The EMBO Journal Vol.17 No.19 pp.5844–5852, 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

Mei-Ling Li, Bertha Cecilia Ramirez[1] and Robert M.Krug[2]

Department of Molecular Biology and Biochemistry, Rutgers University, Piscataway, NJ 08855, USA

[1]Present address: Universite Paul Sabatier, 31062 Toulouse Cedex, France

[2]Corresponding author
e-mail: krug@waksman.rutgers.edu

The capped RNA primers required for the initiation of influenza virus mRNA synthesis are produced by the viral polymerase itself, which consists of three proteins PB1, PB2 and PA. Production of primers is activated only when the 5′- and 3′-terminal sequences of virion RNA (vRNA) bind sequentially to the polymerase, indicating that vRNA molecules function not only as templates for mRNA synthesis but also as essential cofactors which activate catalytic functions. Using thio U-substituted RNA and UV crosslinking, we demonstrate that the 5′ and 3′ sequences of vRNA bind to different amino acid sequences in the same protein subunit, the PB1 protein. Mutagenesis experiments proved that these two amino acid sequences constitute the functional RNA-binding sites. The 5′ sequence of vRNA binds to an amino acid sequence centered around two arginine residues at positions 571 and 572, causing an allosteric alteration which activates two new functions of the polymerase complex. In addition to the PB2 protein subunit acquiring the ability to bind 5′-capped ends of RNAs, the PB1 protein itself acquires the ability to bind the 3′ sequence of vRNA, via a ribonucleoprotein 1 (RNP1)-like motif, amino acids 249–256, which contains two phenylalanine residues required for binding. Binding to this site induces a second allosteric alteration which results in the activation of the endonuclease that produces the capped RNA primers needed for mRNA synthesis. Hence, the PB1 protein plays a central role in the catalytic activity of the viral polymerase, not only in the catalysis of RNA-chain elongation but also in the activation of the enzyme activities that produce capped RNA primers.

Keywords: capped RNA primers/influenza virus polymerase/PB1 protein/RNA as cofactor/RNA-binding sites

Introduction

The RNA-dependent RNA polymerase of influenza virus, which is comprised of three viral polymerase (P) proteins, PB1, PB2 and PA, catalyzes the synthesis of viral mRNAs and the replication of genomic RNAs in infected cells (reviewed in Krug *et al*., 1989). The distinctive feature of influenza virus mRNA synthesis is that it requires initiation by 5′-capped RNA fragments which are cleaved from host-cell RNA polymerase II transcripts. The production of these primers, i.e. the cleavage of capped host-cell RNAs, is catalyzed by the viral polymerase complex itself. The PB2 protein subunit of the polymerase serves an essential role: it binds specifically to the 5′-cap structure of cellular mRNAs. An endonuclease then cleaves these capped RNAs 10–13 nucleotides from their 5′ ends. It has not yet been clearly established which P protein(s) catalyze endonuclease cleavage. The capped RNA fragments produced by the endonuclease serve as the primers for viral mRNA synthesis. The PB1 protein subunit catalyzes nucleotide addition during mRNA-chain elongation.

The complex of the three P proteins acquires cap binding and endonuclease activities only when influenza virion RNA (vRNA) is present (Hagen *et al*., 1994; Cianci *et al*., 1995). First, binding of the common 5′-terminal sequence of the vRNA segments activates the capped RNA-binding activity of the PB2 protein. Subsequent binding of the common 3′-terminal sequence of the vRNA segments activates endonuclease activity, thereby enabling the polymerase complex to catalyze capped RNA-primed mRNA synthesis. Consequently, the 5′- and 3′-terminal sequences of vRNA function as essential cofactors which activate the catalytic activities of the P-protein complex that produce capped RNA primers. Via this control mechanism the production of primers is activated only when the template for viral mRNA synthesis, namely vRNA, is present.

Our goal is to determine the mechanism by which the binding of these two virus-specific RNA sequences activates the catalytic activity of the P-protein complex for the production of primer RNAs. Here we identify the specific binding sites of these two RNA sequences in the P-protein complex, using thio U-substituted RNA and UV crosslinking. Replacement of specific amino acids in the identified sites with alanines abrogated both RNA binding and the activation of catalytic activity, proving that these are indeed the functional RNA-binding sites. Surprisingly, both RNA-binding sites are on the same protein subunit, the PB1 protein. The two RNA-binding sites in the PB1 protein, which are separated from each other by ~300 amino acids, are on opposite sides of the consensus sequence for the active site for RNA polymerization (Poch *et al*., 1989; present study). We conclude that binding of the two terminal vRNA sequences to two different amino acid sequences in the PB1 protein causes the polymerase complex to undergo two sequential allosteric alterations that first activate capped RNA-binding activity, followed by the activation of endonuclease activity.

Results

Identification of the site in the P-protein complex to which the 5′-terminal sequence of vRNA binds

Our strategy was to take advantage of the very high efficiency with which thio U residues crosslink with amino

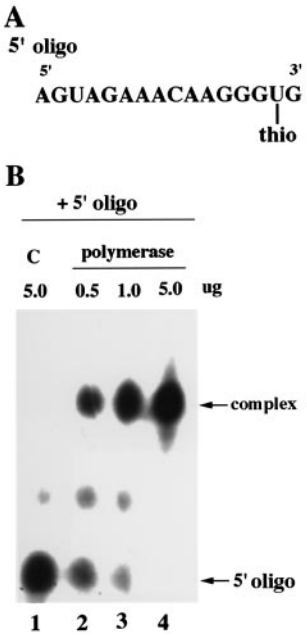

Fig. 1. The oligoribonucleotide that contains thio U and the 5′-terminal sequence of vRNA binds to recombinant influenza virus polymerase complexes. (**A**) Sequence of the thio U-containing oligoribonucleotide. The first 13 nucleotides at the 5′ end constitute the common sequence at the 5′ ends of the eight influenza vRNA segments. Nucleotides 14–16 contain the sequence at the 5′ end of the vRNA segment encoding the two nonstructural (NS) proteins. (**B**) Gel-shift assay for binding. The oligoribonucleotide (^{32}P-end-labeled: 10 ng, 1×10^5 c.p.m.) was incubated with increasing amounts of a nuclear extract from cells expressing the three P proteins (lanes 2–4) or with 5 μg of a nuclear extract from cells expressing T7 RNA polymerase (C, lane 1), and protein–oligoribonucleotide complexes were separated from free oligoribonucleotides by electrophoresis on 4% non-denaturing gels. The arrows denote the positions of free 5′ oligoribonucleotide and of the oligoribonucleotide–polymerase complexes.

