Distinct regions of influenza virus PB1 polymerase subunit recognize vRNA and cRNA templates

Susana González and Juan Ortín[1]

Centro Nacional de Biotecnología (CSIC), Campus de Cantoblanco, 28049 Madrid, Spain

[1]Corresponding author
e-mail: jortin@cnb.uam.es

The influenza virus RNA polymerase is a heterotrimer comprising the PB1, PB2 and PA subunits. PB1 is the core of the complex and accounts for the polymerase activity. We have studied the interaction of PB1 with model cRNA template by *in vitro* binding and Northwestern analyses. The binding to model cRNA was specific and showed an apparent K_d of ~7 × 10⁻⁸ M. In contrast to the interaction with vRNA, PB1 was able to bind equally the 5′ and 3′ arm of the cRNA panhandle. The N-terminal 139 amino acids of PB1 and sequences between positions 267 and 493 proved positive for binding to cRNA, whereas the interaction with vRNA template previously was mapped to the N- and C-terminal regions. Competition experiments using the 5′ and 3′ arms of either the vRNA or cRNA panhandle indicated that the N-terminal binding site is shared by both templates. The data indicate that the PB1 RNA-binding sites are constituted by: (i) residues located at the N-terminus (probably common for vRNA and cRNA binding) and, either (ii) residues from the central part of PB1 (for cRNA) or (iii) residues from the C-terminal region of PB1 (for vRNA), and suggest that PB1 undergoes a conformational change upon binding to cRNA versus vRNA templates.
Keywords: cRNA/influenza virus/PB1 subunit/ polymerase/RNA-binding sites

Introduction

The influenza A viruses are members of the family Orthomyxoviridae that produce epidemics of respiratory infections every year and occasionally severe pandemics. Their genome consists of eight single-stranded RNA segments of negative polarity with a coding capacity for 10 proteins (for reviews, see Krug *et al*., 1989; Lamb, 1989). The transcription and replication of these RNA segments take place in the nucleus of the infected cells (Herz *et al*., 1981; Jackson *et al*., 1982) and are mediated by ribonucleoprotein (RNP) complexes in which the vRNA template is associated with the nucleoprotein (NP) and the three P proteins (PB2, PB1 and PA) (reviewed in Krug *et al*., 1989; Lamb, 1989). Replication of vRNA molecules takes place by generation of full-length RNA copies of positive polarity (cRNA) that form RNP complexes analogous to those present in the virions (Hay, 1982). Transcription is initiated by cap stealing of cellular hnRNAs (Krug *et al*., 1979). The capped primers are

elongated by the polymerase present in the vRNPs to generate viral mRNAs that are polyadenylated at an oligo(U) signal located next to the RNA panhandle structure at the 5′ terminus of the vRNA templates (Robertson *et al*., 1981; Poon *et al*., 1999). Polyadenylation appears to require the proximity of the panhandle structure (Luo *et al*., 1991) and the interaction of the polymerase with the conserved 5′-terminal sequences of the template (Poon *et al*., 1998; Pritlove *et al*., 1998, 1999).

The viral polymerase consists of a heterotrimer formed by the PB1, PB2 and PA proteins (Kato *et al*., 1985; Detjen *et al*., 1987; Digard *et al*., 1989; Honda *et al*., 1990). All three subunits are required for viral RNA replication (Perales and Ortín, 1997). A map of intersubunit contact regions has been established. Thus, the N-terminal sequences of PB1 interact with the C-terminal region of the PA subunit, while sequences next to the C-terminus of PB1 interact with the N-terminal region of the PB2 subunit (Pérez and Donis, 1995; González *et al*., 1996; Perales *et al*., 1996; Toyoda *et al*., 1996; Zürcher *et al*., 1996). The interaction of the polymerase with vRNA template has been studied using virion cores or the enzyme complex reconstituted by co-expression of the subunits from vaccinia recombinant viruses. Both PB1 and PB2 subunits of the virion core could be cross-linked to the 3′-terminal sequence of vRNA (Fodor *et al*., 1993) and all three subunits were cross-linked to the vRNA 5′-terminal sequence (Fodor *et al*., 1994). The enzyme complex bound to the 5′-terminal sequence with higher affinity than to the 3′-terminal one (Tiley *et al*., 1994).

In recent years, our knowledge about the roles of the different subunits in the transcription and replication processes has improved considerably. The PB2 subunit has cap-binding and cap-dependent endonuclease activities (Ulmanen *et al*., 1981; Blaas *et al*., 1982; Licheng *et al*., 1995; Shi *et al*., 1996). Consistent with these findings, cap primer-dependent *in vitro* RNA synthesis is affected by mutations in the PB2 gene (Perales *et al*., 1996; P.Gastaminza, B.Perales and J.Ortín, unpublished results). Nevertheless, both transcription and cap-dependent endonuclease activity require the presence of the three subunits of the polymerase and the RNA template (Hagen *et al*., 1994; Cianci *et al*., 1995). Genetic evidence (reviewed in Mahy, 1983) suggests a role for the PA subunit in vRNA synthesis. The PA subunit is a phosphoprotein (Sanz-Ezquerro *et al*., 1998) whose expression by transfection leads to the degradation of co-expressed proteins (Sanz-Ezquerro *et al*., 1995). The regions of the PA subunit responsible for this activity map to the N-terminal third of the protein (Sanz-Ezquerro *et al*., 1996), close to the nuclear localization signal (NLS) (Nieto *et al*., 1994). The PB1 protein is responsible for the polymerase activity. It contains amino acid motifs typical of RNA-dependent RNA polymerases (Poch *et al*., 1990), and mutations of

the conserved residues abolish the transcriptional activity (Biswas and Nayak, 1994). Furthermore, extracts from baculovirus-infected cells expressing PB1 show some polymerase activity *in vitro* (Kobayashi *et al.*, 1996). Several functional domains have been identified in the PB1 subunit. Thus, in addition to the regions responsible for the polymerase activity (Biswas and Nayak, 1994), the locations of the NLS and the putative nucleotide-binding domains have been described (Nath and Nayak, 1990; Asano and Ishihama, 1997). Likewise, the protein regions involved in binding to the vRNA template have been identified (Li *et al.*, 1998; González and Ortín, 1999). Two different regions of the protein, located at the N- and C-terminus, appear to interact with both arms of the panhandle structure of the template, the interaction with the 5′ arm being of higher affinity than that with the 3′ arm (González and Ortín, 1999). In this report, we have extended these studies by analyzing the interaction of the isolated PB1 subunit with the cRNA template. The individual PB1 protein bound cRNA specifically, with an apparent K_d of ~7×10^{-8} M. PB1 bound both the 5′ and the 3′ arm of the panhandle with similar affinities. Although the N-terminal region of the protein was also responsible for the binding to the cRNA template, the central region of the protein, rather than the C-terminal portion, was involved in the recognition of the cRNA template.

