabeled NP were mixed with the PB2 fusion proteins, or as controls, with nonfused MBP or GST, GST–NP, GST fused to C-terminal sequences of PB1 (GST-1N683 and -1N712), or GST fused to an irrelevant papillomavirus protein (GST-16E6). After

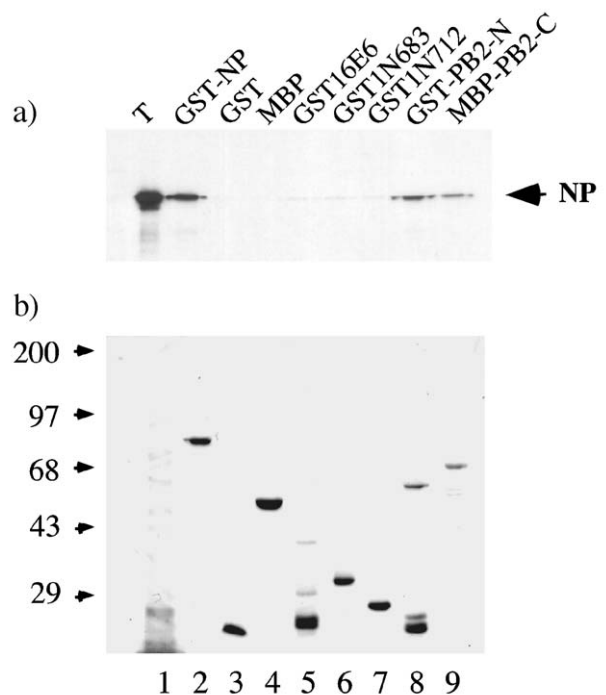


Fig. 3. Ability of PB2 fusion proteins to bind NP. GST or MBP fusion proteins (approximately 0.3 μ g per reaction) were tested for their ability to bind radiolabeled in vitro translated NP. Aliquots of NP were analyzed by SDS-PAGE and (a) autoradiography, (b) staining of the same gel with Coomassie brilliant blue before (T) and after precipitation with the indicated fusion proteins. Arrows indicate NP and the positions of molecular mass markers (kDa) as labeled.

incubation, the fusion proteins were precipitated by the addition of glutathione sepharose or amylose resin as appropriate, washed, and bound protein examined by SDS-PAGE. Coomassie-blue staining of total protein showed that the fusion proteins were precipitated in approximately equal quantities (Fig. 3b). Autoradiography confirmed that as previously shown (Elton et al., 1999a), radiolabeled NP bound efficiently to GST–NP (Fig. 3a, lane 2), but not to GST or MBP alone (lanes 3 and 4). Only trace quantities of NP bound to GST fused to human papillomavirus type 16 E6 protein or to GST fused to C-terminal sequences from influenza virus PB1 protein (lanes 5–7). However, substantial quantities of NP were bound by the N- and C-termini of PB2 fused to GST and MBP, respectively (lanes 8 and 9). This confirms the results of experiments analyzing the binding of radiolabeled PB2 fragments to GST–NP (Fig. 1), and shows that both N- and C-terminal regions of PB2 specifically bind NP.

Relationship of NP- and PB1-binding regions of PB2

PB2 also interacts with the PB1 subunit of the RNA polymerase (Digard et al., 1989), and similarly to one of the NP-binding regions identified above, a PB1-binding region has been identified in the N-terminal 249 amino acids of PB2 (Ohtsu et al., 2002; Toyoda et al., 1996). We therefore wished to determine what relationship the NP-binding domains of PB2 had to the sequences important for binding PB1. Accordingly, we examined the ability of the PB2 fusion proteins to bind PB1. Radiolabeled PB1 from extracts of microinjected *Xenopus* oocytes (Fig. 4a, lane 1) was incubated with the various fusion proteins and examined for binding as above. As a positive control, an aliquot of the cell extract was immunoprecipitated with anti-PB1 sera (Fig. 4a, lane 2). No detectable PB1 was bound by GST or MBP alone (lanes 3 and 7, respectively) or by irrelevant fusion proteins such as GST16E6 or MBP fused to the coxsackie/adenovirus receptor (data not shown). In contrast, PB1 was efficiently bound by the fusion protein containing sequences from the N-terminus of PB2 (lane 5), confirming the results of Toyoda et al. (1996). However, PB1 also bound to the fusion proteins containing C-terminal residues of PB2, either linked to GST (lane 4) or MBP (lane 6). Identical results were obtained using PB1 synthesized in *E. coli* or BHK cells as a target (data not shown), suggesting the existence of additional PB1-binding sites in PB2 to that already identified (Ohtsu et al., 2002; Toyoda et al., 1996).

To further examine the possibility of additional PB1-binding sites in PB2, we employed a co-immunoprecipitation assay using material from cells co-expressing both proteins. *Xenopus* oocytes were microinjected with synthetic mRNAs derived from plasmids pKT1 and 2, and incubated in the presence of ³⁵S-methionine. Cell extracts were then analyzed by SDS-PAGE and autoradiography after immunoprecipitation with antisera specific for PB1 or PB2. When extracts from oocytes co-expressing wild-type

PB1 and PB2 were analyzed, both polypeptides were immunoprecipitated by either antiserum (Fig. 4b, lanes 3 and 4), indicating that the two proteins were associated. The coprecipitation was specific, as no precipitation of PB1 or PB2 by the heterologous antiserum was observed when the polypeptides were expressed in isolation (Fig. 4b, lanes 1, 2, 5, and 6), and neither were significant quantities of *Xenopus* polypeptides precipitated with either P protein. This reproduces our earlier finding that PB1 and PB2 interact in the absence of PA (Digard et al., 1989). Next, we tested the effect of removing the previously identified N-terminal PB1-binding domain from PB2. A mutant PB2 molecule lacking the first 282 amino acids still efficiently and specifically co-immunoprecipitated with PB1 (Fig. 4c, lanes 1–4). Thus, the minimal PB1-binding domain identified previously (Toyoda et al., 1996) is not the only PB2 sequence capable of interacting with PB1. In confirmation of the results obtained with the GST-PB2 fusion proteins, further N-terminal truncation of PB2 indicated that the amino-terminal two-thirds of the protein was dispensable for PB1 binding. Mutants lacking sequences upstream of amino acid 487 still specifically coprecipitated with PB1 (N400, data not shown but summarized in Fig. 2, N488; Fig. 4c, lanes 5–8). Further truncation of PB2 resulted in a polypeptide that still bound PB1, but in a less stable fashion as both polypeptides were immunoprecipitated when anti-PB2 sera were used (Fig. 4c, N580, lane 12), but not when anti-PB1 sera were used (lane 11, possibly due to disruption of a weak interaction by the latter serum). However, removal of C-terminal sequences from an already N-terminally deleted mutant resulted in polypeptides that no longer bound PB1, as evidenced by the lack of cross-precipitation of the two P proteins (Fig. 4c, 283–683, lanes 15 and 16, 283–590, 29–258 summarized in Fig. 2). Thus, PB2 contains an additional PB1-binding site located toward its C-terminus. Progressive C-terminal deletion mutagenesis of PB2 confirmed the presence of a PB1-binding site at the N-terminus of the polypeptide, as proteins truncated at amino acids 683, 590, 400, 320, 269, or 131 all still coprecipitated with PB1 (N269, N131, Fig. 1a, lanes 7–12; N683, N590, N400; N320, summarized in Fig. 2). Overall therefore, PB2 contains two independent binding sites for PB1, located similarly to the NP-binding sites at the N- and C-termini of the polypeptide (Fig. 2).