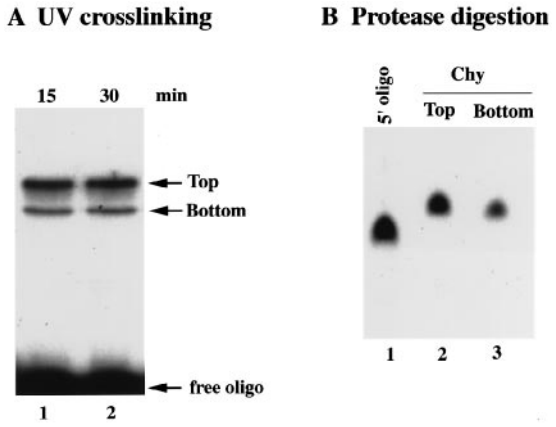

Fig. 2. Isolation of the polymerase peptide that is crosslinked to the oligoribonucleotide containing thio U and the 5′-terminal sequence of vRNA. (**A**) This oligoribonucleotide (^{32}P-end-labeled) was incubated with a nuclear extract from cells expressing the three P proteins. After this binding reaction and subsequent exposure to 366 nm UV light for the times indicated (15 or 30 min), the reaction mixtures were electrophoresed on a 7% denaturing gel to separate the crosslinked protein–oligoribonucleotide complexes from free oligoribonucleotide. (**B**) The two radiolabeled protein bands from (A) were digested with chymotrypsin, and the resulting products were analyzed by electrophoresis on a 18% denaturing gel (lanes 2–5). Lane 1: the ^{32}P-end-labeled oligoribonucleotide prior to UV crosslinking.

acids after exposure to UV light (Favre *et al*., 1998; Wang and Rana, 1998). Thio U was inserted at position 15 of a 16 nucleotide-long oligoribonucleotide which contains the common 5′-terminal 13 nucleotide-long sequence of the vRNA segments (Figure 1A). This oligonucleotide was labeled with ^{32}P at its 5′ end and was added to a preparation of recombinant influenza virus RNA polymerase. To prepare this polymerase, HeLa cells were infected with three vaccinia virus vectors expressing the PB1, PB2 and PA proteins; the nuclear extract from these cells contains the influenza virus polymerase (Hagen *et al*., 1994; Cianci *et al*., 1995). The thio U-containing oligoribonucleotide, like those lacking thio U, bound to polymerase complexes in these extracts, as determined in a gel-shift assay (Figure 1B, lanes 2–4). Nuclear extracts from cells infected with a control vaccinia virus plasmid that expressed the T7 RNA polymerase did not cause a gel shift (lane 1). Also, in confirmation of previous results (Hagen *et al*., 1994; Cianci *et al*., 1995), gel shift of this oligoribonucleotide required the nuclear extracts to contain all three P proteins (data not shown).

To identify the binding site of the labeled, thio U-containing oligonucleotide, it was incubated with the polymerase preparation, and the mixture was exposed to UV light. Gel electrophoresis indicated that two protein bands were radiolabeled and hence crosslinked to the oligoribonucleotide (Figure 2A). The presence of two labeled protein species was consistent with two possibilities: either two different P proteins were crosslinked to the oligoribonucleotide, or the slower protein species was a breakdown product of the larger species. To distinguish between these two possibilities, both protein species were separately digested with chymotrypsin, and the resulting products were analyzed by gel electrophoresis (Figure 2B). The labeled chymotrypsin digestion products migrated slightly slower than the labeled oligoribonucleotide, consistent with the presence of a small peptide covalently attached to the oligoribonucleotide. The chymotrypsin digestion products of both the top and bottom bands contained a single peptide that was microsequenced. The two crosslinked peptides had the same sequence, indicating that the bottom band was a breakdown product of the top band. The sequence matched a sequence in the PB1 protein subunit, extending from amino acid 560 to 574 (Figure 3A).

Two arginines in the 560–574 amino acid sequence of the PB1 protein are required for the binding of the 5′-terminal sequence of vRNA to the polymerase complex

The 560–574 amino acid sequence of the PB1 protein contains four arginine (R) residues which are potential candidates for interacting with the negatively charged oligoribonucleotide containing the 5′-terminal sequence of vRNA (Figure 3A). To determine whether any of these arginines were required for RNA binding, each arginine codon was individually replaced with an alanine (A) codon by site-directed mutagenesis, and the resulting mutated PB1 gene was inserted into a vaccinia virus vector (Elroy-Stein and Moss, 1996). HeLa cells were then coinfected with vaccinia virus vectors expressing the wild-type PB2 and PA proteins, and one of the mutant PB1 proteins. The polymerase complexes in the nuclear extracts from these cells were assayed for their ability to bind the oligoribo-

