

A new promoter-binding site in the PB1 subunit of the influenza A virus polymerase

Tanis E. Jung and George G. Brownlee

Correspondence
George G. Brownlee
george.brownlee@path.ox.ac.uk

Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK

The influenza A virus RNA-dependent RNA polymerase consists of three subunits PB1, PB2 and PA. The 5' and 3' terminal sequences of the viral RNA (vRNA) form the viral promoter and are bound by the PB1 subunit. The putative promoter-binding sites of the PB1 subunit have been mapped in previous studies but with contradictory results. The aim of the current study was to investigate the function of two evolutionary conserved regions in PB1 – from aa 233 to 249 and 269 to 281, which lie immediately N- and C-terminal, respectively, of a previously proposed binding site for the 3' end of the vRNA promoter. The previously proposed binding site extended from aa 249 to 256 and centred on two phenylalanine residues (F251 and F254). However, the fact that F251 is required for polymerase activity was not confirmed here. Instead, it was proposed that the 233–249 region contains a new 5' vRNA promoter-binding site, and arginine residues crucial for this activity were characterized. However, residues 269–281 were unlikely to be directly involved in promoter binding. These results are discussed in relation to the previous studies and a new model for vRNA promoter binding to the influenza RNA polymerase is presented.

Received 23 August 2005
Accepted 8 November 2005

INTRODUCTION

Influenza A virus belongs to the family *Orthomyxoviridae*, a family of viruses with negative-sense, segmented RNA genome (Lamb & Krug, 2001). Each gene segment is associated with a single molecule of the RNA-dependent RNA polymerase and multiple copies of the nucleoprotein (NP) forming ribonucleoprotein complexes (RNPs) (Fodor & Brownlee, 2002; Lamb & Krug, 2001). The polymerase is composed of PB1, PB2 and PA subunits that catalyse both replication and transcription (Fodor & Brownlee, 2002; Lamb & Krug, 2001; Portela *et al.*, 1999). PB1 functions as the 'classic polymerase' responsible for polymerization and endonuclease cleavage (Fodor & Brownlee, 2002; Lamb & Krug, 2001). PB2 has cap-binding activity (Fechter *et al.*, 2003; Fodor & Brownlee, 2002; Lamb & Krug, 2001; Neumann *et al.*, 2004), while PA is involved in replication, elongation and also endonuclease activity (Fodor *et al.*, 2002, 2003; Huarte *et al.*, 2003; Kawaguchi *et al.*, 2005).

The terminal 13 and 12 nt of the 5' and 3' ends of the influenza A gene segments are conserved and form the viral RNA (vRNA) promoter (Fodor & Brownlee, 2002). The binding site of the promoter has been mapped to the PB1 subunit of the polymerase (Gonzalez & Ortin, 1999; Li *et al.*, 1998). Gonzalez & Ortin (1999) proposed that the 5' end of

the vRNA promoter bound in a single promoter-binding site formed by the N- (1–83) and C-terminal (494–757) regions of PB1. Li *et al.* (1998), however, suggested that two distinct regions in PB1 were involved. One (249–256) bound the 3' end of the vRNA promoter and two phenylalanine residues, F251 and F254, within this region were essential. Another region, centred on R571 and R572, was involved in binding the 5' end of the viral promoter.

Here, we investigated two evolutionary conserved regions in PB1, extending from aa 233 to 249 and 269 to 281, which were immediately N- and C-terminal to the previously proposed binding site for the 3' end of the vRNA promoter (Li *et al.*, 1998). We determined by mutagenesis whether these regions were involved in promoter binding and re-examined the role of F251 and F254.

METHODS

Plasmids. All plasmids were described previously (Fodor *et al.*, 2002; Subbarao *et al.*, 2003) except for pcDNA-PA-(His6)TAP, which encodes the PA protein of influenza A/WSN/33 virus tagged at its C terminus with a tandem affinity purification (TAP) tag (Puig *et al.*, 2001) modified to include six histidine residues in place of the calmodulin-binding peptide. The C-terminal tag retains the tobacco etch virus (TEV) cleavage site and two protein A domains. All constructs were confirmed by sequencing.

Mutants. pcDNA-PB1 point and multiple mutations were introduced by site-directed mutagenesis and confirmed by sequencing.

Supplementary figure showing primer extension data of steady-state levels of mRNA and cRNA isolated from cells expressing influenza A virus polymerase and vRNA-like CAT-RNA is available in JGV Online.

Transfection, RNA isolation and analysis of vRNA, cRNA and mRNA by primer extension. Transfection of human kidney 293T cells with pcDNA-PB1, pcDNA-PB2, pcDNA-PA, pcDNA-NP (from influenza virus A/WSN/33) and pPOLI-CAT-RT plasmids, RNA isolation and primer extension of the CAT reporter vRNA, cRNA and mRNA were as described previously (Fodor *et al.*, 2002). Fractionation was on 6% PAGE in 7 M urea, followed by autoradiography.

Viral rescue. Influenza viruses were rescued and RNA sequenced as described previously (Fodor *et al.*, 1999, 2002).

Preparation of partially purified recombinant His-tagged influenza A virus polymerase. Recombinant PA-(His₆)TAP-tagged influenza A virus polymerases were partially purified by nickel-agarose affinity chromatography, essentially as in Crow *et al.* (2004) except that pcDNA-PB1, pcDNA-PB2 and pcDNA-PA-(His₆)TAP plasmids were used. The PA-(His₆)TAP-tagged subunit fractionated at 100 kDa on SDS-PAGE because of the additional 167 residues, and TEV cleavage was omitted. PA-His₆-tagged polymerase was prepared similarly, except that pcDNA-PA-His₆ (Fodor *et al.*, 2002) replaced pcDNA-PA-(His₆)TAP.

ApG-primed transcription. Reactions were performed with recombinant polymerase and short 15 and 14 nt synthetic 5' and 3' ends of a model vRNA promoter (for sequences see below) *in vitro* as described previously (Fodor *et al.*, 2002).

UV cross-linking assay. A UV-cross-linking assay was performed essentially as in Crow *et al.* (2004) except that reactions included 4 ng degraded yeast RNA μl^{-1} . Two microlitres partially purified (His₆)TAP-tagged polymerase were mixed with 1 pmol 5' end of the vRNA (5'-AGUAGAAACAAGGCC-3') (Dharmacon) and approximately 0.1 pmol (100 000 c.p.m.) [³²P]-labelled 3' end of the vRNA (5'-GGCCUGCUUUUGCU-3') (Dharmacon) or with [³²P]-labelled 5' end of the vRNA alone, in 10 μl containing 10 mM HEPES (pH 8.0), 50 mM NaCl, 2 mM MgCl₂, 0.5 mM EGTA, 1 mM DTT, 0.8 U RNasin (Promega) and 10% glycerol (v/v). After UV cross-linking (254 nm), products were denatured in SDS and separated on 8% SDS-PAGE. Gels were dried and autoradiographed. Quantification was carried out by using Fuji FLA-500 image analysis.