Competitive protein–protein interactions among PB2, NP, and PB1

The experiments described above identified two separate binding sites in PB2 for each of NP and PB1 that mapped to similar regions of PB2. Although differences in the binding activities of certain PB2 polypeptides were noticed (e.g., 283–590; Fig. 2), the N-terminal 269 and C-terminal 180 amino acids of PB2 encompass both NP and PB1 binding activity (Figs. 1–4). Thus, it was of interest to determine whether these regions of PB2 could bind both polypeptides

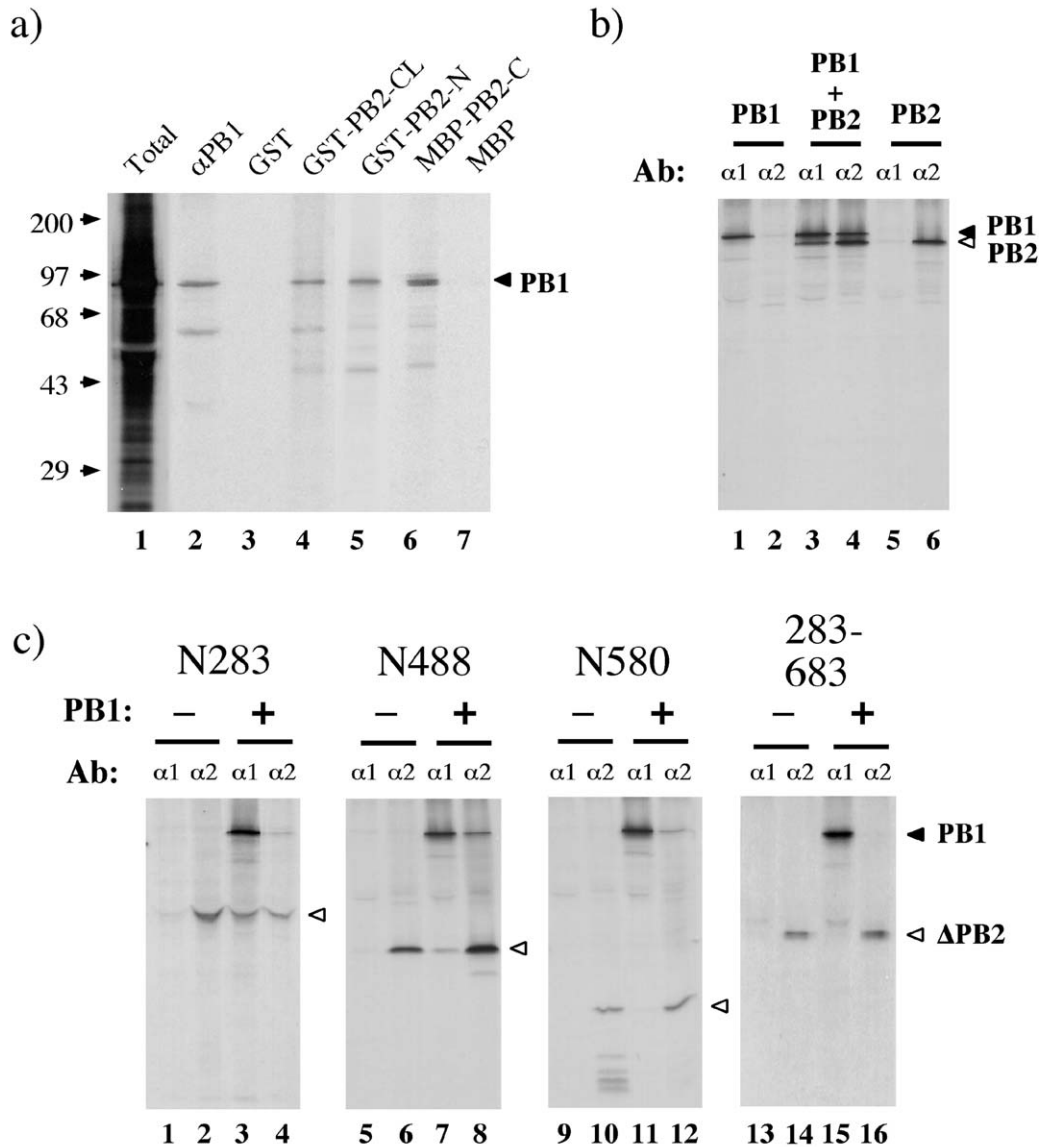


Fig. 4. PB1-binding regions of PB2. (a) Radiolabeled oocyte lysates containing PB1 were analyzed by SDS-PAGE and autoradiography before (Total) or after precipitation with F1 anti-PB1 serum (α -PB1) or the indicated fusion proteins. (b, c) Co-immunoprecipitation analysis of PB2–PB1 interactions. SDS-PAGE and autoradiographic analysis of polypeptides immunoprecipitated from lysates of oocytes microinjected with the indicated combinations of mRNAs encoding wild-type PB1 and wild-type (b) or truncated (c) PB2. Samples were immunoprecipitated with anti-PB1 (α 1) or anti-PB2 (α 2) sera. Black arrows indicate PB1, white arrows PB2 derived polypeptides. The positions of molecular mass markers (kDa) are also shown in (a).