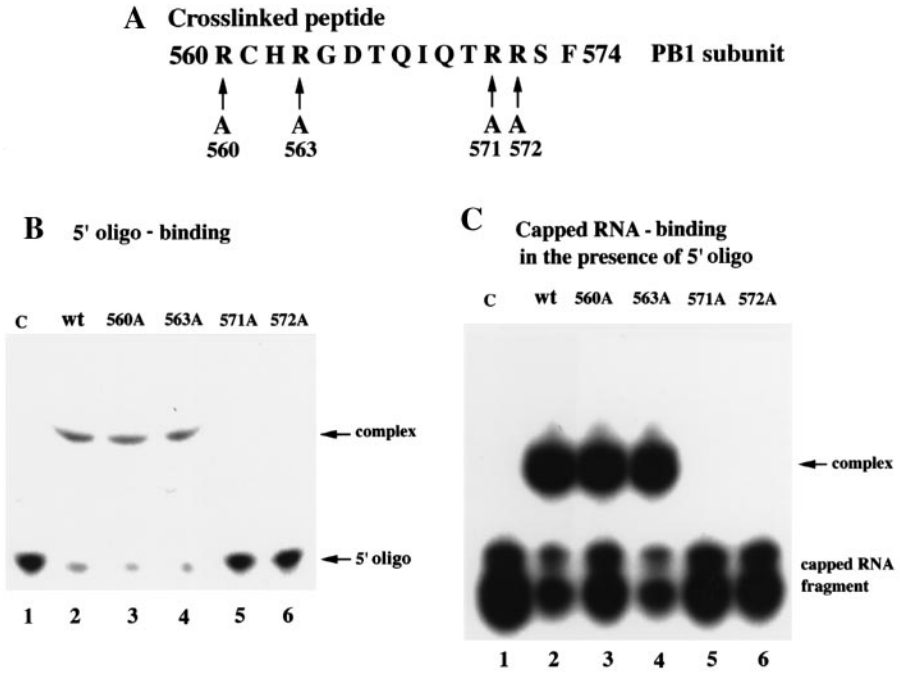

Fig. 3. Identification of the functional polymerase binding site for the 5′-terminal sequence of vRNA. (**A**) Amino acid sequence of the peptide in the PB1 protein that crosslinks to the oligoribonucleotide containing thio U and the 5′-terminal sequence of vRNA. The positions of the four arginines that were individually replaced with an alanine are denoted. (**B**) Binding of the 5′ oligoribonucleotide (lacking thio U) to polymerase complexes containing a wild-type PB1 protein or one of the mutant PB1 proteins. This oligoribonucleotide (^{32}P-end-labeled) was incubated with a nuclear extract from cells expressing either the wild-type (lane 2) or mutant polymerase complexes (lanes 3–6), and protein–oligoribonucleotide complexes were separated from free oligoribonucleotide by electrophoresis on 4% non-denaturing gels. The arrows denote the positions of free oligoribonucleotide and of the protein–oligoribonucleotide complexes. Lane 1 (C): 5′ oligoribonucleotide alone. (**C**) Binding of capped RNA fragments to polymerase complexes containing a wild-type PB1 protein or one of the mutant PB1 proteins. Subsequent to the binding of the unlabeled 5′ oligoribonucleotide to the polymerase complexes, the 13 nucleotide-long-capped AlMV RNA 4 RNA fragment containing a $m^7G^{32}pppG_m$ 5′ end (10 ng, 1×10^5 c.p.m.) was incubated with the wild-type (lane 2) or mutant polymerase complexes (lanes 3–6). The arrows denote the positions of free capped RNA fragments and of the capped RNA polymerase complexes. Lane 1 (C): capped RNA fragment alone.

nucleotide containing the 5′-terminal sequence of vRNA. In addition, the activity of these complexes for binding capped RNAs was determined, using as substrate the 13 nucleotide-long capped RNA fragment which had been cleaved from alfalfa mosaic virus (AlMV) RNA 4 by the influenza virion endonuclease itself. This capped RNA fragment, which contained ^{32}P only in its cap, binds to the polymerase complex but is not subsequently cleaved by the endonuclease activity of this complex (Plotch *et al*., 1981; Ulmanen *et al*., 1981).

Polymerase complexes that contained a PB1 protein in which the arginine at either position 560 or 563 was replaced with alanine behaved like wild-type polymerase complexes. They bound the oligoribonucleotide containing the 5′-terminal sequence of vRNA (Figure 3B, lanes 3 and 4), and as a consequence acquired the ability to bind a capped RNA fragment (Figure 3C, lanes 3 and 4). In contrast, polymerase complexes that contained a PB1 protein in which the arginine at either position 571 or 572 was replaced with alanine did not behave like wild-type. They did not bind the oligoribonucleotide containing the 5′-terminal sequence of vRNA (Figure 3B, lanes 5 and 6), and consequently did not acquire the ability to bind a capped RNA fragment (Figure 3C, lanes 5 and 6). The latter two amino acid replacements did not cause an inhibition of an earlier step: these mutant PB1 proteins interacted with the other two P proteins to form polymerase complexes (Figure 4). Anti-PB1 antiserum co-immunoprecipitated the PA and PB2 proteins, whether the PB1 protein

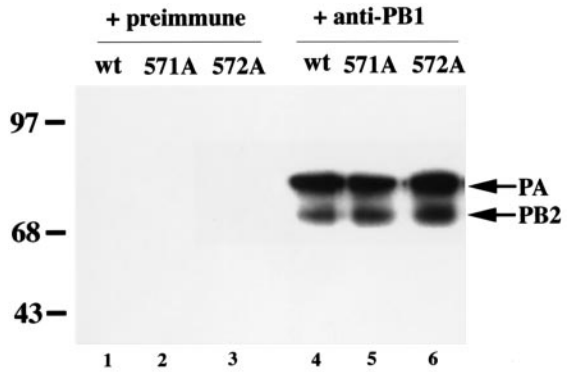

Fig. 4. PB1 proteins containing an alanine substitutions at either position 571 or 572 interact with the PB2 and PA proteins to form polymerase complexes. Nuclear extracts from cells expressing the PB2 and PA proteins and either wild-type PB1 protein (lanes 1 and 4) or one of the mutant PB1 proteins (lanes 2, 3, 5 and 6) were immunoprecipitated with control (preimmune) antiserum (lanes 1–3) or with anti-PB1 antiserum (lanes 4–6). After denaturing gel electrophoresis, the proteins were transferred to a nitrocellulose filter which was then probed with both anti-PB2 and anti-PA antisera. The identity of the two proteins was established by probing with a single antiserum, either the anti-PB2 or the anti-PA antiserum.

was wild-type or contained an alanine substitution at position 571 or 572. We conclude that the arginines at positions 571 and 572 of the PB1 protein are required for the binding of the 5′-terminal sequence of vRNA to the polymerase complex and for the consequent acquisition of the ability to bind capped RNAs.

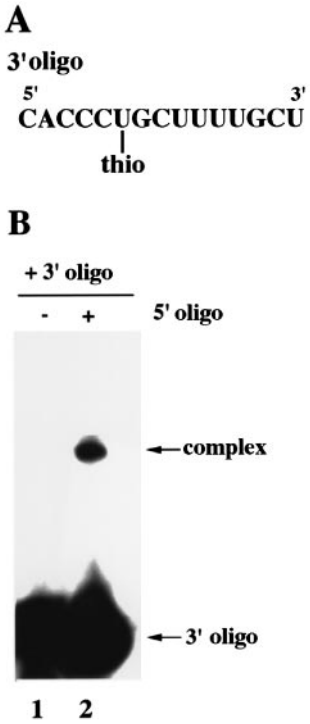

Fig. 5. The oligoribonucleotide that contains thio U and the 3′-terminal sequence of vRNA binds to recombinant influenza virus polymerase complexes. (**A**) Sequence of the thio U-containing oligoribonucleotide. The first 12 nucleotides at the 3′ end constitute the common sequence at the 3′ ends of the eight influenza vRNA segments. Nucleotides 13–15 contain the sequence at the 3′ end of the vRNA segment encoding the two NS proteins. (**B**) Gel-shift assay for binding. This oligoribonucleotide (^{32}P-end-labeled: 10 ng, 1×10^5 c.p.m.) was incubated with a nuclear extract from cells expressing the three P proteins in the absence (lane 1) or the presence of the oligoribonucleotide (unlabeled) containing the 5′-terminal sequence of vRNA (lane 2). Protein–oligoribonucleotide complexes were separated from free oligoribonucleotide by electrophoresis on 4% non-denaturing gels. The arrows denote the positions of free oligoribonucleotide and of the protein–oligoribonucleotide complexes.