Results

The PB1 subunit of the polymerase specifically binds virus complementary RNA

We have shown previously that the PB1 subunit of influenza virus RNA polymerase is able to bind specifically a vRNA analog probe (González and Ortín, 1999). To study whether isolated PB1 is also able to bind cRNA, we followed a similar experimental strategy. The protein was expressed by transfection of PB1 cDNA, cloned under control of the T7 promoter, into vaccinia–T7 virus-infected COS-1 cells. The infected–transfected cells were labeled continuously with [^{35}S]methionine and [^{35}S]cysteine, and soluble extracts were prepared. The recombinant PB1 protein present was immunoprecipitated with specific antibodies and the immunoprecipitates were used for the *in vitro* binding of a cRNA labeled probe (cNSZ, containing a deleted *CAT* gene between the termini of the NS segment; Perales and Ortín, 1997). As a control, an unrelated probe (G3N) of similar length was used. The binding was carried out in the presence of excess yeast RNA and, after extensive washing, the complexes were split into two identical fractions. The protein present in one of the fractions was analyzed by SDS–PAGE and the bound RNA was isolated from the other fraction and analyzed by polyacrylamide–urea gel electrophoresis. The results are presented in Figure 1. It is clear that PB1-containing immunoprecipitates were capable of retaining the specific cNSZ probe (Figure 1A, left panel), but not the unrelated G3N probe (Figure 1A, right panel). As shown in the bottom panels of Figure 1A, the PB1-specific immunoprecipitates contained PB1, but the control immunoprecipitates did not.

To test further the specificity of PB1–cRNA binding, competition experiments were carried out. Parallel binding
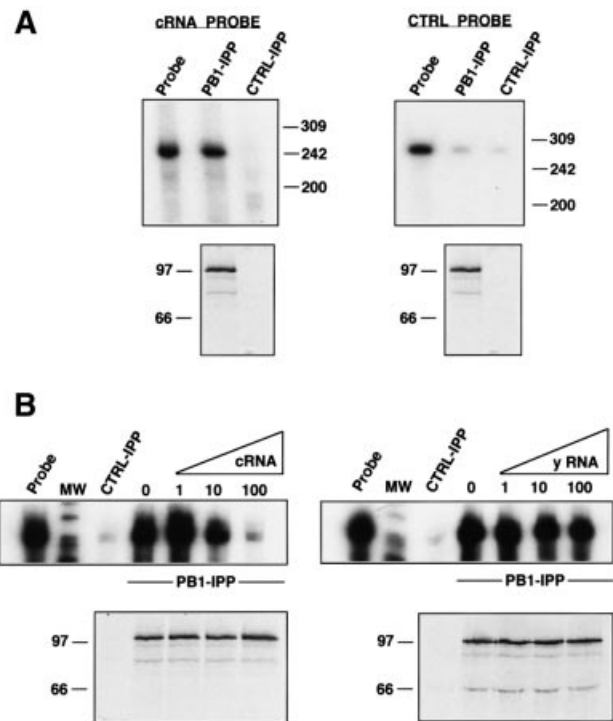

Fig. 1. Specificity of PB1 binding to cRNA. Cultures of COS-1 cells were infected with vaccinia–T7 virus and transfected with either pGorfB1 plasmid or pGEM3 plasmid as a control. The cultures were labeled with [^{35}S]methionine/cysteine and soluble extracts were prepared as described in Materials and methods. (**A**) The extracts were used for immunoprecipitation with an anti-PB1 antiserum, and the immunoprecipitates (B1-IPP or CTRL-IPP) were incubated with ^{32}P-labeled cNSZ probe (cRNA PROBE) or control G3N probe (CTRL PROBE) (sp. act. ~10^8 c.p.m./µg) in the presence of a 250-fold excess of yeast RNA. The RNA from a fraction of the complexes was isolated and analyzed by denaturing gel electrophoresis (top panels) while the protein present was isolated and visualized by SDS–PAGE and autoradiography (bottom panels). (**B**) The binding assay was performed as indicated in (A), except that increasing amounts of unlabeled cNSZ probe (cRNA) (1, 10 or 100 ng; i.e. 10- to 1000-fold molar excess) or yeast RNA (yRNA) were included in the assay. The RNA retained was isolated and analyzed as indicated above (top panels). The proteins present in the immunoprecipitates are shown in the bottom panels. Numbers to the left indicate the size of protein markers in kDa. Numbers to the right indicate the length of RNA markers (MW) in nucleotides. In this and subsequent figures, the lanes indicated as Probe show one-tenth of the amount of probe that was included in the binding test.

tests were performed in which increasing amounts of unlabeled cNSZ RNA or unlabeled yeast RNA were added, in addition to the labeled cNSZ probe, and the retention of the latter was tested as indicated above. The results presented in Figure 1B indicate that the homologous unlabeled probe competed out the labeled probe (Figure 1B, left panel) while an equivalent excess of unrelated RNA did not (Figure 1B, right panel). The presence of equivalent quantities of PB1 in the specific immunoprecipitates was ascertained by SDS–PAGE analysis of the same samples and is presented in the lower panels of Figure 1B.

To estimate the affinity of the interaction of cRNA with isolated PB1, we performed binding experiments in which increasing amounts of PB1-containing extracts were used for immunoprecipitation. The absolute quantity of PB1 present in each binding assay was determined by using as a standard total cell extract containing PB1, labeled in
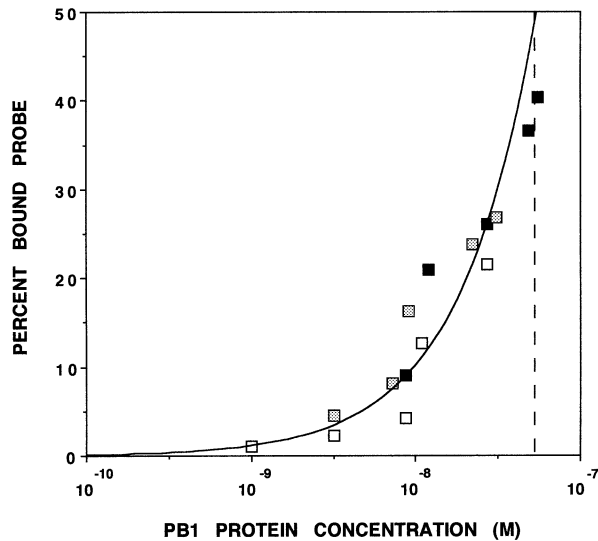
Fig. 2. Affinity of binding of PB1 to cRNA. *In vitro* RNA-binding assays were carried out as described in the legend to Figure 1, using cNSZ probe at a concentration of 10^{-11} M. Extracts containing increasing amounts of PB1 were used, and the actual concentrations of PB1 present in each assay were determined as indicated in the text. The percentage probe retained after washing was determined by Cerenkov counting. Different symbols represent the results of three independent experiments. The curve is the best exponential fit of the data.

Fig. 3. Northwestern assay with the cNSZ probe. Cultures of COS-1 cells were doubly infected with vaccinia–T7 and VPB1 viruses (VAC-B1), or singly infected with vaccinia–T7 virus (VAC-T7) as a control. Extracts prepared as indicated in Materials and methods were separated by SDS–PAGE and transferred to nitrocellulose filters. After renaturation, the filters were incubated with the cNSZ probe (cRNA) or the control G3N probe (CTRL). (**A**) After washing, the signal was revealed by autoradiography. (**B**) The same filters were developed by Western blot with an anti-PB1 serum. Numbers to the left indicate the size of protein molecular weight markers in kDa.