at once, or whether the interactions were competitive. To examine this possibility for the C-terminus of PB2, we tested the ability of sepharose-bead immobilized GST–NP to bind radiolabeled PB1 in the presence and absence of soluble MBP–PB2–C. As we have previously shown (Medcalf et al., 1999), radiolabeled PB1 from *Xenopus* oocytes bound to GST–NP but not to GST alone (Fig. 5a, lanes 2 and 3). Binding was not reduced by the addition of a fivefold molar excess (with respect to GST–NP) of MBP (Fig. 5a, lane 1). However, the addition of equimolar and higher ratios of MBP–PB2–C reduced the amount of PB1 precipitated (lanes 5–7), suggesting that PB1 was preferentially binding to the fragment of PB2 and was no longer

available to interact with NP. Similar results were obtained when PB1 expressed in *E. coli* or BHK cells was used as the target (data not shown), and when the amount of bound PB1 was quantified, it was found that on average, a twofold molar excess of the PB2 fragment inhibited the NP–PB1 interaction by around 50%, and that a sixfold excess was sufficient to almost abolish binding (Fig. 5b). Next, we carried out the analogous experiment with the N-terminus of PB2. Because the N-terminal region of PB2 was available as a GST fusion, we tested its ability to interfere with the interaction between immobilized MBP–NP and PB1. PB1 bound efficiently to MBP–NP in the absence of GST–PB2–N (Fig. 5a, lane 8) but not to MBP alone (lane 13). The

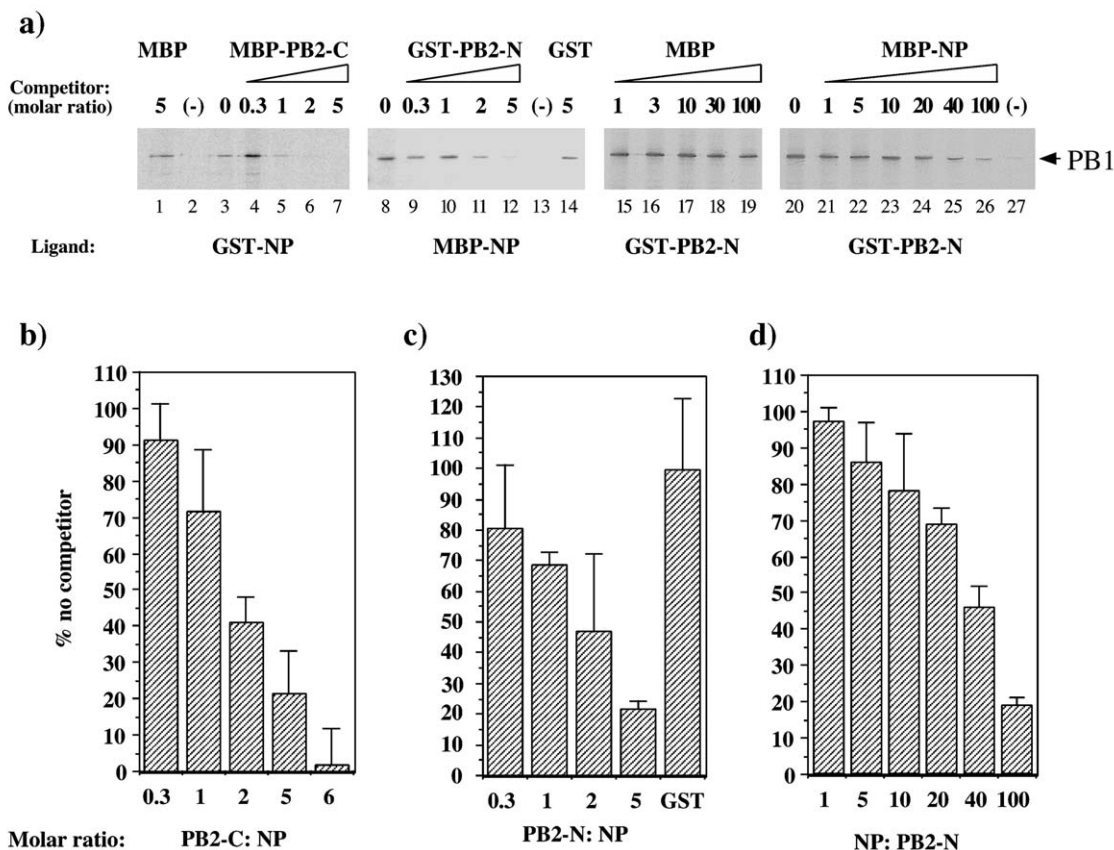


Fig. 5. Competitive protein–protein interactions among PB2, NP, and PB1. (a) The ability of immobilized GST [(-)], GST–NP, MBP–NP, or GST–PB2–N (150 nM) to bind radiolabeled PB1 synthesized in *Xenopus* oocytes in the presence and absence of the indicated amounts of competitor polypeptides was tested. Bound PB1 (arrow) was detected by SDS–PAGE and autoradiography. (b, c, d). Bound PB1 was quantified by densitometry and plotted as a percentage of the amount bound in the absence of competitor. Values plotted are (b) the mean \pm SE from five independent experiments utilizing PB1 synthesized in oocytes, BHK cells, or *E. coli* as a target, (c, d) the mean \pm SE from three independent experiments utilizing PB1 synthesized in oocytes.

addition of a fivefold molar excess of GST had little effect on the NP–PB1 interaction (lane 14). However, the addition of increasing amounts of GST–PB2–N to the reactions caused a titratable decrease in PB1 binding (lanes 9–12) indicating that it too was able to interfere with the NP–PB1 interaction. In replicate experiments, a twofold molar excess of the N-terminus of PB2 inhibited the NP–PB1 interaction by around 50% and a fivefold excess by 80% (Fig. 5c). Thus, we conclude that both N- and C-terminal sequences of PB2 can bind either NP or PB1, but not both simultaneously. Next, we carried out the reciprocal experiment of testing the ability of purified soluble NP to interfere with the interaction between PB1 and immobilized PB2. As before, radiolabeled PB1 bound to GST–PB2–N but not to GST (Fig. 5a, lanes 20 and 27, respectively). Addition of up to a 100-fold molar excess of MBP to the reaction had little effect on the PB2–PB1 interaction (Fig. 5a, lanes 15–19). In contrast, binding decreased as MBP–NP was titrated into the reactions (lanes 21–26), with a 40-fold molar excess (with respect to GST–PB2–N) causing a decrease in binding of around 50% (Fig. 5d). This further confirms the competitive nature of the interactions among NP, PB2, and PB1. In

addition, the finding that NP is a much less effective competitor of the PB1–PB2 interaction than PB2 is of NP–PB1 binding suggests that PB1 binds to PB2 with higher affinity than it does NP.