Western blot analysis. Partially purified (His₆)TAP-tagged polymerases were blotted and then probed (Fodor *et al.*, 2002) with rabbit polyclonal anti-PB1 antibodies raised against fragment 1–180 of PB1 of influenza A/WSN/33 and/or anti-PB2 antibodies (Carr *et al.*, 2005). Both antibodies detected the PA-(His₆)TAP-tagged subunit.

RESULTS

Evolutionary similarity suggested an extended RNA-binding domain in PB1

An alignment of the PB1 protein sequences of influenza A, B, C and Thogoto virus revealed several regions of strong sequence similarity, indicating their importance for polymerase structure and/or function. Interestingly, one evolutionary conserved region in PB1 (Fig. 1) extending from residues 233 to 281 included the proposed binding site for the 3' end of the vRNA promoter (aa 249–256) – referred to here as 3A (Li *et al.*, 1998). We refer to the region flanking 3A N-terminal as N1 and flanking 3A C-terminal as C1 (Fig. 1). Interestingly, the regions flanking site 3A displayed a higher degree of evolutionary conservation than 3A

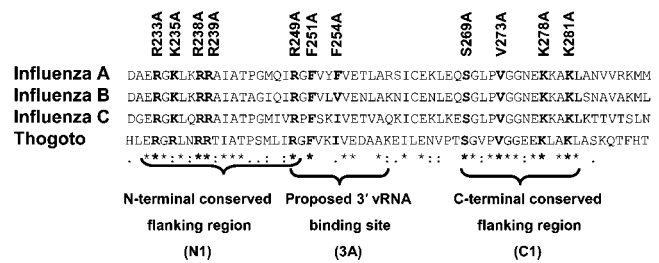


Fig. 1. Alignment of amino acid sequences of PB1 subunits of influenza A, B and C viruses and Thogoto virus for region 230–290 of influenza A/WSN/33. PB1 sequences of A/WSN/33, B/Norway/1/84, C/Johannesburg/1/66 and Thogoto virus (GenBank accession nos P11V33, AAF06870, AAF89738 and O41353) were aligned with CLUSTAL W (GCG). The proposed 3' end of the vRNA-binding site (3A), its conserved N- (N1) and C-terminal (C1) flanking regions are shown. Sites 3A and N1 overlap since residue R249 was implicated in 3' end of vRNA binding (Li *et al.*, 1998). Mutations are in bold. Asterisk (*), identical; colon (:), conserved and dot (.), semi-conserved residues.

itself. This suggested that site 3A may be more extensive than previously described (Li *et al.*, 1998). In order to investigate this hypothesis we initially constructed three sets of point mutants: first, we introduced alanine substitutions at all positions containing an evolutionary conserved positively charged amino acid in the N1 and C1 regions. We targeted the positively charged amino acids in these regions because of their potential to bind the negatively charged RNA promoter. Second, we introduced alanine substitutions for two conserved but uncharged amino acids, S269 and V273 in the C1 region, for comparison with the alanine mutants of the charged amino acids. Third, we constructed alanine mutations at positions F251 and F254 in region 3A to act as presumptive negative controls, since these two amino acids were previously described as being essential for 3' vRNA promoter binding (Li *et al.*, 1998).

Positively charged amino acids within the 233–281 region of PB1 were essential for replication and transcription *in vivo*

Primer extension assays were performed to specifically measure the levels of steady-state vRNA, mRNA and cRNA in transfected 293T cells expressing the wild-type or mutant influenza polymerases and NP with vRNA-like CAT reporter RNA (Methods). Fig. 2(a) shows that all seven PB1 mutations involving an alanine substitution for a positively charged amino acid resulted in a complete loss of RNA replication and transcription (Fig. 2a, compare lanes 4–7, 11 and 12 with lane 1, and lane 15 with 13). In contrast, mutants S269A and V273A (Fig. 2a, compare lanes 9 and 10 with lane 1) showed 31 and 17% activity, respectively (averaging the mean of the vRNA, cRNA