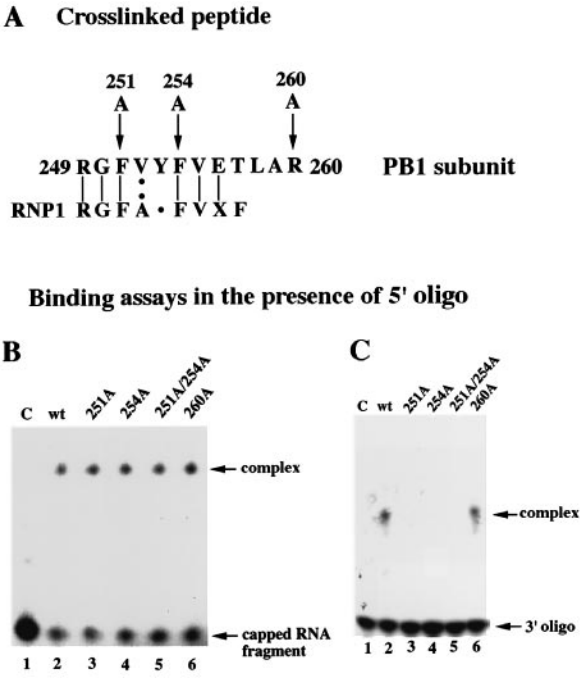

Fig. 6. Identification of the functional polymerase binding site for the 3′-terminal sequence of vRNA. (**A**) Amino acid sequence of the peptide in the PB1 protein that crosslinks to the oligoribonucleotide containing thio U and the 3′-terminal sequence of vRNA. The positions of the two phenylalanines and the single arginine that were individually replaced with an alanine are denoted. (**B**) Binding of capped RNA fragments to polymerase complexes containing a wild-type PB1 protein or one of the mutant PB1 proteins. Subsequent to the binding of the unlabeled 5′ oligoribonucleotide to the polymerase complexes, the 13 nucleotide-long capped AlMV RNA 4 RNA fragment containing a $m^7G^{32}pppG_m$ 5′ end (10 ng, 1×10^5 c.p.m.) was incubated with the wild-type (lane 2) or mutant polymerase complexes (lanes 3–6). The arrows denote the positions of free capped RNA fragments and of the capped RNA–protein complexes. Lane 1 (C): capped RNA fragment alone. (**C**) Binding of the 3′ oligoribonucleotide (lacking thio U) to polymerase complexes containing a wild-type PB1 protein or one of the mutant PB1 proteins. Subsequent to the binding of the unlabeled 5′ oligoribonucleotide to the polymerase complexes, the oligoribonucleotide containing the 3′-terminal sequence of vRNA (^{32}P-end-labeled) was incubated with a nuclear extract from cells expressing either the wild-type (lane 2) or mutant polymerase complexes (lanes 3–6), and protein–oligoribonucleotide complexes were separated from free oligoribonucleotide by electrophoresis on 4% non-denaturing gels. The arrows denote the positions of free oligoribonucleotide and of the protein–oligoribonucleotide complexes. Lane 1 (C): 3′ oligoribonucleotide alone.

Identification of the site in the P-protein complex to which the 3′-terminal sequence of vRNA binds

For these experiments, thio U was inserted at position 10 of a 15 nucleotide-long oligoribonucleotide that contains the common 3′-terminal 12 nucleotide sequence of the vRNA segments (Figure 5A). This oligoribonucleotide (5′-^{32}P-labeled) bound to polymerase complexes, but binding was dependent on the prior addition of an unlabeled oligonucleotide containing the 5′-terminal sequence of vRNA (Figure 5B). This dependence, which was previously observed with comparable oligoribonucleotides lacking thio U (Cianci *et al*., 1995), verifies that the binding of vRNA sequences to the polymerase complex is sequential: first, the 5′-terminal sequence of vRNA, followed by the 3′-terminal sequence of vRNA.

To identify the binding site of the oligonucleotide containing the 3′-terminal sequence, the polymerase preparation was first incubated with an unlabeled oligoribonucleotide containing the 5′-terminal sequence of vRNA and lacking thio U. The labeled, thio U-containing oligonucleotide containing the 3′-terminal sequence of vRNA was then added, and the mixture was exposed to UV light. The single protein species which was crosslinked to the labeled oligoribonucleotide was isolated by gel electrophoresis and digested with trypsin to yield a product containing a small peptide covalently attached to the labeled oligoribonucleotide (data not shown). The sequence of this peptide matched a sequence in the PB1 protein subunit, extending from amino acid 250 to 260 (Figure 6A).

Two phenylalanines in the 250–260 amino acid sequence of the PB1 protein are required for the binding of the 3′-terminal sequence of vRNA to the polymerase complex

The amino acid sequence of the PB1 protein from amino acid 249 to 256 has a strong homology with the ribonucleoprotein 1 (RNP1) motif, which is found in many RNA-binding proteins (Figure 6A). Because phenylalanines (F) mediate RNA binding by the RNP1 motif (Burd and