Fig. 4. Interaction of PB1 with the 5′ and the 3′ arm of the cRNA panhandle. *In vitro* RNA-binding assays were carried out as described in Materials and methods and in the legend to Figure 1, except that 3′ probe, 5′ probe or a mixture of both were used. The 3′ probe always contained a shorter oligonucleotide probably generated by premature termination of transcription. The top panel shows the analysis of the RNA retained. The bottom panel shows the analysis of the proteins present in the immunoprecipitates. Numbers to the left indicate the size of protein markers in kDa. Numbers to the right indicate the length of RNA markers in nucleotides.

parallel, derived from COS-1 cells doubly infected with vaccinia–T7 and VPB1 recombinant virus, as described (González and Ortín, 1999). Such an extract contains a high proportion of PB1 that can be quantitated by Coomassie Blue staining using bovine serum albumin (BSA) as a reference. The proportion of the added cNSZ probe that was retained by the PB1-specific immunoprecipitates was determined by counting the associated Cerenkov radiation. The cumulative data from three separate experiments (Figure 2) led to an estimation of 7×10^{-8} M for the K_d. This is only an estimation because the determination of the absolute concentration of PB1 is technically difficult and the maximal concentration of PB1 obtained was not sufficient to achieve saturation in the binding reaction.

The binding assays described above indicate a specific binding of cRNA to the PB1-containing immunoprecipitates but do not demonstrate a direct interaction of PB1 with the cNSZ probe. To address such a question directly, we carried out Northwestern assays. Extracts derived from COS-1 cells doubly infected with vaccinia–T7 and VPB1 viruses were prepared and separated by SDS–PAGE as described in Materials and methods. The separated proteins were renatured after their transfer to a nitrocellulose membrane and probed with either cNSZ or the G3N unrelated probe. After autoradiography, the filter was probed by Western blot with antibodies specific for PB1. The results are shown in Figure 3. In addition to unspecific reactive bands, a major reactive band was observed in extracts of VPB1-infected cells when the cNSZ probe was used (Figure 3A, left panel), but not when the unspecific G3N probe was used (Figure 3A, right panel). This signal had a mobility corresponding to the size of PB1 and could be superimposed on the signal of PB1 by Western blot of the same filters (Figure 3B).
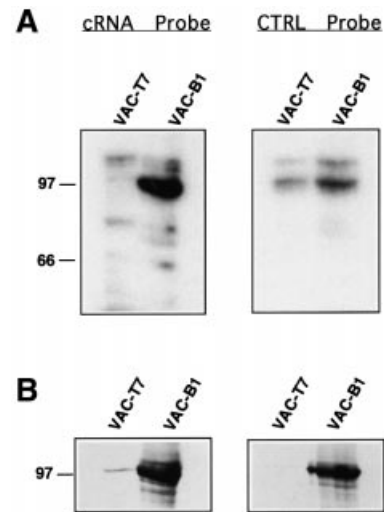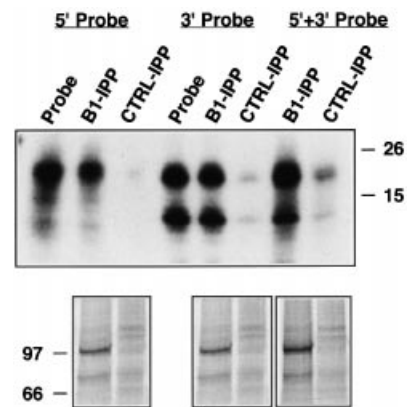
PB1 binds both the 5′ and the 3′ arm of the cRNA panhandle

We have demonstrated previously that PB1 binds preferentially to the 5′ arm of the vRNA panhandle (González and Ortín, 1999), although binding to the 3′ arm is also detectable. To study whether a similar situation exists with regard to the PB1–cRNA binding, we prepared short probes corresponding to the 5′ and 3′ arms of the cRNA panhandle and carried out *in vitro* binding experiments analogous to those reported above for the cNSZ probe. As shown in Figure 4, both the 5′ and 3′ arm probes were retained by PB1-specific immunoprecipitates, and binding
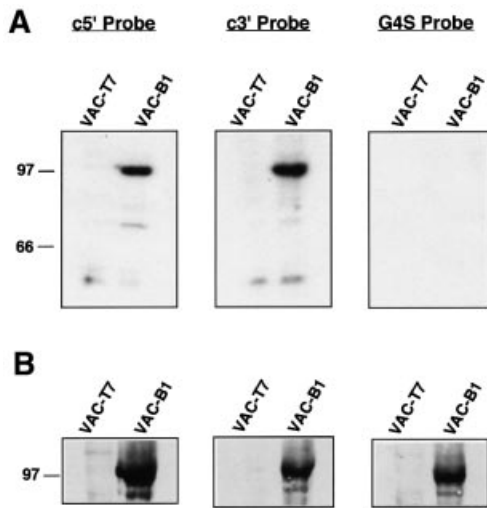
Fig. 5. Northwestern assay with the 5′ and the 3′ arm of the cRNA panhandle. Cultures of COS-1 cells were doubly infected with vaccinia–T7 and VPB1 viruses (VAC-B1), or singly infected with vaccinia–T7 virus (VAC-T7) as a control. Extracts were processed as indicated in Materials and methods and in the legend to Figure 3. The filters were incubated with either 5′ probe (c5′), 3′ probe (c3′) or the control G4S probe. (**A**) After washing, the signal was revealed by autoradiography. (**B**) The same filters were developed by Western blot with an anti-PB1 serum. Numbers to the left indicate the size of protein molecular weight markers in kDa.

to a 5′ + 3′ annealed probe was not enhanced as compared with the individual interactions. Under the same experimental conditions, no detectable binding was observed when an unrelated probe of similar length was used (G4S probe, 26 nucleotides, data not shown).

The binding of either the 5′ or the 3′ arm sequences to PB1 was also tested by Northwestern assays. Extracts obtained from cells doubly infected with vaccinia–T7 and VPB1 recombinant viruses, or only with vaccinia–T7 as a control, were separated by SDS–PAGE, renatured and tested for binding with either the 5′ arm, the 3′ arm or a control G4S probe. The results clearly indicated a direct interaction of PB1 with both 5′ and 3′ probes but not with the unrelated probe (Figure 5A). The presence of PB1 in the extracts from doubly infected cells was ascertained by Western blot and is presented in Figure 5B.