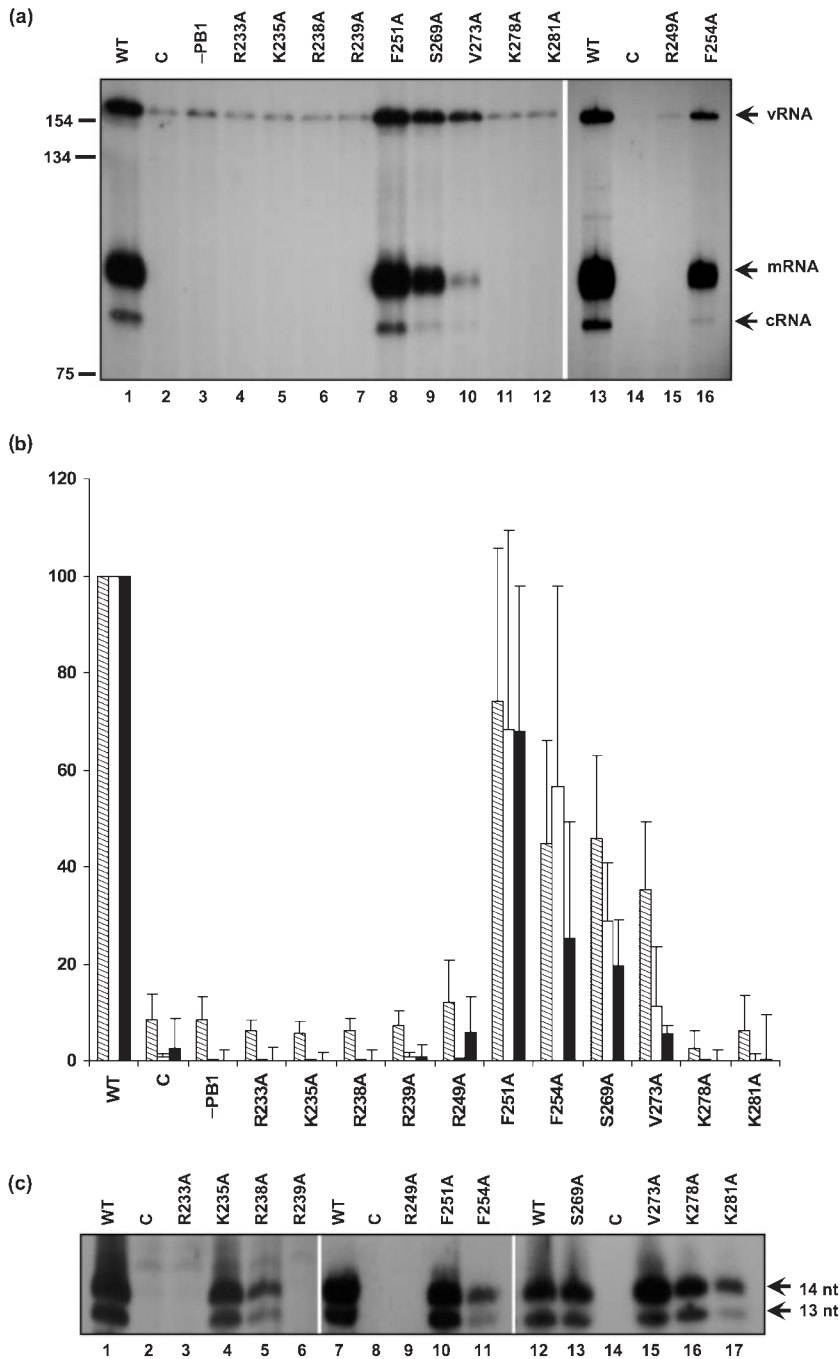


Fig. 2. RNA synthesis by influenza virus RNA polymerase with mutant PB1 subunits *in vivo* and *in vitro*. (a) *In vivo* primer extension of vRNA, mRNA and cRNA isolated from cells expressing polymerase, NP and vRNA-like CAT-RNA. 293T cells were transfected with wild-type (WT) or mutant pcDNA-PB1 and NP-encoding plasmids, with pPOLI-CAT-RT only (C) or with the same plasmids as for WT, except for pcDNA-PB1 (-PB1). Positions of vRNA, mRNA and cRNA signals and sizes of a [32 P]-labelled 1 kb DNA ladder are indicated. (b) Quantification of (a) expressed as percentage of wild-type (mean \pm SD, $n=4$). Hatched, solid white and solid black bars are vRNA, mRNA and cRNA, respectively. (c) *In vitro* ApG-primed transcription with wild-type (WT) or mutant PA-His₆-tagged polymerases from 293T cells, or control (C) extracts from untransfected cells. The two longest products (14 and 13 nt) are indicated.

and mRNA levels), compared with wild-type (Fig. 2b), suggesting that they were not absolutely essential for replication and transcription. Interestingly, mutants F251A and F254A were active *in vivo* (Fig. 2a, compare lane 8 with lane 1 and lane 16 with lane 13). Quantification showed that F251A and F254A had a mean of 70 and 42% of wild-type activity, respectively (Fig. 2b). If these mutants had been unable to bind the 3' end of the vRNA promoter, as proposed (Li *et al.*, 1998), they should also have been unable to synthesize mRNA and cRNA from the vRNA template.

ApG-primed transcription of PB1 mutants *in vitro*

To study further the role of the conserved amino acids in the N1 and C1 regions in PB1, we tested the effect of the alanine substitutions on polymerase activity *in vitro* by ApG-primed transcription (Methods). Activity was measured by the incorporation of α [32 P]GTP into a 14 nt long, [32 P]-labelled product from a model vRNA template (Methods). Consistent with the *in vivo* results, mutants R233A, R239A and R249A showed no detectable activity (Fig. 2c). However,

mutants F251A, S269A and V273A were indistinguishable from wild-type, although F254A had intermediate activity (Fig. 2c). Other mutants that exhibited a complete loss of activity *in vivo* showed some activity *in vitro*. Thus, mutants K235A, R238A, K278A and K281A had low to intermediate activity compared with wild-type (see Fig. 2b and Discussion).

Promoter-binding activity of PB1 mutants assayed by UV cross-linking *in vitro*

Next, we tested the role of the positively charged amino acids in the N1 and C1 region of PB1 in polymerase–promoter interactions. We used UV cross-linking – a procedure known to cross-link all three subunits of the polymerase to the vRNA promoter (Fodor *et al.*, 1993). Consistent with this earlier work, Fig. 3(a) (lanes 1 and 2) showed that all three polymerase subunits, PB1, PB2 and PA-(His₆)TAP were cross-linked to the [³²P]-labelled 3' end of the vRNA promoter in the presence of the unlabelled 5' end (Methods). The intensity of the PB1 band was greater than the other two subunits. All three polymerase subunits were specifically cross-linked as determined by competition experiments designed to test the binding of the duplex vRNA promoter to the polymerase using specific and non-specific competitors (Fig. 3c). However, when the polymerase was cross-linked to the [³²P]-labelled 5' end of the vRNA promoter alone, only the PB1 and PA-(His₆)TAP subunits were specifically cross-linked (Fig. 3b, lanes 1 and 2). No band was observed in the PB2 position (Fig. 3b). The faint background band (*) in both lanes 1 and 2 – midway between the PB1 and PB2 positions – is not to be confused with the position of PB2. Moreover, when the polymerase was cross-linked to the [³²P]-labelled 3' end of the vRNA promoter alone no polymerase bands were detected (Fig. 3a, lane 3). Only a faint, unknown band (†) was observed, co-migrating with a

band in one of the negative controls (lane 4), although it was absent in the other control (lane 2), possibly because the 5' end of the vRNA competed with the labelled probe for binding.

UV cross-linking of PB1 mutants to the 5' and 3' ends of the vRNA promoter

Initially, cross-linking analysis was performed using recombinant, partially purified, wild-type or mutant (His₆)TAP-tagged polymerases and the [³²P]-labelled 3' end of the vRNA promoter in the presence of the unlabelled 5' end (Fig. 4). Mutants R233A, R238A, R239A and R249A were impaired in their ability to bind the vRNA promoter (Fig. 4a, compare the intensity of the PB1 band relative to that of the PB2 band within lanes 3 and 5–7 with lane 1). These PB1 mutants cross-linked to the promoter with only approximately 46, 59, 76 and 62% efficiency, respectively, when compared with wild-type (Fig. 4c). These activities were significantly different (>95%, one sample Student's *t* test) from wild-type, except for R239A, which was significant at 94% ($P=0.004, 0.006, 0.058$ and 0.017 for PB1 mutants, R233A, R238A, R239A and R249A, respectively). All other point mutants, including F251A and F254A, were indistinguishable from wild-type. Western blot analysis, with anti-PB2 antibody, showed that approximately equal amounts of mutant and wild-type PB2 and PA-(His₆)TAP were present, except for lanes 12, 15 and 17 where lower yields were present (Fig. 4b and Methods). The amounts of PB1 detected by Western blot analysis with anti-PB1 antibody mirrored those of PB2 but were fainter since this was a lower avidity antibody. However, these lower yields did not affect quantification (Fig. 4c) because PB1 signals were normalized to the in-lane PB2 signal. Moreover, the mutations at positions 233, 238, 239 and 249 did not obviously affect binding to the PA and PB2 subunits.