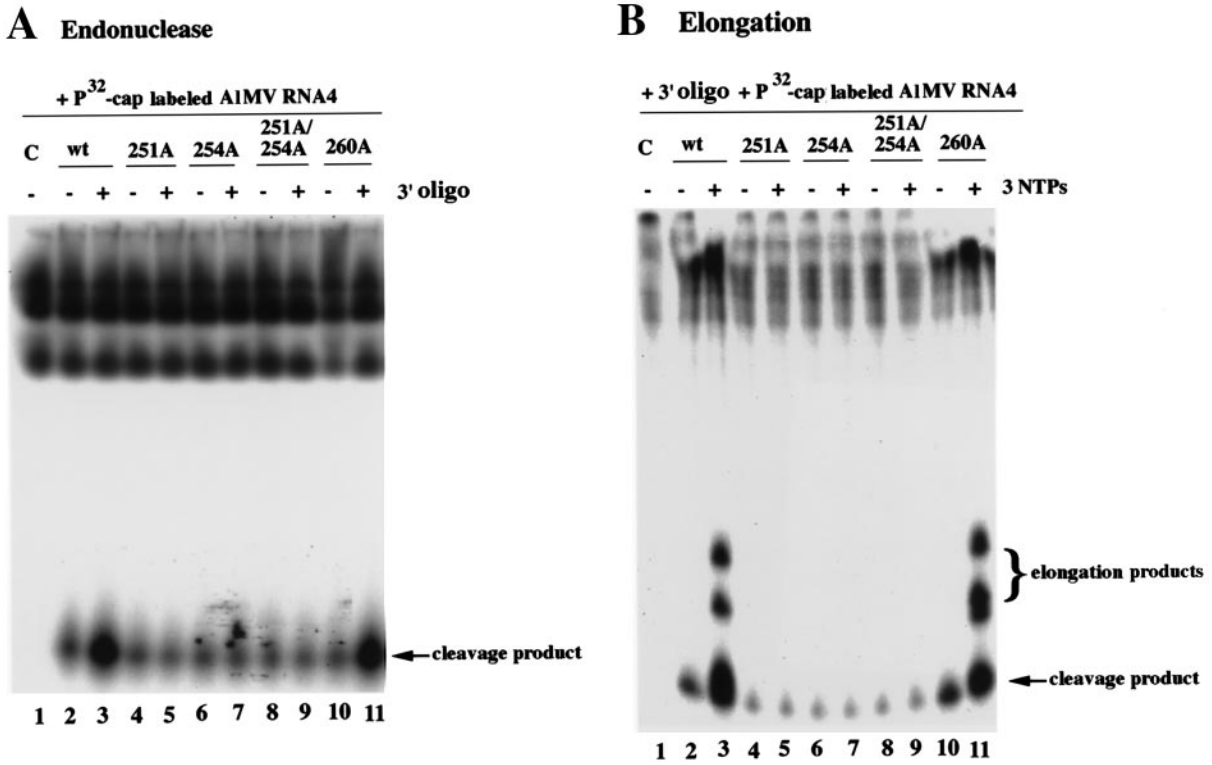

Fig. 7. Enzymatic activities of PB1 mutants in the presence of 5′ oligo. Specific alanine replacements in the PB1 binding site for the 3′ oligoribonucleotide result in polymerase complexes that fail to acquire the cap-dependent endonuclease activity required for RNA-chain elongation. (**A**) Cap-dependent endonuclease activities of wild-type and mutant polymerases. After binding the 5′ oligoribonucleotide to the polymerase complexes, wild-type (lanes 2 and 3) or mutant polymerases (lanes 4–11) were incubated with full-length AlMV RNA 4 containing a $m^7G^{32}pppG_m$ 5′ end, either with (lanes 3, 5, 7, 9 and 11) or without (lanes 2, 4, 6, 8 and 10) prior incubation of the polymerases with the 3′ oligoribonucleotide. The RNA products were separated on a denaturing 20% gel. The position of the specific 13-long cleavage product is indicated by an arrow. Lane 1 (C): full-length AlMV RNA 4 incubated in the absence of a polymerase complex. (**B**) RNA-chain elongation activities of wild-type and mutant polymerases. The endonuclease assays contained the 3′ oligoribonucleotide, and where indicated (lanes 3, 5, 7, 9 and 11) three ribonucleoside triphosphates (ATP, CTP and GTP, each at 1 mM). The positions of the endonuclease cleavage product and the partially elongated RNA chains are denoted. No elongation products were detected in lanes 5, 7 and 9 even when the gel was exposed 20 times longer.

Dreyfuss, 1994), our strategy was to replace the phenylalanines at either position 251 or 254 of the PB1 sequence with alanine. As a control, the arginine at position 260 was replaced with alanine. Each of the mutated PB1 genes was inserted into a vaccinia virus vector (Elroy-Stein and Moss, 1996), which was used to infect HeLa cells together with the two vaccinia virus vectors expressing the wild-type PB2 and PA proteins. The polymerase complexes in the nuclear extracts from these cells were assayed in the presence of an oligonucleotide containing the 5′-terminal sequence of vRNA. Because the binding of this vRNA sequence was not inhibited by these mutations in the PB1 protein, all the viral polymerases, whether containing a wild-type or a mutant PB1 protein, acquired equivalent capped RNA-binding activities (Figure 6B).

Two of the mutations in the PB1 protein, specifically replacement of one or both of the phenylalanines at positions 251 and/or 254 of the PB1 protein with alanine, resulted in polymerases which did not bind an oligonucleotide containing the 3′-terminal sequence of vRNA (Figure 6C). In contrast, replacement of the arginine at position 260 with an alanine had no detectable effect on the binding of this oligoribonucleotide. We conclude that the phenylalanines at positions 251 and 254 of the PB1 protein are required for the binding of the 3′-terminal sequence of vRNA to the polymerase complex.

To measure the cap-dependent endonuclease activity of these viral polymerases, full-length AlMV RNA 4 containing ^{32}P only in its 5′ cap was incubated with each polymerase complex, and the amount of the specific 13 nucleotide-long capped cleavage product was determined (Plotch *et al*., 1981; Figure 7A). All the viral polymerases, whether containing a wild-type or a mutant PB1 protein, cleaved a small amount of the capped RNA precursor in the absence of the oligoribonucleotide containing the 3′-terminal sequence of vRNA (Figure 7A, lanes 2, 4, 6, 8 and 10). However, addition of the 3′ oligoribonucleotide greatly stimulated endonucleolytic cleavage 10- to 20-fold, but only when the polymerase contained either a wild-type PB1 protein or a PB1 protein in which the arginine at position 260 was replaced with an alanine (Figure 7A, compare lanes 3 and 11 to lanes 5, 7 and 9). In contrast, no detectable increase in endonuclease activity occurred with polymerases in which one or both of the phenylalanines at positions 251 and/or 254 of the PB1 protein subunit was replaced with an alanine (Figure 7A, lanes 5, 7 and 9). Only those capped RNA fragments that were cleaved from the precursor in the presence of the oligonucleotide containing the 3′-terminal sequence of vRNA were functional. These fragments served as primers for the minimal amount of RNA-chain elongation which these polymerase complexes are capable of catalyzing in the presence of ribonucleoside triphosphates, whereas those capped RNA fragments that were produced at low

levels in the absence of this oligonucleotide were not elongated (Figure 7B, compare lanes 3 and 11 to lanes 5, 7 and 9). Consequently, a polymerase complex that contained a mutant PB1 protein in which one or both of the phenylalanines at positions 251 and/or 254 was replaced with an alanine did not acquire the cap-dependent endonuclease activity that is required for RNA-chain elongation.

Discussion

Capped RNA primers are required for the initiation of viral mRNA synthesis by the influenza virus RNA-dependent RNA polymerase (Krug *et al*., 1989). The production of these primers, which is catalyzed by the viral polymerase itself, is activated only when vRNA, the template for viral mRNA synthesis, is present (Hagen *et al*., 1994; Cianci *et al*., 1995; present study). This activation results from the sequential binding of two different vRNA sequences to the polymerase: first, the 5′-terminal sequence; then, the 3′-terminal sequence. Here we show that both of these vRNA sequences bind to the same subunit of the polymerase, the PB1 protein, specifically to two amino acid sequences that are ~300 amino acids apart. The 5′-terminal sequence of vRNA binds to an amino acid sequence centered around two arginine residues at positions 571 and 572, and the 3′-terminal sequence binds to an RNP1-like motif extending from amino acids 249–256 (Figure 8A). The PB1 protein, which also catalyzes nucleotide addition during mRNA-chain elongation (Krug *et al*., 1989), would be expected to contain the consensus sequence for the active site for RNA polymerization. The sequence S-D-D at amino acids 444–446, which is located between the two RNA-binding sites, is the prime candidate for this active-site sequence (Poch *et al*., 1989). Thus, the PB1 protein plays a central role in the catalytic activity of the viral polymerase, both in the activation of the enzyme activities that produce capped RNA primers and in the subsequent catalysis of RNA-chain elongation.