Effect of the PB2 protein-binding regions on virus transcription

To further examine the functional role of the PB2 protein-binding regions, we tested their ability to act as trans-dominant inhibitors of virus gene expression, reasoning that they might compete with WT PB2 for NP and PB1 binding. To permit expression of the desired PB2 fragments in mammalian cells, the GST, GST–PB2–N, and GST–PB2–C coding regions were subcloned into plasmid pKT-0 (Tibbles et al., 1995) under the control of a bacteriophage T7 RNA polymerase promoter (see Materials and methods). CV-1 cells were multiply infected with recombinant vaccinia viruses expressing the three influenza virus P proteins (Smith et al., 1987) and the bacteriophage T7 RNA polymerase (vTF-7; Fuerst et al., 1987), and transfected with a synthetic RNA containing a negative sense CAT gene flanked by

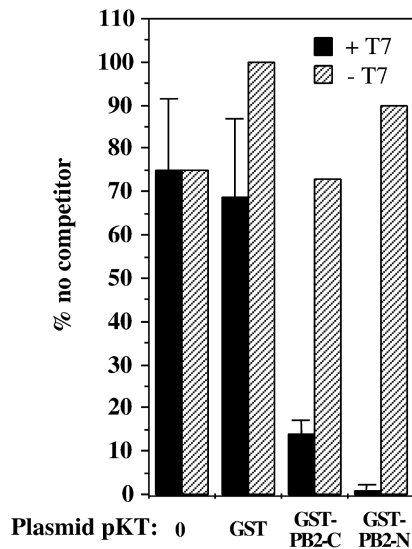


Fig. 6. Ability of GST, GST-PB2-C, and GST-PB2-N to act as trans-dominant inhibitors of virus gene expression. CV-1 cells were infected with recombinant vaccinia viruses expressing the three subunits of the influenza virus RNA polymerase and transfected with 1 μ g each of a synthetic influenza virus RNA segment containing an antisense CAT gene and the indicated pKT plasmid. In some cases, NP expression was obtained from transfection of plasmid pKT5 (1 μ g) and superinfection with a recombinant vaccinia virus expressing T7 RNA polymerase (+T7). Alternatively, T7 RNA polymerase was omitted and NP expression was obtained from a recombinant vaccinia virus (–T7). The resulting accumulation of CAT polypeptide was quantified and plotted as a percentage of the value obtained in the absence of a competitor plasmid. (+T7) Values are the average \pm SE from three independent experiments, (–T7) values are from a single experiment.

influenza virus terminal sequences from segment 1 (PB2-CAT; generous gift of Dr. Mark Krystal) as well as with plasmid pKT5, encoding influenza virus NP (Digard et al., 1999) and the pKT GST plasmids. In certain reactions, plasmid pKT 5 and vaccinia virus vTF-7 were omitted and replaced with NP-VAC (Smith et al., 1987) to obtain expression of the influenza virus RNP polypeptides in the absence of T7 RNA polymerase. In the absence of competitor plasmids, the WT influenza RNP polypeptides transcribed the PB2-CAT RNA to give rise to CAT polypeptide expression, which was then quantified by ELISA. After subtraction of the background expression obtained in the absence of an NP

gene, this value was used as a baseline with which to compare virus gene expression in the presence of the competitor plasmids. Co-transfection of plasmids pKT-0 or pKT-GST had relatively little effect on CAT synthesis (Fig. 6), reducing expression less than twofold. However, co-transfection of plasmids expressing the N- or C-termini of PB2 fused to GST reduced expression dramatically: to around 15% of normal levels in GST-PB2-C and 2% with GST-PB2-N (Fig. 6). This inhibition depended on expression of the PB2 fragments and could not be ascribed to trivial interference with the transfection procedure, as no substantial inhibition was observed from co-transfection of any of the plasmids in the absence of T7 RNA polymerase (Fig. 6). Thus, both the N- and C-terminal protein binding domains of PB2 act as trans-dominant inhibitors of viral gene expression.