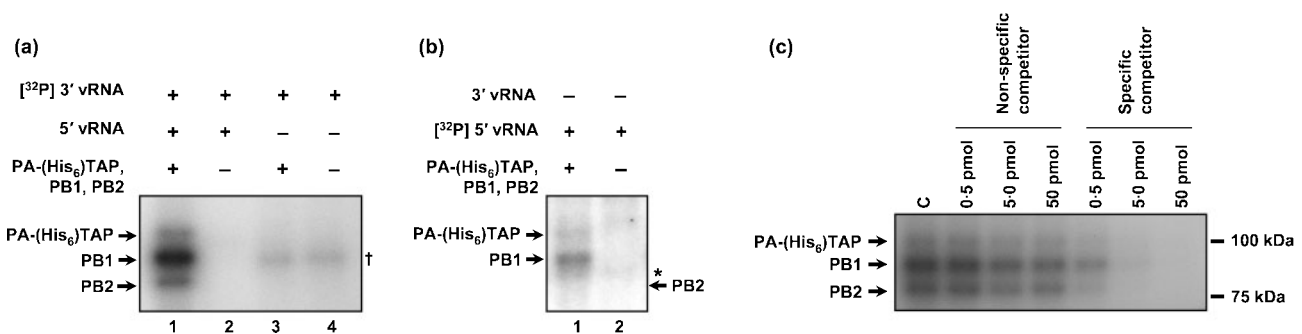


Fig. 3. UV cross-linking of polymerase to the vRNA promoter. PA-(His₆)TAP-tagged polymerase (+) from 293T cells or from untransfected cells (-) were cross-linked to (a) [³²P]-labelled 3' end of the vRNA with or without unlabelled 5'-end vRNA or (b) [³²P]-labelled 5' end of the vRNA alone. Asterisk (*) and cross (†) represent unknown background bands. The estimated position of the cross-linked PB2 band, based on its relative mobility to the PB1 band in (a) lane 1, is arrowed. (c) Competitive cross-linking of the polymerase to the vRNA promoter. PA-(His₆)TAP-tagged polymerase from 293T cells were cross-linked to [³²P]-labelled 3' end of the vRNA in the presence of 5' end of the vRNA. C, No competitor; amounts per 10 μl of specific (3' end of the vRNA) and non-specific (yeast RNA) competitors, added prior to the addition of polymerase, are indicated. Positions of the PA-(His₆)TAP, PB1 and PB2 and size markers are indicated.

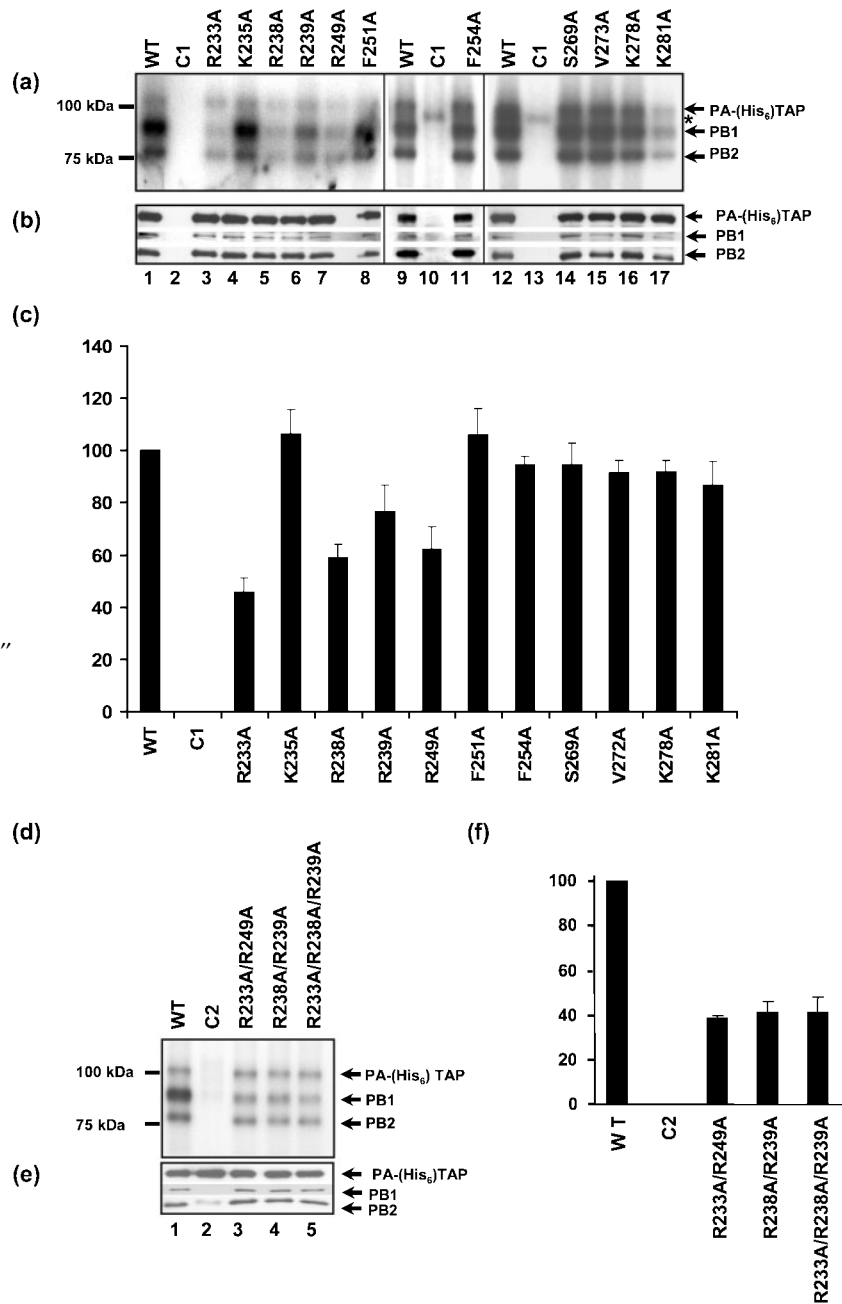


Fig. 4. UV cross-linking of polymerase with mutant PB1 subunits to the 5' and 3' ends of the vRNA promoter. (a) PA-(His₆)TAP-tagged polymerase, (WT) or mutant, or control extracts (C1) from untransfected 293T cells were cross-linked to [³²P]-labelled 3' end of the vRNA in the presence of unlabelled 5' end of the vRNA. Positions of the PA-(His₆)TAP, PB1 and PB2 bands and sizes of protein standards are indicated. Asterisk (*) represents a background band of unknown origin in lanes 10 and 13. (b) Western blot analyses of polymerase preparations showing the PA-(His₆)TAP, PB1 and PB2 bands, detected with a mixture of anti-PB1 and anti-PB2 antibodies. The three panels in (a) and (b) are from different experiments. (c) Quantification of mutant PB1 promoter-binding activity expressed as a percentage of wild-type. PB1 signals (of a, above) were normalized using the in-lane PB2 signal to correct for minor variations in protein concentration between samples and expressed as percentage of wild-type (mean ± SD, n=3). (d) UV cross-linking, as in (a) above, of PA-(His₆)TAP polymerase containing PB1 double and triple point mutations. C2 is from 293T cells transfected without pcDNA-PB1. (e) Western blot analyses of polymerase with anti-PB1 and anti-PB2 antibodies as in (b) above, except that PB1 was detected in a separate experiment from PB2 and PA-(His₆)TAP. (f) Quantification of mutant relative to wild-type PB1 binding (as a percentage), normalizing to the in-lane PB2 band as in (c) above (mean ± SD, n=3).