The amino acid sequence to which the 5′-terminal sequence of vRNA binds is conserved in all sequenced influenza A virus PB1 proteins. This sequence does not exhibit significant homology to any sequence in the GCG or NCBI database, indicating that it is a novel motif for specific RNA binding. On the other hand, the sequence to which the 3′-terminal sequence of vRNA binds has a strong homology with the RNP1 motif found in many RNA-binding proteins (Burd and Dreyfuss, 1994; present study). In fact, this sequence was identified as an RNP1 motif in a previous computer search of PB1 sequences (Fodor *et al*., 1993). This PB1 sequence, RGFV<u>Y</u>FVE (amino acids 249–256), differs from the consensus RNP1 motif only by the presence of an additional amino acid, the underlined Y residue. In addition, the RNP1-like motif of the PB1 protein, which is conserved in the PB1 proteins of other influenza A viruses, shares several properties with the consensus RNP1 motif (Nagai *et al*., 1990; Burd and Dreyfuss, 1994): its binding activity is dependent on one or more phenylalanine residues; and it is predicted to form a β-sheet structure, the known structure of consensus RNP1 motifs (present study). In many of the other RNA-binding proteins the RNP1 motif is part of a larger domain, the RNA recognition motif (RRM) (Burd and Dreyfuss, 1994). As with consensus RNP1 motifs which are part of RRMs, the RNP-like motif of the PB1 protein is adjacent to a downstream region that is predicted to be an α helix (amino acids 277–290). However, the PB1 protein sequence does not contain the upstream RNP2 motif that is found in the larger RRM domain of other proteins. Instead, the PB1 sequence contains a potential RNP2 motif that is far downstream (amino acids 500–505) from its RNP1-like sequence. Perhaps the PB1 protein contains a novel RRM in which the RNP1 and RNP2 motifs are inverted and are separated from each other by a large amino acid sequence.

Our results, along with those of other investigators (Hagen *et al*., 1994; Cianci *et al*., 1995), indicate that the binding of the two terminal vRNA sequences to the polymerase complex induces two allosteric alterations in this enzyme complex (Figure 8B). In the absence of vRNA the complex of the three P proteins is enzymatically inactive, but contains a functional site on the PB1 subunit for the binding of the 5′-terminal sequence of vRNA which is centered around two arginine residues at positions 571 and 572. Upon the binding of the 5′-terminal sequence to this site on the PB1 protein, two new functions of the polymerase complex are activated, presumably as a result of allosteric alterations of the complex. One function is acquired by a different subunit, the PB2 protein, which is now capable of binding the 5′-capped ends of RNAs. The other new function is acquired by the PB1 protein itself: a functional RNA-binding site that is specific for the 3′-terminal sequence of vRNA, i.e. the RNP1-like motif extending from amino acid 249 to 256. When the latter RNA binding occurs, a second allosteric alteration is induced, resulting in the activation of the enzymatic activity that endonucleolytically cleaves capped RNAs 10–13 nucleotides from their 5′ ends. The resulting capped RNA fragments serve as primers for mRNA synthesis, as demonstrated here by the addition of nucleotides to the 3′ ends of the fragments. Nucleotide addition is catalyzed by the PB1 protein (Krug *et al*., 1989), presumably utilizing the consensus sequence for the active site of RNA polymerization centered around amino acids 444–446 (Poch *et al*., 1989). This RNA polymerase site, which is not able to initiate chains *de novo*, requires that a capped RNA primer be provided for the initiation of mRNA synthesis (Krug *et al*., 1989). It should be noted that this mechanism is consistent with the recent demonstration that the juxtaposition of the 5′ and 3′ ends of vRNA molecules in the nucleocapsids of influenza virions requires the presence of the polymerase (Klumpp *et al*., 1997).

The activation of the production of capped RNA primers requires the sequential binding of two different vRNA sequences to two different amino acid sequences in the PB1 protein (Hagen *et al*., 1994; Cianci *et al*., 1995; present study). Specific RNA binding to the first site activates only one activity, the binding of a capped RNA to the PB2 protein. The activation of this binding activity alone without the activation of the endonuclease does in fact occur (Shih *et al*., 1995; Shih and Krug, 1996). Such a partial activation enables P-protein complexes to serve two other functions in addition to the catalysis of mRNA and genome RNA synthesis. For these other functions, activation of capped RNA binding results from the binding of an alternative virus-specific RNA sequence, specifically