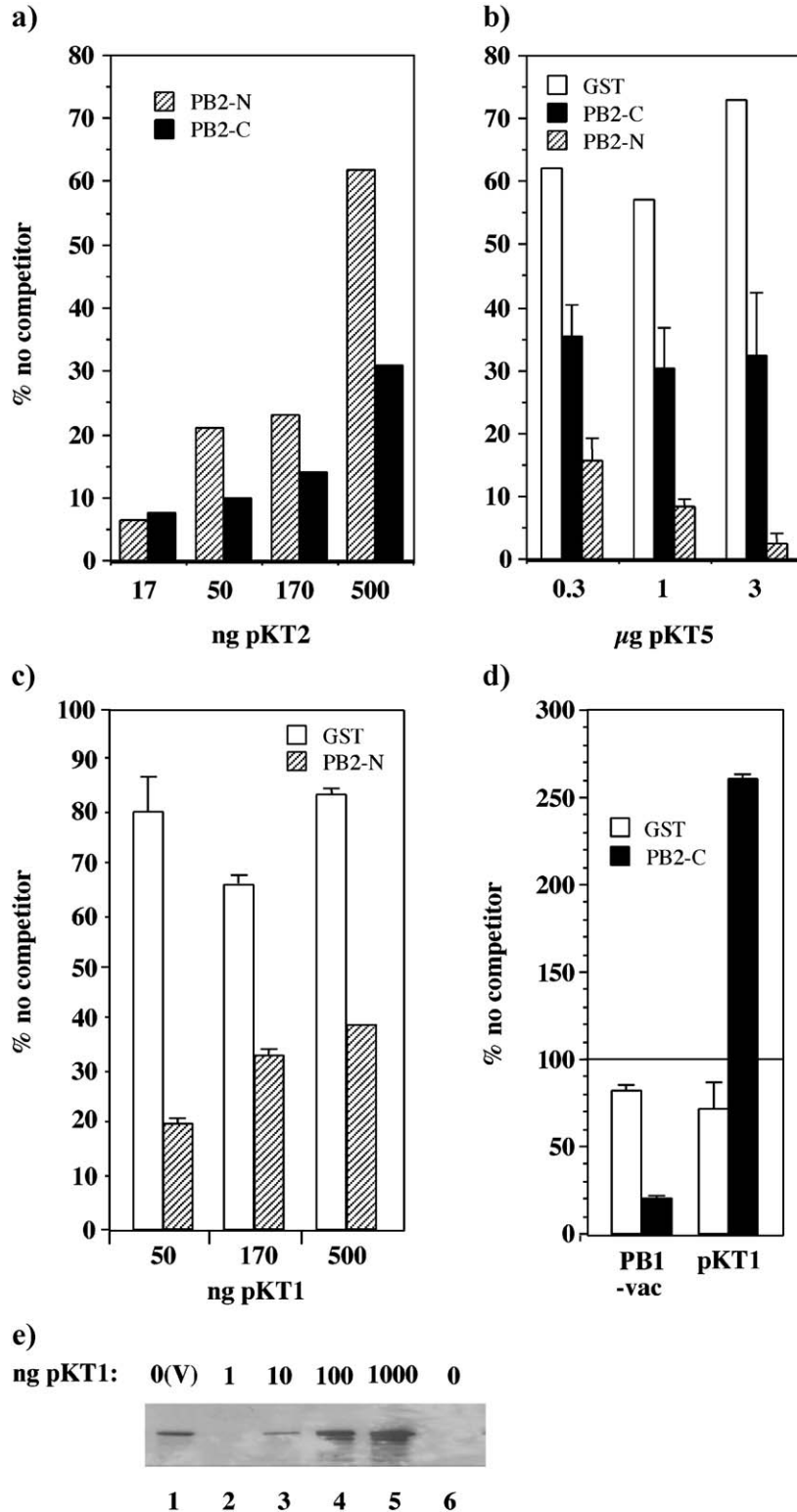
Dominant-negative inhibition by the PB2 fragments is most readily explained by the interaction of the defective mutants with other essential components of the transcriptase, either by binding to and inactivating RNPs or by sequestration of limiting factors and preventing the assembly of active RNPs. Indeed, the N-terminal 124 amino acids of PB2 have previously been shown to act as a dominant negative inhibitor of virus transcription and the interference plausibly ascribed to competition for PB1 (Perales et al., 1996). To further examine this issue, we examined the effect of varying the levels of WT PB2, NP, and PB1 on trans-dominant inhibition by the PB2 fragments. First, we tested the effects of varying the concentration of WT PB2. Cells were infected with the recombinant vaccinia viruses, transfected with the negative sense CAT RNA and plasmid pKT5 in the presence or absence of the pKT-GST-PB2 plasmids as before, except that WT PB2 was supplied by the transfection of varying amounts of plasmid pKT2. The cells were subsequently lysed and CAT accumulation measured as a percentage of that observed in the absence of any competitor plasmid. Parallel Western blot analysis of PB2 levels confirmed that increasing the dose of plasmid pKT2 did lead to the synthesis of greater amounts of PB2 (data not shown). At low doses of WT PB2, both GST-PB2 fusions caused a greater than 90% reduction in CAT accumulation (Fig. 7a). However, this inhibition was markedly decreased by the co-transfection of larger amounts of plasmid pKT2, in the N-terminal fragment of PB2, to levels similar to that observed

Fig. 7. Effect of varying PB2, NP, or PB1 concentration on the inhibitory activities of GST, GST-PB2-C, and GST-PB2-N. CV-1 cells were infected with vac-T7 and vac-PA, transfected with pKT5 and the synthetic influenza CAT segment, in the absence or presence of pKTGST, pKTGST-PB2-C (1 μ g), or pKTGST-PB2-N (300 ng) as labeled. PB1 and PB2 were supplied by infection with vac-PB1 and vac-PB2, respectively, except in specific experiments where they were replaced with plasmids pKT1 or pKT2 as indicated. NP was supplied by transfection of plasmid pKT5 (1 μ g except where labeled otherwise). Cells were harvested after 24–36 h and CAT accumulation measured by ELISA. Results are expressed as the % CAT accumulation observed in the absence of a transfected pKTGST-family plasmid. (a) Effect of varying PB2 levels by transfection of the indicated amounts of pKT2. (b) Effect of varying NP levels by transfecting the indicated amounts of pKT5. The mean and SE from three independent experiments are plotted for pKT GST-PB2-C and pKTGST-PB2-N, while the result from a single experiment is shown for pKT-GST. (c) Effect of varying PB1 concentration by transfecting the indicated amounts of pKT1 on inhibition by GST-PB2-N. The average and range of two experiments are shown. (d) Effect of PB1 expression method on inhibition by GST-PB2-C. PB1 was supplied either by transfection of pKT1 or by infection with vac-PB1 (as labeled). The average and range of two experiments using 50 and 170 ng pKT1 or vac-PB1 MOIs of 3 and 10 are shown. (e) PB1 expression levels. Lysates from equal numbers of cells transfected with the indicated amounts of plasmid pKT1 or infected (V) with vac-PB1 (MOI 3) were analyzed by SDS-PAGE and Western blotting with anti-PB1 serum.

with GST only (Fig. 7a). This is consistent with both domains of PB2 competing with WT PB2 for other transcription factors.

Next, we carried out a similar experiment where the amount of NP was varied by transfecting varying amounts

of plasmid pKT5. Expression of GST alone resulted in only minimal decreases in CAT accumulation, regardless of the NP dose (Fig. 7b). In contrast, co-expression of GST-PB2-C caused inhibition of CAT accumulation by around 70%, although this was similarly independent of NP



expression levels (Fig. 7b). Expression of GST-PB2-N likewise caused inhibition of CAT accumulation, by up to 95%. Unexpectedly however, trans-dominant inhibition by GST-PB2-N was partially relieved by decreasing the amount of NP and was exacerbated by increasing the amount (Fig. 7b). In replicate experiments, a tenfold increase in the amount of NP plasmid transfected caused on average a sixfold increase in the level of inhibition caused by a constant amount of pKTGST-PB2-N. This NP-dependent variation is consistent with the *in vitro* data indicating a protein–protein interaction between the N-terminus of PB2 and NP (Figs. 1, 4, and 5).