Mapping the PB1 sequences required for binding to cRNA

To obtain information about the regions of PB1 involved in binding to cRNA, several deleted versions of the gene were prepared. In every case, the mutant proteins were expressed in mammalian cells as His-tagged recombinants to allow their immunoprecipitation with a common antiserum recognizing the tag present in them (see Figure 6A for a diagram). The corresponding immunoprecipitates (Figure 6D) were used for *in vitro* binding to vNSZ, cNSZ probe or an unrelated probe as reported above for wild-type PB1. The results of these experiments are presented in Figure 6. Similarly to the results obtained for wild-type PB1, no retention was observed for the unspecific probe (data not shown). As reported previously (González and Ortín, 1999), vNSZ RNA was retained by the N-terminal region of PB1 (mutants PB1-Δ267–757 and PB1-Δ139–757) and also by the C-terminal region (mutant PB1-Δ1–493) (Figure 6C).
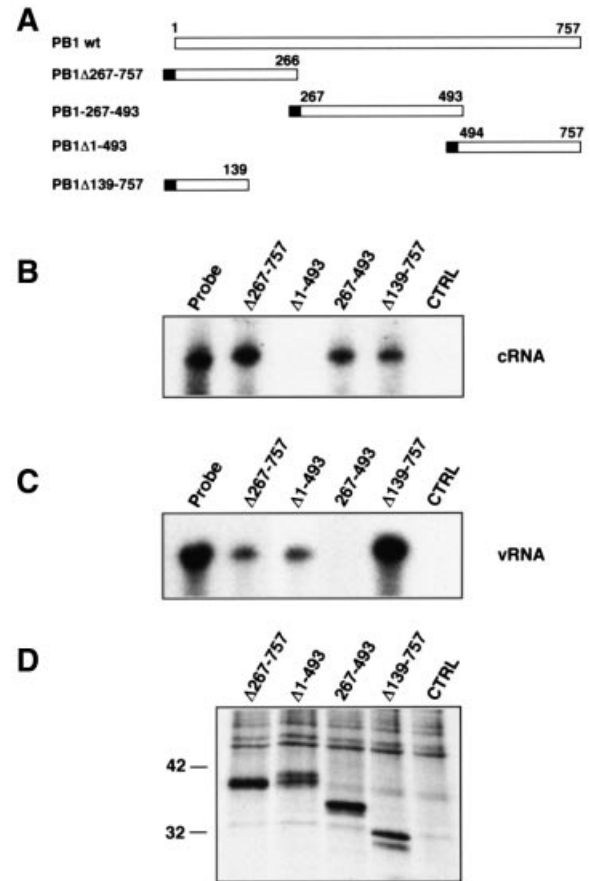

Fig. 6. Binding of mutant PB1 proteins to cRNA. Cultures of COS-1 cells were infected with vaccinia–T7 virus and transfected with the mutant plasmids indicated in (**A**) or pRSET plasmid as a control (CTRL). The RNA-binding assay was carried out as indicated in Materials and methods and in the legend to Figure 1. The mutants were expressed as His-tagged proteins (denoted by black boxes to the left of each bar) and immunoprecipitated with anti-HisNS1 serum. (**B**) Analysis of the cRNA retained. (**C**) Analysis of the vRNA retained. (**D**) Analysis of the proteins present in the immunoprecipitates. Numbers indicate the size of protein molecular weight markers in kDa.

Binding to the cNSZ probe was also positive for mutants containing the N-terminal regions of PB1 (mutants PB1-Δ267–757 and PB1-Δ139–757) but not the C-terminal region (mutant PB1-Δ1–493). Instead, positive binding was detected with mutant PB1-267–493 (Figure 6B). The level of probe retention was similar for wild-type PB1 and for the PB1 mutants that proved positive for binding (compare Figures 1A and 6B). The results presented clearly indicate that although the N-terminal sequences of PB1 appear to bind either vNSZ or cNSZ probes, distinct regions elsewhere in the protein are involved in the recognition of vRNA and cRNA (see below).

Since PB1 is able to bind both the 5′ and the 3′ arm of the cRNA panhandle (Figure 4), we wished to know whether the two separate sequences of PB1 protein involved in cRNA binding, defined by mutants PB1-267–493 and PB1-Δ139–757, were involved in the recognition of either arm of the panhandle or, alternatively, if each arm was recognized by both binding regions of PB1. Hence, *in vitro* binding experiments were carried out using either 5′ or 3′ arm probes and the various deletion mutants described above. The results presented in Figure 7 further
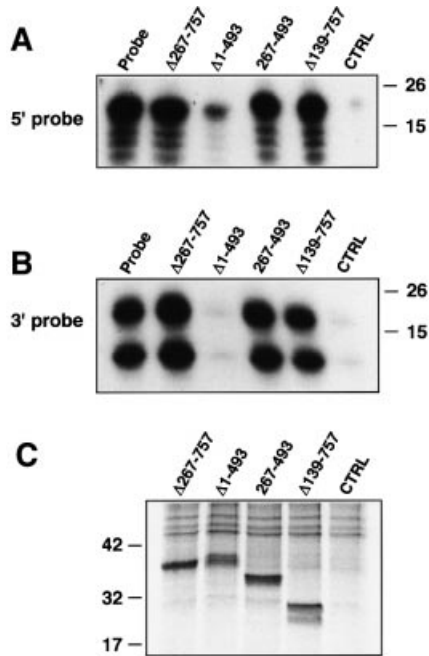
Fig. 7. Binding of mutant PB1 proteins to the 5′ and the 3′ arm of the cRNA panhandle. Cultures of COS-1 cells were infected with vaccinia–T7 virus and transfected with the mutant plasmids indicated in Figure 6A or pRSET plasmid as a control (CTRL). The RNA-binding assay was carried out as indicated in Materials and methods and in the legend to Figure 1. Analysis of the RNA retained when using (**A**) the 5′ probe and (**B**) the 3′ probe. (**C**) Analysis of the proteins present in the immunoprecipitates. Numbers to the right indicate the length of RNA markers in nucleotides. Numbers to the left indicate the size of protein molecular weight markers in kDa.
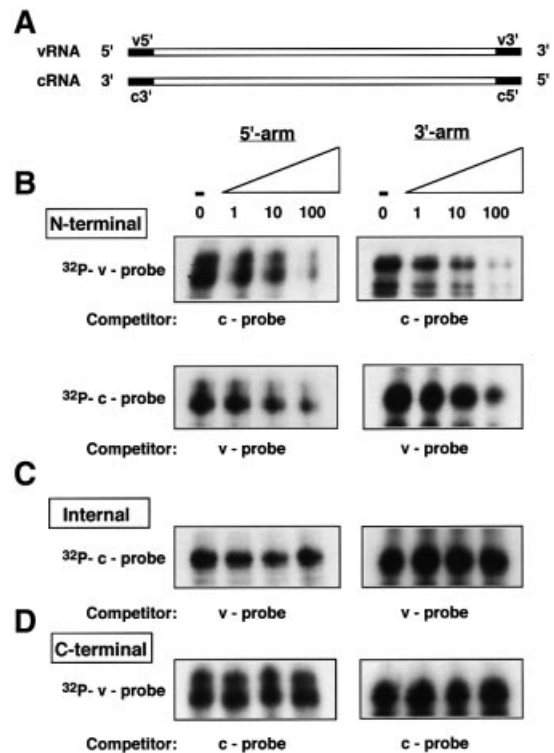

Fig. 8. Competition assays. Cultures of COS-1 cells were infected with vaccinia–T7 virus and transfected with the mutant plasmids pRPB1Δ267–757 (N-terminal), pRPB1-267-493 (internal) or plasmid pRPB1Δ1–493 (C-terminal). The RNA-binding assay was carried out as indicated in Materials and methods and in the legend to Figure 1 using the labeled probes indicated in the figure. (**A**) Diagram indicating the probes used in the competition experiments. Results obtained for binding to the (**B**) PB1Δ267–757 mutant, (**C**) the PB1-267–493 mutant and (**D**) the PB1Δ1–493 mutant. In each case, the indicated amounts (in ng) of competing unlabeled probes were included in the binding tests.

substantiate the conclusion derived from Figure 6 with regard to the mapping of the two independent regions of PB1 involved in cRNA binding and indicate that they both interact with each arm of the cRNA panhandle. Thus, N-terminal and internal sequences of PB1 (mutants PB1-Δ139–757, PB1-Δ267–757 and PB1-267–493) are able to retain either probe, while the C-terminal sequences (mutant PB1-Δ1–493) are not (Figure 7A and B).