To confirm the importance of residues 233, 238, 239 and 249 for promoter binding, we constructed two double and one triple point mutants. Fig. 4(d and f) show that cross-linking of the promoter to the PB1 subunit of both double (R233A/R249A and R238A/R239A) and triple point mutants (R233A/R238A/R239A) was significantly reduced to about 40% of wild-type levels. The double and triple point mutants showed no significant further decrease in PB1 signal compared to the R233A or the R238A mutations alone (Fig. 4c). Thus, the effect of the multiple mutations was not additive. This suggests that each of the point mutants, alone, can significantly disrupt promoter binding. Residual cross-linking to PB1, and notably also to PB2 and

PA, is not significantly impaired in the double and triple mutants (Fig. 4d and Discussion).

UV cross-linking of PB1 mutants to the 5' end of the vRNA promoter

As described above, binding of the 3' end of the vRNA promoter to the polymerase is dependent on binding of the 5' end. Mutant polymerases with impaired binding to the promoter may, therefore, simply be impaired in their ability to bind the 5' end of the vRNA promoter rather than the complete promoter. To distinguish these possibilities, we cross-linked the [³²P]-labelled 5' end of the vRNA

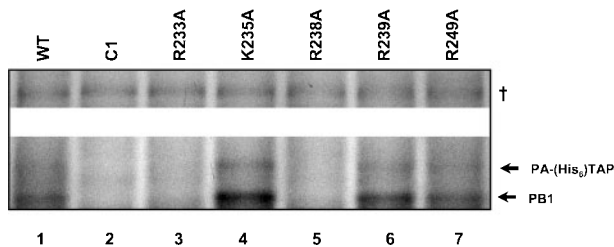


Fig. 5. UV cross-linking of polymerase with selected PB1 mutants to the 5' end of the vRNA promoter. WT, mutant and control (C1) extracts (see Fig. 4) were cross-linked to [32 P]-labelled 5' end of the vRNA. The positions of the PA-(His₆)TAP and PB1 bands are indicated.

promoter, alone, to polymerases containing point mutants of basic residues 233, 235, 238, 239 and 249 of PB1. The R233A and R238A mutants, but not the K235A, R239A or R249A mutants (Fig. 5), showed reduced cross-linking to PB1. Interestingly, the PA band was also reduced in R233A and R238A (Fig. 5, lanes 3 and 5) (see Discussion). Reduction in promoter binding by the R233A and R238A mutants, could not be ascribed to different yields of the polymerases, since near equal quantities of PB1 and PB2 were present, as confirmed in Western blots with anti-PB1 and anti-PB2 antibodies (Fig. 4b, lanes 1 and 3–5). Accurate quantification of the reduced binding compared with wild-type, however, was impossible because binding to the 5' end of the promoter was weaker than with the complete promoter and there was interference from background host-derived bands. Thus, our results imply that residues R233 and R238 can bind the 5' end of the vRNA promoter, alone. Residues R239 and R249, however, differ (see Discussion).

***In vivo* rescue of influenza virus A/WSN/33 with an F251A mutation in the PB1 subunit**

To investigate whether the F251A mutation within the PB1 subunit of the polymerase was compatible with viral function, we attempted to rescue virus containing this mutation. F251A mutant A/WSN/33 virus was rescued on two separate occasions and the sequence of the mutation in the rescued virus was confirmed (Methods). The plaque size of the isolated infectious virus was indistinguishable from wild-type (Fig. 6) on both occasions that the virus was rescued. Thus, F251 residue was not essential for viral growth in Madin–Darby bovine kidney cells.

An F251A mutation in the PB1 subunit of the polymerase of influenza A/PR/8/34 is indistinguishable from wild-type PB1 of A/PR/8/34 in replication and transcription *in vivo*

We constructed an F251A mutation in the PB1 segment of influenza A/PR/8/34 and tested the transcription and replication of reconstituted RNP complex in 293T cells using a CAT reporter construct by primer extension. The F251A mutation in the PB1 subunit derived from influenza

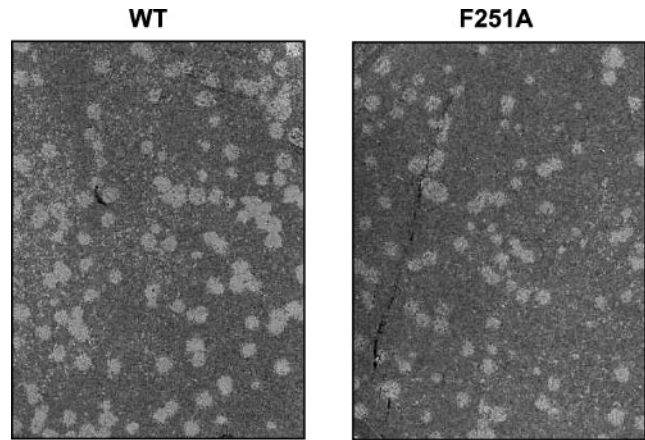


Fig. 6. Plaque size of rescued recombinant influenza A/WSN/33 virus with the F251A mutation in the PB1 protein (magnified $\times 2.5$). Recombinant wild-type (WT) and mutant (F251A) influenza viruses were rescued using reverse genetics. Viruses were propagated in Madin–Darby bovine kidney cells and stained with Coomassie blue.

A/PR/8/34, in a background of PB2, PA and NP derived from influenza A/WSN/33, gave similar mRNA and cRNA yields to the wild-type, influenza A/PR/8/34, PB1 subunit in the same background (Supplementary Figure, lanes 2 and 3, available in JGV Online).

DISCUSSION

The aim of the current study was to investigate the function of two evolutionary conserved regions in PB1, one extending from aa 233 to 249 (N1) and the other from aa 269 to 281 (C1). Because these regions lay immediately N- and C-terminal to a previously proposed binding site (3A) for the 3' end of the vRNA promoter (Li *et al.*, 1998) (Fig. 1), we postulated that they may also be involved in promoter binding.