When the matching experiment was carried out for PB1, increasing the dose of plasmid pKT1 from 50 to 500 ng/transfection resulted in a decrease in inhibition by pKTGST-PB2-N (Fig. 7c). The decrease was relatively small, but reproducible as in three independent experiments a tenfold increase in transfected PB1 plasmid increased CAT expression by 2.0 ± 0.1 -fold. In addition, a similar magnitude decrease in inhibition was obtained by increasing the MOI of PB1-VAC tenfold (data not shown). When the same methodology was applied to the C-terminal fragment of PB2, an unexpected but clear qualitative difference in the effect of GST-PB2-C on influenza virus gene expression that depended on the source of PB1 became apparent. While co-transfection of pKTGST-PB2-C reproducibly inhibited CAT expression when PB1 was expressed from PB1-VAC (Figs. 6 and 7a, b, d), it stimulated it when PB1 was supplied by transfection of plasmid pKT1 (Fig. 7d). In four replicate experiments, pKTGST-PB2-C stimulated transcription driven by the optimal amount of pKT1 (50 ng) to an average of 200% of the baseline value. Western blot analysis of the PB1 content of cells indicated that T7 RNA polymerase-driven expression from even moderate amounts of plasmid pKT1 produced higher quantities of PB1 than infection with the recombinant vaccinia virus PB1-VAC (Fig. 7e, compare lanes 1 and 4). However, immunofluorescence analysis showed that although infection with vac-PB1 resulted in PB1 expression in almost every cell, transfection with plasmid pKT1 was less than 10% efficient (data not shown). Thus, plasmid-driven expression of PB1 results in substantially higher levels of PB1 per cell. This therefore suggests that the modulatory effect of the C-terminal domain of PB2 on virus transcription is dependent upon PB1 levels, again, consistent with the *in vitro* protein–protein interactions.

Discussion

Assembly and transcriptional activity of influenza virus RNPs depend on a complex network of protein–protein and protein–RNA interactions among the three subunits of the polymerase, NP, and both viral and cellular RNAs. In the absence of any high-resolution structural information

for the RNPs, many molecular–genetic studies have been directed at mapping the functional regions of the protein components. In addition to biochemical and mutational analysis of catalytic centers (Asano and Ishihama, 1997; Biswas and Nayak, 1994; Li et al., 2001), several studies have examined sequences involved in protein–RNA contacts (Albo et al., 1995; Elton et al., 1999b; Fechter et al., 2003; Gonzalez and Ortin, 1999a, 1999b; Honda et al., 1999; Kobayashi et al., 1994; Li et al., 1998, 2001) and protein–protein contacts in the RNP. These latter analyses have identified regions of the P proteins involved in forming the polymerase heterotrimer (Biswas and Nayak, 1996; Gonzalez et al., 1996; Ohtsu et al., 2002; Perez and Donis, 1995; Toyoda et al., 1996; Zurcher et al., 1996), and NP sequences involved in forming homo-oligomers and contacting PB2 (Biswas et al., 1998; Elton et al., 1999a). In this study, we extend knowledge of RNP protein–protein contacts by identifying for the first time regions of the polymerase that bind NP. These data permit the drawing of a more detailed functional map of the PB2 protein, containing information on NP-interacting sequences and a hitherto unidentified PB1-binding domain (Fig. 8). Our finding that two separate regions of PB2 are capable of binding NP is consistent with that of Biswas et al. (1998) that conversely, multiple regions of NP interact with PB2. The identification of a novel C-terminal PB1-binding domain in PB2 contradicts an earlier study that found no evidence for PB1-binding activity in N-terminally deleted PB2 fragments (Toyoda et al., 1996). However, we note that a subsequent study from the same laboratory using identical expression and immunoprecipitation conditions and some of the original plasmid constructs did detect coprecipitation of PB1 and mutant PB2 molecules lacking up to the first 205 amino acids (Ohtsu et al., 2002). Further N-terminal truncations were not analyzed in the second study and the data were interpreted in light of the known N-terminal PB1-binding sequence. However, the data are equally consistent with the results presented here indicating the presence of a second PB1-binding site in PB2. The definition of a second PB1-binding site in PB2 may well provide an explanation for why the study of Biswas and Nayak (1996) identified a different region of PB1 as important for binding PB2 compared to the studies of Toyoda et al. (1996) and Gonzalez et al. (1996). Our results confirm that the C-terminus of PB1 does bind the N-terminus of PB2 (Fig. 1a, lanes 7–12), suggesting that conversely, the C-terminal binding domain of PB2 might interact with the more N-terminal region of PB1 identified by Biswas and Nayak (1996).

The NP- and PB1-binding sites identified here show considerable overlap with each other at the sequence level, which a series of competitive binding experiments showed to also operate at the functional level (Fig. 5). The binding sites also show a degree of overlap with sequences proposed by others as being involved in mRNA cap binding (Fig. 8;

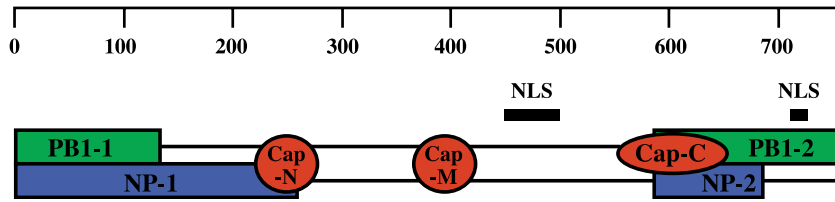


Fig. 8. Functional domains of PB2. The minimal identified sequences capable of binding PB1 (green boxes) and NP (blue boxes) are depicted relative to a linear representation (scale bar calibrated in amino acids) of the PB2 molecule, while sequences implicated in cap binding are shown in red. Black bars indicate sequences implicated in the nuclear localization of PB2. NP- and PB1-binding domains: this study. N-terminal (N), middle (M), and C-terminal (C) cap-binding sequences: de la Luna et al. (1989), Fechter et al. (2003), Honda et al. (1999), Li et al. (2001). NLS sequences: Mukaigawa and Nayak (1991).

De la Luna et al., 1989; Honda et al., 1999; Li et al., 2001). However, we found no evidence for cap- or RNA-binding activity in the purified PB2 fusion proteins employed in this study (data not shown).

Both NP/PB1-binding domains of PB2 acted as trans-dominant inhibitors of the viral RNA polymerase in a recombinant virus gene expression assay, further confirming their functional importance (Fig. 6). A previous study noted the inhibitory effect of co-expressing an N-terminal fragment of PB2 on RNP function and proposed that the inhibition resulted from competition with the WT protein for PB1 (Perales et al., 1996). Supporting this hypothesis, we found that the inhibitory potential of the N- and C-terminal fragments of PB2 depended on the amount of WT PB2 and PB1 (Fig. 7). However, the inhibitory activity of the N-terminal PB2 fragment also depended upon the expression levels of NP, consistent with its identification as an NP-binding domain. The somewhat surprising finding that increased expression levels of NP potentiated its inhibitory activity might reflect the mutually incompatible triangle of protein–protein interactions among the PB1, PB2, and NP polypeptides, or that the N-terminal fragment of PB2 only inhibits virus transcription when complexed with NP. It may also result from suboptimal or unbalanced expression levels of the individual RNP components in the recombinant systems employed here. This could also explain the finding that the C-terminal domain of PB2 is inhibitory in the presence of (relatively) low levels of PB1 but stimulatory in the presence of high amounts of the protein (Fig. 7d). Overall however, the abilities of the N- and C-terminal PB2 domains to modulate RNP transcriptional activity in a manner dependent on the concentration of NP and PB1 suggest that the protein–protein interactions identified here are functionally important.