The PB1-binding sites for vRNA and cRNA are distinct and partially overlap

The mapping of the binding of vNSZ and cNSZ probes on PB1 indicated that the N-terminal region of the protein interacts with both RNAs while distinct sequences elsewhere in the protein contribute to the binding of vRNA and cRNA (González and Ortín, 1999; Figures 6 and 7). To ascertain whether the N-terminal region contains a single RNA-binding site, common to both vRNA and cRNA, or two distinct interaction sites, competition experiments were carried out. Each of the PB1 mutant proteins was used in binding experiments in which a labeled probe corresponding to the 5′ or the 3′ arm of either positive or negative polarity RNA (v5′, v3′, c5′ or c3′ probes) (Figure 8A) was competed with excess unlabeled homologous or heterologous probes. In every case, the homologous unlabeled probe was able to compete out binding (data not shown), in a way similar to the data presented in Figure 1B for the cNSZ probe. When the N-terminal sequences of PB1 were used for binding (mutant PB1-Δ267–757), the binding of labeled v5′ or v3′ probes

was competed with excess unlabeled c5′ or c3′ probes, respectively (Figure 8A). Likewise, the binding of labeled c5′ or c3′ probes was competed with unlabeled v5′ or v3′ probes, respectively (Figure 8B). These results indicate that a binding site common to both vRNA and cRNA exists at the N-terminal end of the PB1 molecule. In contrast, the binding of v5′ or v3′ labeled probes to the C-terminal sequence of PB1 (mutant PB1-Δ1–493) was not competed with excess unlabeled c5′ or c3′ unlabeled probes (Figure 8C). Similarly, no competition of c5′ or c3′ binding to the internal sequences of PB1 protein (mutant PB1-267–493) was observed when excess unlabeled v5′ or v3′ probes were used, respectively (Figure 8D). These results are in agreement with the binding of vRNA or cRNA panhandle arms to these regions of the protein (González and Ortín, 1999; Figures 6 and 7).

Discussion

The process of replication of influenza virus RNA implies first the synthesis of a complementary ribonucleoprotein (cRNP) that serves as a replication intermediate (Hay, 1982; reviewed in Krug *et al.*, 1989; Portela *et al.*, 1999). This involves the shift of template and the recognition of the cRNA by the newly synthesized polymerase and NP.
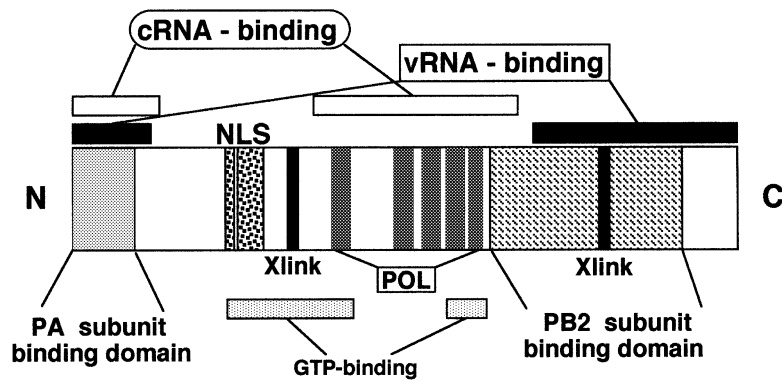
Fig. 9. Model for the PB1 protein. The figure shows a diagram of PB1 indicating the regions involved in its various biological properties. Xlink shows the sequences that were cross-linked to thio-U-modified vRNA probes (Li *et al*., 1998).

Since we showed previously that the isolated PB1 subunit binds a vRNA template with high affinity (González and Ortín, 1999), a binding that might reflect in part the interaction that takes place in the vRNP, we set out to study the interaction of the isolated PB1 protein with the alternative template, cRNA.

The PB1 subunit of the polymerase interacts with cRNA

The interaction experiments described demonstrate that the PB1 subunit of the polymerase is able to bind specifically a cRNA probe. The specificity of the interaction was validated by using an unrelated probe and by competition experiments with homologous and heterologous RNAs (Figure 1). Furthermore, a PB1–cRNA direct interaction was established by Northwestern assays (Figures 3 and 5). The specificity of binding was confirmed further by the interaction of PB1 with short probes corresponding to the 5′ and 3′ arms of the cRNA panhandle (Figure 4), but not with an unrelated probe of similar length (data not shown).

A substantial binding affinity could be inferred for the PB1–cRNA interaction, as the estimated K_d was ~7× 10^{-8} M (Figure 2), in the range of a typical RNA-binding protein (10^{-7}–10^{-9} M; Draper, 1995) (González and Ortín, 1999). Since a dramatic shift from cRNA to vRNA synthesis occurs early in the influenza virus infection (Enami *et al*., 1985), the simplest explanation for this template shift would be that the viral polymerase would have a higher affinity for cRNA than for vRNA binding, but this situation is not reflected in the interaction of these RNAs with isolated PB1 (González and Ortín, 1999; this study). It is tempting to speculate that the other subunits of the polymerase make a more important contribution to the polymerase–cRNA interaction than to the polymerase–vRNA interaction. In addition to the vRNA to cRNA template shift, it is remarkable that the newly synthesized vRNA is not used for cRNA synthesis (Enami *et al*., 1985). This fact suggests that other viral or cellular factor(s) might alter the newly synthesized vRNPs so as to direct their utilization for transcription and avoid further formation of cRNPs. In this context, it is worth mentioning that NS1 protein has been found associated with transcription–replication complexes in the infected cell (Marión *et al*., 1997a), but is absent from virions.

Another difference between vRNA and cRNA binding

to PB1 protein is reflected in the different interaction of the 5′ and 3′ arms of the vRNA and cRNA panhandles. For vRNA, a preferential interaction with the 5′ arm was found, whereas binding of the 3′ arm was dependent on the previous interaction of the 5′ sequences (González and Ortín, 1999). This situation might be the basis of the activation of capped primer binding mediated by the interaction with the 5′ arm (Hagen *et al*., 1994; Cianci *et al*., 1995). In the case of cRNA, either arm of the panhandle was equally able to interact with PB1, and no synergistic effect was observed when both arms were used for interaction *in vitro* (Figure 4).

Suggestion for a conformational change in PB1 protein upon interaction with either vRNA or cRNA

The mapping of the PB1 sequences involved in the recognition of a vRNA probe indicated that two regions of the protein were active in binding *in vitro*: the N-terminal 83 amino acids and the C-terminal 264 amino acids (González and Ortín, 1999). These results are partly contradictory to those presented in another report (Li *et al*., 1998), in which the interaction of the polymerase complex with vRNA was tested by cross-linking of thio-U-substituted RNA. These authors could cross-link the 5′ probe to a sequence located at around position 250 in the PB1 molecule. This discrepancy could be attributed to the fact that the positions modified with thio-U (position 15 in the 5′ arm and position 10 in the 3′ arm) are a few nucleotides away from the site of interaction mapped in the vRNA panhandle (Tiley *et al*., 1994; Klumpp *et al*., 1997). The evidence presented here indicates that an analogous N-terminal region of the protein interacts with cRNA (positions 1–139) (Figures 6 and 7) but downstream sequences different from those reported for vRNA binding are involved in cRNA recognition (positions 267–493 instead of positions 493–757). Furthermore, the competition experiments presented in Figure 8 indicate that the binding sites for cRNA downstream of position 267 do not overlap, i.e. no competition is observed when the heterologous probes are used, but the binding site close to the N-terminus is common for vRNA and cRNA binding, i.e. competition is observed in both directions. Since the 5′ and the 3′ arm of either vRNA or cRNA panhandles are recognized by both the N-terminus of PB1 and sequences downstream in the protein (González and

Ortín, 1999; Figure 7), we envisage the binding pockets as composed of: (i) residues located at the N-terminus (probably common for vRNA and cRNA binding) and, either (ii) residues from the central part of PB1 (for cRNA binding) or (iii) residues from the C-terminal region of PB1 (for vRNA binding). Such a structural design would imply that PB1 undergoes a conformational change upon binding to cRNA versus vRNA templates (Figure 9).