To test the function of the evolutionary conserved regions N1 and C1 of PB1, we constructed alanine mutants of the evolutionary conserved residues. Initially, measurements of the levels of steady-state vRNA, mRNA and cRNA in transfected 293T cells expressing the wild-type or mutant influenza polymerases and NP with a vRNA-like CAT reporter showed that all positively charged amino acids in the N1 and C1 regions in PB1 were essential for polymerase activity. In contrast, two further other amino acids, S269 and V273, in the C1 region were not absolutely essential (Fig. 2). ApG-primed transcription (Fig. 2c) confirmed that residues R233, R239 and R249 in the N1 region were essential. However, residues S269 and V273 in the C1 region were not essential for activity *in vitro*, contrasting with their partial activity *in vivo*. Also, polymerases with the K235A and R238A mutations in N1 and the K278A and K281A mutations in C1, exhibited low to intermediate activity *in vitro*, although

they were inactive *in vivo*. We presume that the *in vivo* assay is more sensitive to a minor inhibition of replication or transcription than the *in vitro* assay, because minor inhibition of replication in one cycle of vRNA→cRNA synthesis would be amplified *in vivo* by successive rounds of synthesis. Thus, a minor defect in the initiation of replication *in vitro* of K235A and R238A in N1 and K278A and K281A in C1 could result in undetectable activity *in vivo*.

Cross-linking of the polymerase to the promoter showed (Fig. 4) that mutations R233A, R238A, R239A and R249A, but not K235A, were impaired in binding the vRNA promoter *in vitro*, even though the R238A mutant had shown some ApG-primed transcription activity (Fig. 2c). On the other hand, the K235A, K278A and K281A mutant polymerases appeared to be similar to wild-type in promoter binding (Fig. 4c), so that an alternative explanation for the *in vivo* defect of these mutations seems more likely. Possibly they affect the rate of elongation of the polymerase (Fodor *et al.*, 2003).

Thus, we have identified a novel promoter-binding site in the N1 region in PB1 where promoter binding is mediated by positively charged amino acids R233, R238, R239 and R249. However, until the detailed three-dimensional structure of the polymerase, complexed with its promoter is known, we cannot exclude the possibility that the proposed N1-binding site is only indirectly involved in promoter binding. In contrast, cross-linking of mutants in region C1 showed that mutations S269A, V273A, K278A and K281A had no detectable effect on promoter binding (Fig. 4), although they did affect ApG-primed transcription (Fig. 2c). Therefore, we conclude that the C1 region in PB1 is unlikely to be a major promoter-binding site.

We then tested whether the N1 region specifically bound the 5' or 3' end of the vRNA promoter by cross-linking polymerase to the 5' end of the vRNA promoter in the absence of the 3' end. Polymerase preparations with the R233A and R238A mutations were impaired in PB1 promoter binding, although we were unable to quantify the degree of inhibition because of its low efficiency (Fig. 5). Intriguingly, the R239A and R249A mutants did not significantly impair PB1 binding to the 5' end of the vRNA promoter. This contrasted with the results obtained with the complete promoter, where we observed that residues R233, R238, R239 and R249 were all involved (Fig. 4c). How do we explain the different cross-linking results (Figs 4 and 5) with the 5' end of the vRNA promoter compared with the complete promoter? The results with the R233A and R238A mutations suggest that residues R233 and R238 can bind the 5' end of the promoter alone (Fig. 5). The fact that the R239A and R249A mutants only affected PB1 binding if the 3' end of the promoter was present suggested that the secondary structure of the promoter – thought to be in a corkscrew structure – might be involved (reviewed by Fodor & Brownlee, 2002). Thus, we speculate that residues R239 and R249 in PB1 may bind the duplex region of the promoter, formed by intrastrand base pairing between the

two ends of the promoter. The formation of additional contacts between PB1 and the duplex region of the promoter is consistent with the observed higher efficiency of cross-linking to the complete promoter compared with the 5' end alone. Additional stabilization of promoter binding to PB1 would result from binding to PB2 that occurs only when the 3' end of the promoter is present (Fig. 3). Thus, in the absence of the 3' end, it is also perhaps not surprising that the R233A and R238A PB1 mutant polymerases inhibit PA as well as PB1 binding (Fig. 5).

Surprisingly, two residues, F251 and F254 previously proposed to be essential for 3' vRNA binding in region 3A (Li *et al.*, 1998) were not absolutely required for polymerase activity *in vivo* and *in vitro*, or for promoter binding (Figs 2, 4 and 6). Polymerase containing the F251A mutation showed no statistically significant difference from wild-type in all assays tested, whether *in vivo* or *in vitro*. Significantly, a virus containing the F251A mutation in its PB1 subunit was rescued by reverse genetics and was indistinguishable from wild-type in plaque morphology (Fig. 6). However, polymerase with the F254A mutation inhibited transcription and replication partially when assayed by primer extension and ApG-primed transcription (Fig. 2), but no inhibition of promoter binding was detected (Fig. 4). Thus, we have not been able to confirm that site 3A, and in particular residue 251, is part of a 3' promoter-binding site, although it remains possible that residue 254 plays a minor role.

Interestingly, none of the mutations studied here completely abolished binding of the promoter to the PB1 subunit of the polymerase. Binding was still significant, at about 40% of wild-type, when cross-linking was performed even with double and triple point mutants of the N1 region (Fig. 4d). This residual binding is consistent with known binding sites elsewhere in PB1 – around residues 571 and 572 (Li *et al.*, 1998) and possibly other regions of PB1 (Gonzalez & Ortin, 1999). Binding of PB2 and PA to the promoter (Crescenzo-Chaigne *et al.*, 2002; Fodor *et al.*, 1993, 1994) was also not affected by mutations in the N1 region of PB1, except when the 5' end alone was used (Fig. 5).

A possible criticism of our cross-linking experiments is that the C-terminal (His₆)TAP-tag on the PA subunit may have interfered with promoter binding (Figs 2–5). However, such TAP tags are known to be compatible with polymerase-mediated transcription and replication *in vivo* (Deng *et al.*, 2005). Furthermore, electron microscopy studies have shown that the C terminus of PA is superficial and available for tagging (Area *et al.*, 2004).

The most obvious reason for the discrepancy in cross-linking between our data and Li *et al.* (1998) relates to their use of a modified U residue (4 thioU) and its position in the promoter. 4 thioU was specifically cross-linked at 366 nm (Li *et al.*, 1998), whereas UV cross-links unmodified nucleotides non-specifically at the shorter wavelengths used here. This probably explains why no cross-linking to PB2 or

PA was observed (Li *et al.*, 1998), whereas it had been previously established that all three subunits of the polymerase could be UV cross-linked, at shorter wavelengths, to the vRNA promoter (Fodor *et al.*, 1993, 1994).

Intriguingly, Li *et al.* (1998) could not exclude that promoter binding to site 3A in PB1 was mediated by the 5' end of the vRNA promoter binding to residues in site N1, since N1 and 3A are adjacent (Fig. 1). Moreover, a 5' end was necessary to cross-link the 4 thioU-containing 3' end of the promoter. The 4 thioU-containing 5' end of the promoter may have failed to cross-link to site N1, because cross-linking was performed in the absence of the 3' end, and may have been below their level of detection (Li *et al.*, 1998). Alternatively, the position of the 4 thioU – flanking the promoter at nt 15– may have been too distant from the promoter to cross-link to site N1.