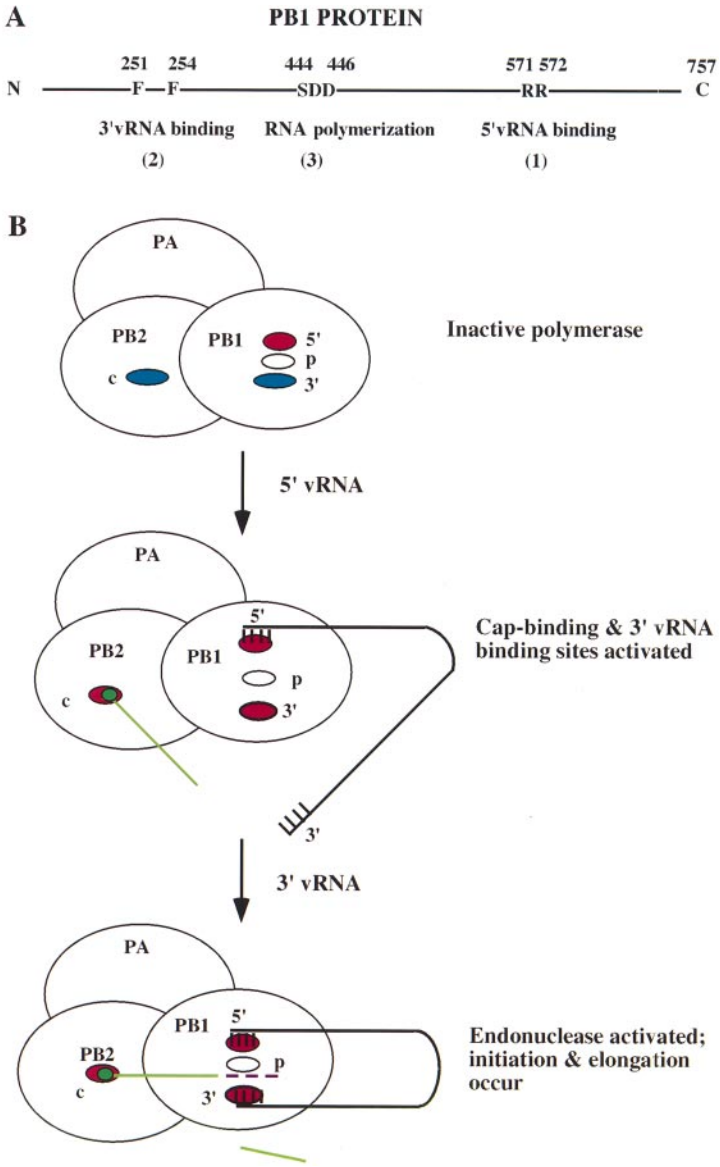

Fig. 8. The central role of the PB1 protein in the activation of the catalytic functions of the influenza virus polymerase. (**A**) Relative positions of the three functional amino acid sequences of the PB1 protein: the binding site for the 3′-terminal sequence of vRNA (designated as 3′ vRNA), the presumed catalytic site for RNA polymerization, and the binding site for the 5′-terminal sequence of vRNA (designated as 5′ vRNA). The numbers above these amino acid sequences denote their positions in the PB1 protein. The numbers in parentheses denote the sequential order that these sequences function during viral mRNA synthesis. (**B**) Model for the activation of capped primer production by the influenza virus polymerase complex. This model is described in the text. 5′, binding site for the 5′ end of vRNA; 3′, binding site for the 3′ end of vRNA; c, binding site for capped RNA; p, polymerase active site. Blue denotes an inactive site, and red denotes an active site. The p site is shown with neither color, because it is not known whether it is constitutively active or requires activation. One possibility is that it possesses constitutive activity, but that this activity is inapparent because capped RNA primers have to be provided before the P site can initiate mRNA synthesis. Capped RNA is shown in green; its 5′ cap structure is denoted by an oval at one end.

the sequence in all viral mRNAs that is immediately 3′ to the 5′-terminal sequence supplied by the cellular capped RNA primers. Presumably this RNA sequence also binds to the PB1 sequence centered around the two arginine residues at positions 571 and 572. When this viral mRNA sequence binds to P-protein complexes, their PB2 protein subunits are activated and bind to the 5′ cap structures of the viral mRNAs. Consequently, partially activated P-protein complexes are bound to the 5′ ends of all viral mRNAs (Shih *et al*., 1995; Shih and Krug, 1996). These complexes selectively protect viral, but not cellular, mRNAs from the endonucleolytic cleavage that is catalyzed by other, fully activated P-protein complexes, so that only cellular, and not viral, mRNAs are cleaved for the production of capped RNA primers (Shih and Krug, 1996). In addition, the partially activated P-protein complexes that are bound to the 5′ end of one viral mRNA, the M1 mRNA, regulate its alternative splicing by blocking one of its 5′ splice sites (Shih *et al*., 1995).

During mRNA synthesis influenza vRNA molecules function not only as templates, but also as essential cofactors which activate the catalytic activities of P-protein complexes that produce capped RNA primers. Specific RNA sequences also act as cofactors for other enzymes. For example, the polymerase of another virus, hepatitis B virus, also requires activation by a specific

viral RNA sequence, a stem–loop at the 5′ end of the precursor of the genomic RNA (Tavis and Ganem, 1996; Tavis *et al*., 1998). In addition, telomerases, the cellular enzymes which maintain the telomeres at the ends of eukaryotic chromosomes, contain a short RNA component that not only serves as a template but is also necessary for the catalytic activity of the enzyme (Gilley and Blackburn, 1996; Bhattacharyya and Blackburn, 1997; Prescott and Blackburn, 1997; Autexier and Greider, 1998). Some tRNA synthetases also have an RNA cofactor (Lin *et al*., 1996; Hale *et al*., 1997). Thus, isoleucyl-tRNA synthetase incorrectly activates the closely related amino acid, valine, with a relatively high frequency. The correction of this error is mediated by a specific sequence in the cognate (isoleucyl) tRNA molecule itself which induces the synthetase to edit out the incorrectly activated valine. We anticipate that there will be other examples of specific RNA sequences that function as cofactors of enzymes.

Materials and methods

Preparation of recombinant influenza virus polymerase complexes

Recombinant vaccinia virus vectors individually encoding the influenza virus PB1, PB2 and PA proteins (Vac-PB1, Vac-PB2 and Vac-PA) or encoding T7 RNA polymerase (VTF7-3) were grown in HeLa cells (Smith *et al*., 1987; Cianci *et al*., 1995). To construct recombinant vaccinia virus vectors containing a mutant PB1 gene, the desired mutation was introduced by PCR into a PB1 gene which was in the plasmid transfer vector pGS77. The entire mutant PB1 gene was sequenced to rule out the presence of extraneous mutations. The insertion of the mutant gene into vaccinia virus was carried out as described by Elroy-Stein and Moss (1996). The pGS77 vector containing the mutant PB1 gene was transfected into CV-1 cells that had been infected with vaccinia virus for 1.5 h. After 2 days, cell-associated vaccinia virus was collected, and then used to infect HuTK$^-$143B cells that are deficient in thymidine kinase. These cells were grown under agarose containing 5-bromodeoxyuridine (BrdU), conditions under which only the TK$^-$ recombinant virus will form plaques. Recombinant viruses were obtained after three plaque purifications. HeLa cells were infected three times with Vac-PB2, Vac-PA, and either Vac-PB1 encoding wild-type PB1 protein or a vaccinia virus vector encoding one of the mutant PB1 proteins (Smith *et al*., 1987; Cianci *et al*., 1995). As a control, other HeLa cells were infected with VTF7-3. At 20–24 h after infection, nuclear extracts were prepared and used as the source of the influenza virus polymerase complex (Fiering *et al*., 1990; Hagen *et al*., 1994).

Preparation of labeled oligoribonucleotides and RNAs

Oligoribonucleotides containing the 5′- or 3′-terminal sequence of vRNA were synthesized using an oligonucleotide synthesizer. Where indicated, thio U was introduced at a particular position of the oligoribonucleotide (Shah *et al*., 1994). The purity of each oligoribonucleotide was established by gel electrophoresis. Oligoribonucleotides were labeled at their 5′ ends using polynucleotide kinase and [γ-^{32}P]ATP. To prepare an RNA substrate containing a labeled 5′ cap, the 5′ terminal m^{7}G of AlMV RNA 4 was removed by β-elimination, and the RNA was then incubated with the vaccinia virus capping enzyme (guanyltransferase and 7-methyltransferase) and 2′-*O*-methyltransferase in the presence of [α-^{32}P]GTP to produce a m^{7}G^{32}pppG$_m$ 5′ end (Plotch *et al*., 1981). The substrate for capped RNA binding was prepared by incubating the full-length AlMV RNA 4 (containing a labeled 5′ cap) with the polymerase complexes in detergent-treated influenza virions (Plotch *et al*., 1981; Ulmanen *et al*., 1981). The 13 nucleotide-long-capped RNA fragment produced by the virion endonuclease was isolated by gel electrophoresis.