It is noteworthy that the region of PB1 involved in interaction with PA protein (Pérez and Donis, 1995; González *et al*., 1996; Toyoda *et al*., 1996) overlaps with the region mapped as responsible for vRNA binding (González and Ortín, 1999) and for cRNA binding (Figures 6 and 7; see Figure 9 for a diagram). This fact, together with the possibility of cross-linking PA to vRNA (Fodor *et al*., 1994), suggests that the interaction between both subunits might be mediated by protein–protein contacts as well as by protein–RNA–protein bridges. If that were the case, it could be predicted that the affinity of the PB1–PA interaction would be increased by addition of a vRNA or cRNA panhandle. Conversely, the affinity of the PB1–RNA interactions could be increased by the presence of PA.

On the other hand, there is an overlap between the region of PB1 that interacts with PB2 (González *et al*., 1996; Toyoda *et al*., 1996) and the region mapped as responsible for vRNA binding (González and Ortín, 1999), but not with the region involved in cRNA binding (Figures 6 and 7; see Figure 9 for a diagram). Therefore, it is tempting to speculate that the PB2 subunit participates in the recognition of the vRNA template but not of the cRNA template. The results presented here contribute to the delineation of a complex set of interactions among the influenza virus polymerase subunits and the RNA templates, and suggest a dynamic nature of the binding sites during the virus infection.

Materials and methods

Biological materials

The COS-1 cell line (Gluzman, 1981) was provided by Y.Gluzman and was cultivated as described (Ortín *et al*., 1980). The vaccinia recombinant virus vTF7-3 (Fuerst *et al*., 1987) was a gift of B.Moss. To generate plasmid pGorfB1, the open reading frame of the PB1 gene was amplified by PCR from plasmid pGPB1 (Mena *et al*., 1994), sequenced and inserted into the pGEM vector. The origin of plasmids pRPB1Δ267–757, pRPB1Δ1–493 and pRPB1-267–493 has been described previously (González *et al*., 1996). Plasmid pRPB1Δ139–757 was constructed by digestion of pRPB1Δ267–757 with *Eag*I restriction nuclease and filling in with the Klenow fragment of DNA polymerase I. Recombinant vaccinia virus VPB1 expresses PB1 cDNA under a T7 promoter (González *et al*., 1996; Sanz-Ezquerro *et al*., 1998). The antiserum specific for the N-terminal region of PB1 was prepared by immunization of rabbits with purified HisB1Δ267–757 protein (González *et al*., 1996). A general anti-PB1 protein serum was prepared by immunization of rabbits with purified PB1 protein obtained by isolation from SDS–polyacrylamide gels. The antiserum specific for His-NS1 protein has been described (Marión *et al*., 1997a). Monoclonal antibodies specific for the T7 antigenic tag were purchased from Novagen.

Transfection

Transfections were carried out as described before (González and Ortín, 1999). Briefly, cultures of COS-1 cells (35 mm dishes) were infected with vaccinia–T7 virus. After virus adsorption for 1 h at 37°C, the cultures were washed with Dulbecco's modified Eagle's medium (DMEM) and transfected with 10 µg of pGorfB1, mutants thereof, or pGEM3 as a control. The DNAs and cationic liposomes (2 µl/µg of DNA) were diluted separately to 100 µl of DMEM. Both dilutions were mixed, kept at room temperature for 15 min and added to the culture plates containing 1 ml of DMEM. Cationic liposomes were prepared as described (Rose *et al*., 1991).

RNA probe labeling

The synthesis of the cNSZ probe, which contains a deleted version of the *CAT* gene in positive polarity with the termini of the NS segment, was carried out as described (Perales and Ortín, 1997), using [α-^{32}P]GTP as a precursor. Transcription of the 5′ and 3′ arm probes was carried out as described (Seong and Brownlee, 1992; González and Ortín, 1999). Oligodeoxynucleotides 5′-CACCCTGCTTTTGCTCCTATAGTGAGT-CGTATTAACC-3′ or 5′-AGTAGAAACAAGGGTGCCTATAGTGAG-TCGTATTAACC-3′, that contain the T7 promoter (underlined) fused to the 5′ and the 3′ arm template sequences, respectively, were annealed to a T7 promoter complementary oligodeoxynucleotide (5′-GGTTAA-TACGACTCACTATAGG-3′). By transcription of these DNAs with T7 RNA polymerase, we obtained the 18 or 17 nucleotide, 5′ or 3′ arm probes, of which the 5′-terminal GG were not virus-specific. Two control probes were used: G4S probe (26 nucleotides) was transcribed with T7 RNA polymerase from plasmid pGEM4 digested with *Sma*I. In addition, G3N probe (330 nucleotides) was synthesized by transcription with T7 polymerase of pGEM3 plasmid digested with *Nhe*I.

RNA analyses

For *in vitro* binding of the labeled probes to PB1 protein, cultures of COS-1 cells were transfected with pGorfB1 plasmid, or pGEM3 as a control, and labeled with [^{35}S]methionine/cysteine as described before (González and Ortín, 1999). Soluble extracts were immunoprecipitated with 10 µl of a matrix of anti-PB1 IgG (or anti-HisNS1 IgG for His-tagged PB1 mutants) bound to protein A–Sepharose. The immune complexes were washed three times with RIPA buffer and seven times with TNE–NP-40 buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris–HCl, 1% NP-40, pH 7.5) containing 100 µg/ml yeast RNA. The immune complexes were incubated with ~30 000 c.p.m. of probe in TNE–NP-40 buffer containing 25 ng of yeast RNA for 1 h at 4°C. After washing three times with TNE–NP-40 buffer, the radioactivity retained in the resin was determined by Cerenkov counting. The resin was split into two identical portions that were used to analyze the bound RNA and the protein content. The bound RNA was isolated by boiling with TNE buffer containing 0.5% SDS and incubation with 50 µg/ml proteinase K for 30 min at 56°C in the same buffer. After phenol extraction, the eluted RNA was precipitated with ethanol and analyzed by electrophoresis on a 4 or 18% polyacrylamide sequencing gel, depending on the probe. The protein present in the immune complexes was extracted in Laemmli sample buffer and analyzed by SDS–PAGE and autoradiography. The quantitation of both RNA and protein was performed in a phosphoimager. As a standard for quantitation of PB1 concentrations, we used a total extract of cells doubly infected with vaccinia–T7 and VPB1 viruses and labeled with [^{35}S]methionine/cysteine as indicated below. BSA was used as a standard for protein concentration after Coomassie Blue staining.