What functions might the PB1–PB2–NP interactions play? The finding that each PB2 domain can bind either NP or PB1 but not both simultaneously is suggestive of a regulatory role. We have previously suggested that one function of a P protein–NP interaction might be to ensure processive RNA synthesis (Medcalf et al., 1999). This hypothesis is supported by the finding that NP is necessary for the transcription of full-length genome segments (Honda et al., 1988) and is consistent with an electron microscopic reconstruction of a recombinant RNP, which suggests that

the polymerase complex interacts with two NP molecules simultaneously (Martin-Benito et al., 2001). However, we found no evidence that the N- and C-terminal PB2 fragments could interfere with NP–NP or NP–RNA interactions in vitro (data not shown), which might be predicted for such a processivity function. Alternatively, it has been hypothesized that the protein–protein interaction of NP with the P proteins might function to alter the activity of the polymerase complex to bias it from mRNA transcription toward genome replication (Biswas et al., 1998; Mena et al., 1999). Some studies (but not all; Brownlee and Sharps, 2002; Lee et al., 2002; Perales and Ortin, 1997) have found evidence that complexes of PB1 and PA might be able to synthesize cRNA in the absence of PB2 (Honda et al., 2002; Nakagawa et al., 1995, 1996). In the absence of any evidence for subcomplexes of the P proteins in vivo, it has been suggested that the viral replicase might consist of a trimeric complex in which PB2 has been functionally disabled (Honda et al., 2002). The demonstration here that PB2 contains two separate regions that can bind either PB1 or NP but not both suggests the possibility that a polymerase complex containing one molecule of each P protein and NP could form in which a PB2–PB1 contact was replaced by a PB2–NP interaction. If the absent PB1–PB2 interaction was functionally important for mRNA synthesis, such a tetrameric complex might form a replicase. This hypothesis is consistent with our finding that the co-expression of the individual PB2 fragments inhibited viral gene expression, if one postulates that the PB2 domains functioned as NP analogues. We have been unable to determine whether the PB2 fragments inhibit all forms of viral RNA synthesis or only mRNA transcription because the low efficiency of the transfection system renders unambiguous detection of cRNA difficult. However, the identification of single amino acid substitutions in NP and PB2 that inhibit NP–PB2 binding without affecting other functions of the polypeptides would provide an alternative way of testing the hypothesis that an NP–PB2 interaction promotes genome replication. We note that a recent study identified a series of point mutations in the N-terminal region of PB2 that inhibit genome replication without apparent effects on PB1-binding or mRNA transcription (Gastaminza et al., 2003). In light of the results presented here, it would be interesting to test the effects of these mutations on NP binding.

Materials and methods

Antisera and plasmids

A set of rabbit sera raised against defined regions of PB2 (expressed in *E. coli* as fusion proteins with β -galactosidase) has already been described (Blok et al., 1996). In some cases, a GST–PB1 fusion protein containing the C-terminal 75 amino acids of PB1 (GST-1N683; see later for construction) was used to precipitate N-terminal fragments of PB2, to avoid use of the low titer PB2 F1 serum. The design of this fusion protein is based on the results of our own unpublished experiments mapping the PB1 sequences important for binding PB2, which are in broad agreement with the results of others (Gonzalez et al., 1996; Toyoda et al., 1996). The anti-PB1 sera used were generated by immunizing rabbits with β -galactosidase fusion proteins containing aa 50–370 (F1; Digard et al., 1989) or aa 421–665 (F3). Plasmids containing cDNA copies of the wild-type P protein genes flanked by 5'- and 3'-nontranslated regions of the *Xenopus* β -globin gene, under the control of either bacteriophage SP6 or T7 RNA polymerase promoters (pKT series), have previously been described (Blok et al., 1996; Digard et al., 1989). Deletion mutations were created using convenient restriction enzyme sites in the wild-type P genes, or in some cases, a unique *Sna*BI site introduced by ligating the oligonucleotide TGGTACG-TACCA into a desired restriction enzyme site, and standard recombinant DNA methodology to generate plasmids lacking specified portions of the P genes. In the interest of brevity, details of the cloning steps are not reported here, but are available on request. N-terminally deleted (N) mutants are named according to the first codon of the WT sequence present, C-terminally truncated (C) mutants according to the last. All C-terminal deletion constructs encode between 1 and 6 additional non-influenza-derived residues at the 3'-end of the ORF as a result of the DNA cloning procedures. A plasmid directing the synthesis of a GST–NP fusion protein has been described previously (Digard et al., 1999). GST–PB1 gene fusions were constructed by excising fragments from PB1 cDNA cloned into pKT1 by digestion with either *Sca*I (at nucleotide coordinate 2067) and *Sma*I (3' to PB1 gene), or *Eco*RV (2154 and 3' to PB1 gene), and ligating them into pGEX-4T-1 digested with *Sma*I (Smith and Johnson, 1988; Pharmacia). The resulting plasmids pGEX-1N683 and pGEX-1N712 contained amino acid codons 683 or 712–757, respectively, fused in frame to the 3'-end of the GST open reading frame, separated by a short linker sequence containing a thrombin protease recognition sequence. GST–PB2 gene fusions were constructed similarly using segment 1 cDNA from plasmid pKT2 or pVB2 (Blok et al., 1996). Plasmid pGEX2 N489 utilized *Sca*I (nucleotide 1489) and *Xba*I (3' to PB2 gene and end filled) and pGEX 4T-3 digested with *Sma*I. Plasmid pGEX 2N580 utilized *Asp* 700 (nucleotide 1763) and *Xba*I (end filled) and pGEX 4T-1 digested with *Sma*I. Plasmid pMAL-2N580