The discrepancy between our results and those of Li *et al.* (1998) with the F251A mutation could be linked to the HeLa cells used for the amplification of the vaccinia virus influenza constructs (Li *et al.*, 1998). Such cells, unlike 293T cells used here, are not permissive for influenza virus replication, raising the possibility that host proteins required in 293T cells may be absent in HeLa cells. Differences in the purity of the recombinant polymerase used by Li *et al.* (1998) (nuclear extracts) and here (Ni-affinity, partially purified polymerase) may also contribute. To exclude the possibility that the differences were caused by the different, although closely related, viral strains – A/PR/8/34 (Li *et al.*, 1998) versus A/WSN/33– we tested the F251A mutation in the PB1 segment of A/PR/8/34 (Cambridge strain), but no differences with wild-type were detected (Supplementary Fig. S1 available in JGV Online). However, there still remains the remote possibility that different laboratory strains of influenza A/PR/8/34 could have caused the discrepancy. Significantly, unlike Li *et al.* (1998), our results were confirmed in two crucial *in vivo* assays. First, the F251A mutated polymerase was as efficient as wild-type in transcription and replication in a reconstituted RNP complex in 293T cells (Fig. 2). Second, we were able to rescue authentic recombinant influenza virus with the F251A mutation (Fig. 6).

Our new model (Fig. 7b) differs from the older model (Li *et al.*, 1998), which had proposed two separate regions in PB1 binding the 3' and 5' ends of the vRNA promoter (sites 3A and 5A, respectively, Fig. 7a), as follows: (i) binding of the 5' end of the vRNA promoter is mediated by residues R233 and R238 in our new site N1, in addition to the previously characterized site (5A) (Li *et al.*, 1998). One or more of the unpaired nucleotides forming the tetranucleotide loop of the 5' hairpin loop may be involved in binding to these two sites in PB1 (Fodor *et al.*, 1994; Leahy *et al.*, 2001; Pritlove *et al.*, 1995; Rao *et al.*, 2003). We speculate that residues R239 and R249 of site N1 bind the duplex region of the promoter. (ii) Polymerase binding of the 3' end of the vRNA promoter is mediated primarily through base-pairing with the 5' end, for which there is extensive evidence (Fodor

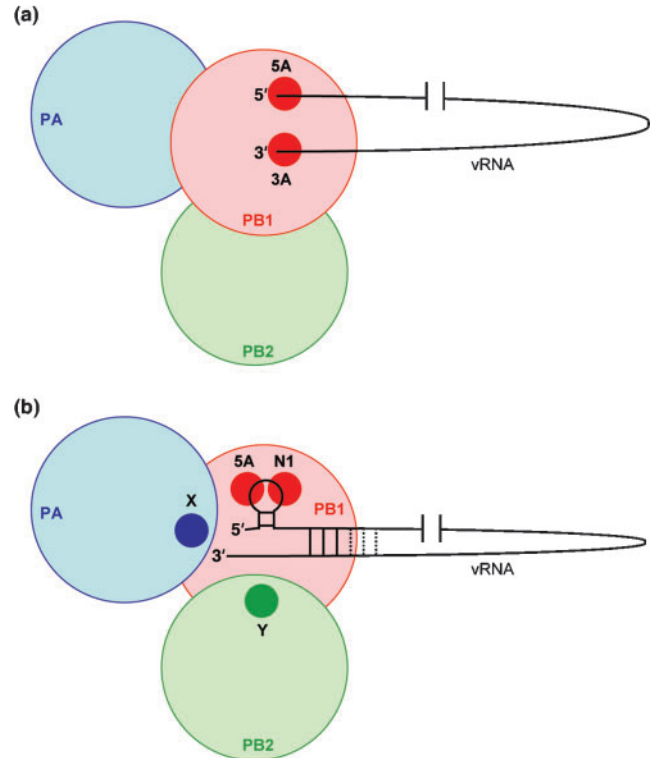


Fig. 7. New and old models of vRNA promoter binding by the influenza virus polymerase. (a) Promoter binding model proposed by Li *et al.* (1998). 3A and 5A denote binding sites for 3' and 5' ends of the vRNA, respectively. (b) Model proposed here. 5A and N1 denote two PB1-binding sites, speculatively binding to the hairpin loop of the 5' end of the vRNA (see text). X and Y are putative binding sites in PA and PB2, although their precise location(s) are unknown. Solid and dotted vertical lines are conserved and segment-specific base-pairs, respectively, between the 5' and 3' ends of the vRNA promoter.

& Brownlee, 2002; Brownlee & Sharps, 2002). (iii) Consistent with earlier work (Fodor *et al.*, 1993, 1994; Tiley *et al.*, 1994), PA binds the promoter, although the exact region is unknown (Fig. 7b, site X). (iv) PB2 is specifically involved in binding the 3' end of the vRNA promoter (Crescenzo-Chaigne *et al.*, 2002) (Fig. 7b, site Y), since only polymerase subunits PA and PB1, and not PB2, were cross-linked to the 5' end of the vRNA promoter (Fig. 3).

Our model does not show a separate binding site in PB1 for the 3' end of the vRNA promoter, since we failed to confirm that residues F251 and F254 (site 3A) were involved (Li *et al.* (1998).

Binding of the vRNA promoter is mediated by arginine residues in both sites 5A and N1, suggesting that they may represent an arginine rich motif (ARM) (Weiss & Narayana, 1998) similar to an HIV TAT ARM (Calnan *et al.*, 1991). ARMs are known to bind hairpin loop structures in RNA

(Legault *et al.*, 1998) consistent with the proposal that residues in sites 5A and N1 of PB1 may bind the 5' hairpin loop of the vRNA promoter.

Previously, the regions in PB1 involved in promoter binding and whether separate binding sites for the 5' and 3' ends of the vRNA promoter existed has proved controversial (Gonzalez & Ortin, 1999; Li *et al.*, 1998). Gonzalez & Ortin (1999) postulated that there was a single promoter-binding site formed by the N-terminal 83 and C-terminal 263 aa of PB1 that overlapped with site 5A proposed by Li *et al.* (1998). The N-terminal 83 aa of PB1 may represent a third binding site for the 5' end of the vRNA promoter, although some doubt remains, since binding was not tested in the heterotrimeric complex. Our study shows that arginine residues in a new N1 region between aa 233 and 249 bind the 5' end of the vRNA promoter. We also throw doubt on previous claims that residue F251 of PB1 is involved in binding the 3' end of the vRNA promoter.

ACKNOWLEDGEMENTS

We thank J. Robinson for sequencing and Simon Carr for anti-PB2 antibody. The work was supported by MRC grants (G9523972 and G9901312 to G. G. B.) and an EPA studentship to T. E. J.