Northwestern assays were carried out with extracts from cells doubly infected with vaccinia–T7 and VPB1 viruses as described previously (González and Ortín, 1999). These extracts were prepared in sample buffer [8% glycerol, 0.1% SDS, 1 mM dithiothreitol (DTT), 0.1% bromophenol blue, 12 mM Tris–HCl, pH 6.8 in phosphate-buffered saline (PBS)] by heating for 10 min at 30°C and centrifugation for 5 min at 10 000 *g* and 4°C. The samples were separated by electrophoresis in SDS–polyacrylamide gels and transferred to nitrocellulose filters in Tris–glycine buffer. The filters were incubated overnight at 4°C in renaturation buffer (50 mM NaCl, 1 mM EDTA, 0.02% each Ficoll, BSA and polyvinylpyrrolidone, 0.1% Triton X-100, 10 mM Tris–HCl, pH 7.5) and further incubated in the same buffer containing labeled probes in the presence of 1 µg/ml yeast RNA. After washing at room temperature with renaturation buffer, the filters were autoradiographed. The filters were processed further for Western blot with anti-PB1 serum as indicated below.

Protein analyses

In vivo labeling of PB1 or its mutant derivatives was carried out as described before (González and Ortín, 1999). At 6 h post-transfection, infected–transfected cultures were washed, starved for 1 h in methionine–cysteine-deficient DMEM medium and [^{35}S]methionine/cysteine was added to a final concentration of 200 µCi/ml in DMEM containing one-tenth the normal methionine/cysteine concentration. After incubation for 16–20 h, soluble extracts were prepared in TNE buffer containing 0.5% deoxycholate by centrifugation for 10 min at 10 000 *g* and 4°C. Total extracts were prepared in Laemmli sample buffer.

Western blotting was carried out as described (Marión *et al*., 1997b).

Briefly, cell extracts were processed by SDS–PAGE and transferred to Immobilon filters that were saturated with 3% BSA for 1 h at room temperature. The filters were incubated with anti-PB1 serum (1:1500 dilution) for 1 h at room temperature. After washing twice for 30 min with PBS containing 0.25% Tween-20, the filters were incubated with a 1:100 000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase. Finally, the filters were washed twice for 30 min as above and developed by enhanced chemiluminescence.

Acknowledgements

References

Asano,Y. and Ishihama,A. (1997) Identification of two nucleotide binding domains on the PB1 subunit of influenza virus RNA polymerase. *J. Biochem.*, **122**, 627–634.

Biswas,S.K. and Nayak,D.P. (1994) Mutational analysis of the conserved motifs of influenza A virus polymerase basic protein 1. *J. Virol.*, **68**, 1819–1826.

Blaas,D., Patzelt,E. and Keuchler,E. (1982) Identification of the cap binding protein of influenza virus. *Nucleic Acids Res.*, **10**, 4803–4812.

Cianci,C., Tiley,L. and Krystal,M. (1995) Differential activation of the influenza virus polymerase via template RNA binding. *J. Virol.*, **69**, 3995–3999.

Detjen,B.M., St. Angelo,C., Katze,M.G. and Krug,R.M. (1987) The three influenza virus polymerase (P) proteins not associated with viral nucleocapsids in the infected cell are in the form of a complex. *J. Virol.*, **61**, 16–22.

Digard,P., Blok,V.C. and Inglis,S.C. (1989) Complex formation between influenza virus polymerase proteins expressed in *Xenopus* oocytes. *Virology*, **171**, 162–169.

Draper,D.E. (1995) Protein–RNA recognition. *Annu. Rev. Biochem.*, **64**, 593–620.

Enami,M., Fukuda,R. and Ishihama,A. (1985) Transcription and replication of eight RNA segments of influenza virus. *Virology*, **142**, 68–77.

Fodor,E., Seong,B.L. and Brownlee,G.G. (1993) Photochemical cross-linking of influenza A polymerase to its virion RNA promoter defines a polymerase binding site at residues 9 to 12 of the promoter. *J. Gen. Virol.*, **74**, 1327–1333.

Fodor,E., Pritlove,D.C. and Brownlee,G.G. (1994) The influenza virus panhandle is involved in the initiation of transcription. *J. Virol.*, **68**, 4092–4096.

Fuerst,T.R., Earl,P.L. and Moss,B. (1987) Use of a hybrid vaccinia virus–T7 RNA polymerase system for expression of target genes. *Mol. Cell. Biol.*, **7**, 2538–2544.

Gluzman,Y. (1981) SV40 transformed simian cells support the replication of early SV40 mutants. *Cell*, **23**, 175–182.

González,S. and Ortín,J. (1999) Characterization of the influenza virus PB1 protein binding to vRNA: two separate regions of the protein contribute to the interaction domain. *J. Virol.*, **73**, 631–637.

González,S., Zürcher,T. and Ortín,J. (1996) Identification of two separate domains in the influenza virus PB1 protein responsible for interaction with the PB2 and PA subunits: a model for the viral RNA polymerase structure. *Nucleic Acids Res.*, **24**, 4456–4463.

Hagen,M., Chung,T.D., Butcher,J.A. and Krystal,M. (1994) Recombinant influenza virus polymerase: requirement of both 5′ and 3′ viral ends for endonuclease activity. *J. Virol.*, **68**, 1509–1515.

Hay,A.J. (1982) Characterization of influenza virus RNA complete transcripts. *Virology*, **116**, 517–522.

Herz,C., Stavnezer,E., Krug,R.M. and Gurney,T. (1981) Influenza virus, an RNA virus, synthesizes its messenger RNA in the nucleus of infected cells. *Cell*, **26**, 391–400.

Honda,A., Mukaigawa,J., Yokoiyama,A., Kato,A., Ueda,S., Nagata,K., Krystal,M., Nayak,D.P. and Ishihama,A. (1990) Purification and molecular structure of RNA polymerase from influenza virus A/PR8. *J. Biochem. (Tokyo)*, **107**, 624–628.

Jackson,D.A., Caton,A.J., McCready,S.J. and Cook,P.R. (1982) Influenza virus RNA is synthesized at fixed sites in the nucleus. *Nature*, **296**, 366–368.

Kato,A., Mizumoto,K. and Ishihama,A. (1985) Purification and enzymatic properties of an RNA polymerase–RNA complex from influenza virus. *Virus Res.*, **3**, 115–127.

Klumpp,K., Ruigrok,R.W. and Baudin,F. (1997) Roles of the influenza virus polymerase and nucleoprotein in forming a functional RNP structure. *EMBO J.*, **16**, 1248–1257.

Kobayashi,M., Toyoda,T. and Ishihama,A. (1996) Influenza virus PB1 protein is the minimal and essential subunit of RNA polymerase. *Arch. Virol.*, **141**, 525–539.

Krug,R.M., Broni,B.A. and Bouloy,M. (1979) Are the 5′-ends of influenza viral mRNAs synthesized *in vivo* donated by host mRNAs? *Cell*, **18**, 329–334.

Krug,R.M., Alonso-Kaplen,F.V., Julkunen,I. and Katze,M.G. (1989) Expression and replication of the influenza virus genome. In Krug,R.M. (ed.), *The Influenza Viruses*. Plenum Press, New York, NY, pp. 89–152.

Lamb,R.A. (1989) The genes and proteins of influenza viruses. In Krug,R.M. (ed.), *The Influenza Viruses*. Plenum Press, New York, NY, pp. 1–87.