was created by a similar strategy except that a 3'-PB2 gene fragment obtained by digestion with *Asp* 700 and *Sa*II was first subcloned into plasmid pGAD424 (Clontech), removed by digestion with *Eco*RI, and then inserted into plasmid pMAL-c2 digested with *Eco*RI. As with the GST PB1 constructs, the GST and PB2 coding regions were separated by a short linker sequence containing a thrombin protease recognition sequence. To express N-terminal sequences of PB2, plasmid pGEX-2C402 was created by digestion of pVB2 with *Bam*HI (5' to PB2 coding region) and *Nco*I (nucleotide 1230, end filled) and ligation of the resulting DNA fragment into pGEX-3X digested with *Bam*HI and *Sma*I. An in frame stop-codon was then introduced after codon 258 by ligation of the commercial oligonucleotide 5'-CTAGTCTAGACTAG-3' (New England Biolabs; *Xba*I linker) into the *Hind*III site (after end-repair) at nucleotide 798 of the PB2 gene. To permit T7 RNA polymerase-driven expression of GST and GST–PB2 fusions, the oligonucleotide primers 5'-CAGGAAACAGTATTCATGTC-3' and 5'-CGCGAGGCAGATCGTCAGTC-3' were used to PCR amplify the appropriate sequences from plasmids pGEX-2C258 and pGEX-2N580 that were then cloned into plasmid pKT-0 (Tibbles et al., 1995) to create plasmids pKTGST-2C258 and pKTGST-2N580, respectively. To create a plasmid that expressed nonfused GST under the control of a T7 RNA polymerase promoter, pKTGST-2C258 was digested with *Bam*HI to excise the PB2 coding region and religated with the addition of the *Xba*I oligonucleotide linker to create plasmid pKT-GST.

Expression and purification of fusion proteins

NP fused to either MBP or GST was purified from *E. coli* containing the appropriate plasmids as described previously (Digard et al., 1999; Frangioni and Neel, 1993). Purified proteins were dialysed against 10 mM HEPES, pH 7.6, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol, and stored at -70°C . Where required, fusion proteins were also left bound to sepharose beads, resuspended in an equal volume of 100% glycerol, and stored at -20°C .

RNA transcription, in vitro translation, microinjection, and immunoprecipitations

Capped RNAs were generated by in vitro transcription of the appropriate linearized plasmids as previously described (Digard et al., 1989). In the case of plasmid constructs that lacked the *Xenopus* β -globin 3' non-coding sequence, polyadenylated RNA was generated by the inclusion of 32 U/ μ l of poly(A) polymerase (GIBCO-BRL) in the transcription reaction. Radiolabeled NP polypeptides were synthesized using a coupled in vitro transcription–translation system as previously described (Elton et al., 1999a). Oocytes were taken from frogs, maintained, and microinjected as previously described (Blok et al., 1996; Digard et al., 1989). Oocytes were injected with around 25 ng of each mRNA in

a maximum volume of 50 nl. After 1 h, the cells were transferred to Modified Barth's Saline containing [³⁵S]-methionine at 1.0 mCi/ml (10 µl/oocyte) and incubated for 5 h. Subsequently, the oocytes were harvested by mechanical disruption into 50 mM Tris–Cl, pH 7.6, 100 mM KCl, 5 mM MgCl₂, 10% glycerol, 1 mM DTT, and the extracts clarified by centrifugation. In some experiments, radiolabeled PB1 was prepared from BHK cells transfected with plasmid pKT1, infected with vaccinia virus vTF-7, and incubated overnight in the presence of [³⁵S]-methionine. For immunoprecipitation, aliquots (5–10 µl, containing the equivalent of half to one oocyte) were diluted into 100 µl of high detergent immunoprecipitation (IP) buffer (50 mM Tris–Cl, pH 7.6, 100 mM KCl, 5 mM MgCl₂, 1 mM DTT, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS), and left on ice for 30 min before the addition of up to 4 µl antiserum. After a further 1 h on ice, 50 µl of a 50% (v/v) suspension of protein-A sepharose (Sigma) in high detergent IP buffer was added and the samples incubated with gentle mixing for 30 min at room temperature. Sepharose-bound material was collected by centrifugation, washed twice with 750 µl of high detergent IP buffer, once with a similar volume of low detergent IP buffer (containing 0.1% Nonidet P-40 as the only detergent) before elution by boiling in SDS-PAGE sample buffer. Radiolabeled proteins were then separated by polyacrylamide gel electrophoresis and detected by autoradiography.

Binding studies with GST fusion proteins

GST fusion proteins (typically 0.3 µg) were diluted in 100 µl of low detergent IP buffer containing 1 mM DTT, mixed with 1–2 µl of [³⁵S]-methionine labeled oocyte lysate, and incubated at room temperature for 1 h before the addition of 40 µl of a 50% (v/v) suspension of glutathione sepharose in PBS. After 30 min incubation with gentle mixing at room temperature, the solid phase was collected by centrifugation, washed three times with 750 µl of low detergent IP buffer, and bound material eluted by boiling in SDS-PAGE sample buffer. Precipitated polypeptides were separated by SDS-PAGE, nonradioactive proteins detected by staining with Coomassie brilliant blue dye (to confirm precipitation of the GST fusion proteins), and radiolabeled polypeptides detected by autoradiography. Binding was quantified by densitometry of the exposed X-ray films as previously described (Digard et al., 1999).

Influenza virus gene expression assay

For in vivo transcription assays, a synthetic influenza genome segment containing an antisense chloramphenicol acetyl transferase (CAT) gene was produced by in vitro transcription of plasmid pPB2CAT9 (generous gift of Dr. Mark Krystal). The three polymerase proteins and NP were supplied by either infection with recombinant vaccinia

viruses (PB1-, PB2-, PA-, and NP-VAC; Smith et al., 1987) or by infection with a vaccinia virus that expresses the bacteriophage T7 RNA polymerase (VTF-7; Fuerst et al., 1987) and transfection with a plasmid containing a cDNA copy of the appropriate influenza virus gene (pKT1, 2, 3, 5 for PB1, PB2, PA, and NP, respectively) under the control of a T7 RNA polymerase promoter. BHK or CV-1 cells (in 6-well plates) were infected with recombinant vaccinia viruses at a multiplicity of infection of 5 of each virus per cell for 2 h at 37 °C. The cells were washed 3 times with serum-free medium before transfection with up to 1 µg of each individual plasmid and 0.5 µg of pPB2CAT9 in vitro transcribed RNA and 10 µg of a cationic liposome mixture (Escort; Sigma-Aldrich), according to the manufacturers' instructions. The cells were incubated at 37 °C for 20 h, washed 3 times with ice-cold PBS, and solubilized in 1 ml of CAT ELISA lysis buffer (Boehringer Mannheim). The lysate was clarified by centrifugation at 14000 × g for 10 min at 4 °C and CAT expression quantified relative to known standards by a commercial enzyme-linked immunosorbent assay (Boehringer Mannheim). Initial titration experiments established that the optimal amounts of each plasmid required were 50 ng for the polymerase subunits and 1 µg for NP (data not shown).

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