REFERENCES

- Area, E., Martin-Benito, J., Gastaminza, P., Torreira, E., Valpuesta, J. M., Carrascosa, J. L. & Ortin, J. (2004). 3D structure of the influenza virus polymerase complex: localization of subunit domains. *Proc Natl Acad Sci U S A* **101**, 308–313.
- Brownlee, G. G. & Sharps, J. L. (2002). The RNA polymerase of influenza A virus is stabilized by interaction with its viral RNA promoter. *J Virol* **76**, 7103–7113.
- Calnan, B. J., Biancalana, S., Hudson, D. & Frankel, A. D. (1991). Analysis of arginine-rich peptides from the HIV Tat protein reveals unusual features of RNA-protein recognition. *Genes Dev* **5**, 201–210.
- Carr, S. M., Carnero, E., Garcia-Sastre, A., Brownlee, G. G. & Fodor, E. (2005). Characterization of a mitochondrial-targeting signal in the PB2 protein of influenza viruses. *Virology* (in press).
- Crescenzo-Chaigne, B., van der Werf, S. & Naffakh, N. (2002). Differential effect of nucleotide substitutions in the 3' arm of the influenza A virus vRNA promoter on transcription/replication by avian and human polymerase complexes is related to the nature of PB2 amino acid 627. *Virology* **303**, 240–252.
- Crow, M., Deng, T., Addley, M. & Brownlee, G. G. (2004). Mutational analysis of the influenza virus cRNA promoter and identification of nucleotides critical for replication. *J Virol* **78**, 6263–6270.
- Deng, T., Sharps, J., Fodor, E. & Brownlee, G. G. (2005). In vitro assembly of PB2 with a PB1-PA dimer supports a new model of assembly of influenza A virus polymerase subunits into a functional trimeric complex. *J Virol* **79**, 8669–8674.
- Fechter, P., Mingay, L., Sharps, J., Chambers, A., Fodor, E. & Brownlee, G. G. (2003). Two aromatic residues in the PB2 subunit of influenza A RNA polymerase are crucial for cap binding. *J Biol Chem* **278**, 20381–20388.
- Fodor, E. & Brownlee, G. G. (2002). Influenza virus replication. In *Influenza*, pp. 1–30. Edited by C. W. Potter. Amsterdam: Elsevier Science.
- Fodor, E., Seong, B. L. & Brownlee, G. G. (1993). Photochemical cross-linking of influenza A polymerase to its virion RNA promoter defines a polymerase binding site at residue 9 to 12 of the promoter. *J Gen Virol* **74**, 1327–1333.
- Fodor, E., Pritlove, D. C. & Brownlee, G. G. (1994). The influenza virus panhandle is involved in the initiation of transcription. *J Virol* **68**, 4092–4096.
- Fodor, E., Devenish, L., Engelhardt, O. G., Palese, P., Brownlee, G. G. & Garcia-Sastre, A. (1999). Rescue of influenza A virus from recombinant DNA. *J Virol* **73**, 9679–9682.
- Fodor, E., Crow, M., Mingay, L. J., Deng, T., Sharps, J., Fechter, P. & Brownlee, G. G. (2002). A single amino acid mutation in the PA subunit of the influenza virus RNA polymerase inhibits endonucleolytic cleavage of capped RNAs. *J Virol* **76**, 8989–9001.
- Fodor, E., Mingay, L. J., Crow, M., Deng, T. & Brownlee, G. G. (2003). A single amino acid mutation in the PA subunit of the influenza virus RNA polymerase promotes the generation of defective interfering RNAs. *J Virol* **77**, 5017–5020.
- Gonzalez, S. & Ortin, J. (1999). Characterization of influenza virus PB1 protein binding to viral RNA: two separate regions of the protein contribute to the interaction domain. *J Virol* **73**, 631–637.
- Huarte, M., Falcon, A., Nakaya, Y., Ortin, J., Garcia-Sastre, A. & Nieto, A. (2003). Threonine 157 of influenza virus PA polymerase subunit modulates RNA replication in infectious viruses. *J Virol* **77**, 6007–6013.
- Kawaguchi, A., Naito, T. & Nagata, K. (2005). Involvement of influenza virus PA subunit in assembly of functional RNA polymerase complexes. *J Virol* **79**, 732–744.
- Lamb, R. A. & Krug, R. M. (2001). *Orthomyxoviridae*: the viruses and their replication. In *Fields Virology*, pp. 1487–1579. Edited by P. M. Howley. Philadelphia: Lippincott Williams & Wilkins.
- Leahy, M. B., Pritlove, D. C., Poon, L. L. & Brownlee, G. G. (2001). Mutagenic analysis of the 5' arm of the influenza A virus virion RNA promoter defines the sequence requirements for endonuclease activity. *J Virol* **75**, 134–142.
- Legault, P., Li, J., Mogridge, J., Kay, L. E. & Greenblatt, J. (1998). NMR structure of the bacteriophage lambda N peptide/boxB RNA complex: recognition of a GNRA fold by an arginine-rich motif. *Cell* **93**, 289–299.
- Li, M. L., Ramirez, B. C. & 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.
- Neumann, G., Brownlee, G. G., Fodor, E. & Kawaoka, Y. (2004). Orthomyxovirus replication, transcription, and polyadenylation. *Curr Top Microbiol Immunol* **283**, 121–143.
- Portela, A., Zurcher, T., Nieto, A. & Ortin, J. (1999). Replication of orthomyxoviruses. *Adv Virus Res* **54**, 319–348.
- Pritlove, D. C., Fodor, E., Seong, B. L. & Brownlee, G. G. (1995). *In vitro* transcription and polymerase binding studies of the termini of influenza A virus complementary RNA: evidence for a cRNA panhandle. *J Gen Virol* **76**, 2205–2213.
- Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M. & Seraphin, B. (2001). The tandem affinity purification (TAP) method: a general procedure of protein complex purification. *Methods* **24**, 218–229.
- Rao, P., Yuan, W. & Krug, R. M. (2003). Crucial role of CA cleavage sites in the cap-snatching mechanism for initiating viral mRNA synthesis. *EMBO J* **22**, 1188–1198.
- Subbarao, K., Chen, H., Swayne, D. & 8 other authors (2003). Evaluation of a genetically modified reassortant H5N1 influenza A

virus vaccine candidate generated by plasmid-based reverse genetics. *Virology* **305**, 192–200.

Tiley, L. S., Hagen, M., Mathews, J. T. & Krystal, M. (1994). Sequence-specific binding of the influenza virus RNA polymerase

to sequences located at the 5' ends of the viral RNAs. *J Virol* **68**, 5108–5116.

Weiss, M. A. & Narayana, N. (1998). RNA recognition by arginine-rich peptide motifs. *Biopolymers* **48**, 167–180.