Li,M.L., Ramírez,B.C. and Krug,R.M. (1998) RNA-dependent activation of primer RNA production by influenza virus polymerase: different regions of the same protein subunit constitute the two required RNA-binding sites. *EMBO J.*, **17**, 5844–5852.

Licheng,S., Summers,D.F., Peng,Q. and Galarza,J.M. (1995) Influenza A virus polymerase subunit PB2 is the endonuclease which cleaves host cell mRNA and functions only as the trimeric enzyme. *Virology*, **208**, 38–47.

Luo,G.X., Luytjes,W., Enami,M. and Palese,P. (1991) The polyadenylation signal of influenza virus RNA involves a stretch of uridines followed by the RNA duplex of the panhandle structure. *J. Virol.*, **65**, 2861–2867.

Mahy,B.W.J. (1983) Mutants of influenza virus. In Palese,P. and Kingsbury,D.W. (eds), *Genetics of Influenza Viruses*. Springer Verlag, Wien, pp. 192–253.

Marión,R.M., Aragón,T., Beloso,A., Nieto,A. and Ortín,J. (1997a) The N-terminal half of the influenza virus NS1 protein is sufficient for nuclear retention of mRNA and enhancement of viral mRNA translation. *Nucleic Acids Res.*, **25**, 4271–4277.

Marión,R.M., Zürcher,T., de la Luna,S. and Ortín,J. (1997b) Influenza virus NS1 protein interacts with viral transcription–replication complexes *in vivo*. *J. Gen. Virol.*, **78**, 2447–2451.

Mena,I., de la Luna,S., Albo,C., Martín,J., Nieto,A., Ortín,J. and Portela,A. (1994) Synthesis of biologically active influenza virus core proteins using a vaccinia–T7 RNA polymerase expression system. *J. Gen. Virol.*, **75**, 2109–2114.

Nath,S.T. and Nayak,D.P. (1990) Function of two discrete regions is required for nuclear localization of polymerase basic protein 1 of A/WSN/33 influenza virus (H1N1). *Mol. Cell. Biol.*, **10**, 4139–4145.

Nieto,A., de la Luna,S., Bárcena,J., Portela,A. and Ortín,J. (1994) Complex structure of the nuclear translocation signal of the influenza virus polymerase PA subunit. *J. Gen. Virol.*, **75**, 29–36.

Ortín,J., Nájera,R., López,C., Dávila,M. and Domingo,E. (1980) Genetic variability of Hong Kong (H3N2) influenza viruses: spontaneous mutations and their location in the viral genome. *Gene*, **11**, 319–331.

Perales,B. and Ortín,J. (1997) The influenza A virus PB2 polymerase subunit is required for the replication of viral RNA. *J. Virol.*, **71**, 1381–1385.

Perales,B., de la Luna,S., Palacios,I. and Ortín,J. (1996) Mutational analysis identifies functional domains in the influenza A PB2 polymerase subunit. *J. Virol.*, **70**, 1678–1686.

Pérez,D.R. and Donis,R.O. (1995) A 48-amino-acid region of influenza A virus PB1 protein is sufficient for complex formation with PA. *J. Virol.*, **69**, 6932–6939.

Poch,O., Sauvaget,I., Delarue,M. and Tordo,N. (1990) Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. *EMBO J.*, **8**, 3867–3874.

Poon,L.L.M., Pritlove,D.C., Sharps,J. and Brownlee,G.G. (1998) The RNA polymerase of influenza virus, bound to the 5′ end of virion RNA, acts *in cis* to polyadenylate mRNA. *J. Virol.*, **72**, 8214–8219.

Poon,L.L.M., Pritlove,D.C., Fodor,E. and Brownlee,G.G. (1999) Direct evidence that the poly(A) tail of influenza A virus mRNA is synthesized by reiterative copying of a U track in the virion RNA template. *J. Virol.*, **73**, 3473–3476.

Portela,A., Zürcher,T., Nieto,A. and Ortín,J. (1999) Replication of Orthomyxoviruses. *Adv. Virus Res.*, in press.

Pritlove,D.C., Poon,L.L.M., Fodor,E., Sharps,J. and Brownlee,G.G.

(1998) Polyadenylation of influenza virus mRNA transcribed *in vitro* from model virion RNA templates: requirement for 5′ conserved sequences. *J. Virol.*, **72**, 1280–1286.

Pritlove,D.C., Poon,L.L.M., Devenish,L.J., Mike,B., Leahy,M.B. and Brownlee,G.G. (1999) A hairpin loop at the 5′ end of influenza A virus virion RNA is required for synthesis of poly(A)$^+$ mRNA *in vitro*. *J. Virol.*, **73**, 2109–2114.

Robertson,J.S., Schubert,M. and Lazzarini,R.A. (1981) Polyadenylation sites for influenza mRNA. *J. Virol.*, **38**, 157–163.

Rose,J.K., Buonocore,L. and Whitt,M.A. (1991) A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. *BioTechniques*, **10**, 520–525.

Sanz-Ezquerro,J.J., de la Luna,S., Ortín,J. and Nieto,A. (1995) Individual expression of influenza virus PA protein induces degradation of co-expressed proteins. *J. Virol.*, **69**, 2420–2426.

Sanz-Ezquerro,J.J., Zürcher,T., de la Luna,S., Ortin,J. and Nieto,A. (1996) The amino-terminal one-third of the influenza virus PA protein is responsible for the induction of proteolysis. *J. Virol.*, **70**, 1905–1911.

Sanz-Ezquerro,J.J., Fernández Santarén,J., Sierra,T., Aragón,T., Ortega,J., Ortín,J., Smith,G.L. and Nieto,A. (1998) The PA influenza polymerase subunit is a phosphorylated protein. *J. Gen. Virol.*, **79**, 471–478.

Seong,B.L. and Brownlee,G.G. (1992) A new method for reconstituting influenza polymerase and RNA *in vitro*: a study of the promoter elements for cRNA and vRNA synthesis *in vitro* and viral rescue *in vivo*. *Virology*, **186**, 247–260.

Shi,L., Galarza,J.M. and Summers,D.F. (1996) Recombinant-baculovirus-expressed PB2 subunit of the influenza A virus RNA polymerase binds cap groups as an isolated subunit. *Virus Res.*, **42**, 1–9.

Tiley,L.S., Hagen,M., Mathews,J.T. and Krystal,M. (1994) Sequence-specific binding of the influenza virus RNA polymerase to sequences located at the 5′-end of the viral RNAs. *J. Virol.*, **68**, 5108–5116.

Toyoda,T., Adyshev,D.M., Kobayashi,M., Iwata,A. and Ishihama,A. (1996) Molecular assembly of the influenza virus RNA polymerase: determination of the subunit–subunit contact sites. *J. Gen. Virol.*, **77**, 2149–2157.

Ulmanen,I., Broni,B.A. and Krug,R.M. (1981) The role of two of the influenza virus core P proteins in recognizing cap 1 structures (m7GpppNm) on RNAs and in initiating viral RNA transcription. *Proc. Natl Acad. Sci. USA*, **78**, 7355–7359.

Zürcher,T., de la Luna,S., Sanz-Ezquerro,J.J., Nieto,A. and Ortín,J. (1996) Mutational analysis of the influenza virus A/Victoria/3/75 PA protein: studies of interaction with PB1 protein and identification of a dominant negative mutant. *J. Gen. Virol.*, **77**, 1745–1749.