UV crosslinking

A nuclear extract containing wild-type polymerase complexes (750 μg) was incubated 25 min at 25°C with either the ^{32}P-end-labeled oligoribonucleotide containing thio U and the 5′-terminal sequence of vRNA (80 ng, 1×10^7 c.p.m.), or an unlabeled oligoribonucleotide (lacking thio U) containing the 5′-terminal sequence of vRNA (80 ng) plus the ^{32}P-end-labeled oligoribonucleotide containing thio U and the 3′-terminal sequence of vRNA (80 ng, 1×10^7 c.p.m.). The reaction, in a final volume of 300 μl, contained 10 mM HEPES pH 7.5, 150 mM KCl, 0.5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 1 unit RNasin and 10% glycerol (binding buffer). After the binding reaction, the mixtures were put on ice and exposed to 366 nm UV light at a distance of 2 cm for 15 or 30 min (Favre *et al*., 1998; Wang and Rana, 1998). The crosslinked protein–oligoribonucleotide complexes were separated from free oligoribonucleotide by electrophoresis on 7% denaturing gels. The gel slices containing the crosslinked complexes were dialyzed overnight to achieve equilibration with either the buffer used for chymotrypsin digestion or the buffer used for trypsin digestion. The proteins in the gel slices were then digested with one of these proteases (0.3 μg) for 18 h at 25°C (chymotrypsin) or 37°C (trypsin), and then the gel slices were placed on the top of an 18% denaturing gel. After electrophoresis, the appropriate band (see Results) was cut out, and either eluted or blotted onto PVDF membranes. This material was microsequenced using automated Edman degradation by the Protein Chemistry Section of the HHMI Biopolymer Facility and W.M.Keck Foundation Biotechnology Resource Laboratory at Yale University.

Assays for the activities of viral polymerase complexes

Assays for the binding of specific oligoribonucleotides to polymerase complexes: in a final volume of 10 μl, a nuclear extract containing wild-type or mutant polymerase complexes (2.5 μg) was incubated for 30 min at 25°C in the binding buffer with either the ^{32}P-end-labeled oligoribonucleotide containing the 5′-terminal sequence of vRNA (10 ng, 1×10^5 c.p.m.), or an unlabeled oligoribonucleotide containing the 5′-terminal sequence of vRNA (10 ng) plus the ^{32}P-end-labeled oligoribonucleotide the 3′-terminal sequence of vRNA (10 ng, 1×10^5 c.p.m.) (Hagen *et al*., 1994; Cianci *et al*., 1995). The assay for the binding of capped RNA fragments to polymerase complexes: following a 30 min reaction for the binding of an unlabeled oligoribonucleotide containing the 5′-terminal sequence of vRNA, the 13 nucleotide-long-capped AlMV RNA 4 RNA fragment containing a m^{7}G^{32}pppG$_m$ 5′ end (10 ng, 1×10^5 c.p.m.) was added, and the mixture was incubated for 30 min at 30°C (Ulmanen *et al*., 1981). For both binding assays, the protein–RNA complexes were separated from free RNA by electrophoresis on 4% non-denaturing gels. The endonuclease assay: following binding of the oligoribonucleotide containing the 5′-terminal sequence of vRNA, and where indicated, the subsequent binding of the oligoribonucleotide containing the 3′-terminal sequence of vRNA, full-length AlMV RNA 4 containing a m^{7}G^{32}pppG$_m$ 5′ end (0.25 ng, 1×10^5 c.p.m.) was added, along with 0.1 μg of tRNA as an additional inhibitor of non-specific nucleases, and the mixture was incubated for 60 min at 30°C (Plotch *et al*., 1981; Hagen *et al*., 1994; Cianci *et al*., 1995). In the assay for RNA-chain elongation, three ribonucleoside triphosphates (ATP, CTP and GTP, each at 1 mM) were added during the cleavage reaction. For both the endonuclease and elongation assays, the RNA products were separated on a denaturing 20% polyacrylamide–7 M urea gel.

Antisera

Rabbit antisera specific for the PB1 protein (Detjen *et al*., 1987), and the PB2 and PA proteins (Tiley *et al*., 1994) were raised against the C-terminal peptides of these proteins. The latter two antisera were kindly provided by Mark Krystal.

Acknowledgements

This investigation was supported by grant AI11772 from the US National Institutes of Health to R.M.K. We thank Tariq Rana for his advice concerning the use of thio U-containing oligoribonucleotides for UV crosslinking, and Mark Krystal for valuable reagents and advice.

References

Autexier,C. and Greider,C.W. (1998) Mutational analysis of the *Tetrahymena* telomerase RNA: identification of residues affecting telomerase activity *in vitro*. *Nucleic Acids Res.*, **26**, 787–795.

Bhattacharyya,A. and Blackburn,E.H. (1997) A functional telomerase RNA swap *in vivo* reveals the importance on nontemplate RNA domains. *Proc. Natl Acad. Sci. USA*, **94**, 2823–2827.

Burd,C.G. and Dreyfuss,G. (1994) Conserved structures and diversity of functions of RNA-binding proteins. *Science*, **265**, 615–621.

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.

Elroy-Stein,O. and Moss,B. (1996) Expression of proteins in mammalian cells using vaccinia viral vector. *Curr. Protocols Mol. Biol.*, 16.15.1–16.19.9.

Favre,A., Saintome,C., Fourrey,J.-L., Clivio,P. and Laugaa,P. (1998) Thionucleobases as intrinsic photoaffinity probes of nucleic acid structure and nucleic acid–protein interactions. *J. Photochem. Photobiol. B*, **42**, 109–124.

Fiering,S., Northrop,J.P., Nolan,G.P., Mattila,P.S., Crabtree,G.R. and Herzenberg,L.A. (1990) Single cell assay of a transcription factor reveals a threshold in transcription activation by signals emanating from the T-cell antigen receptor. *Genes Dev.*, **4**, 1823–1834.

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

Gilley,D. and Blackburn,E.H. (1996) Specific RNA residue interactions required for enzymatic functions of *Tetrahymena* telomerase. *Mol. Cell. Biol.*, **16**, 66–75.

Hagen,M., Chung,T.D.Y., Butcher,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.

Hale,S.P., Auld,D.S., Schmidt,E. and Schimmel,P. (1997) Discrete determination in transfer RNA for editing and aminoacylation. *Science*, **276**, 1250–1252.

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

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

Lin,L., Hale,S.P. and Schimmel,P. (1996) Aminoacylation error correction. *Nature*, **384**, 33–34.

Nagai,K., Oubridge,C., Jessen,T.H., Li,J. and Evans,P.R. (1990) Crystal structure of the RNA-binding domain of the U1 small nuclear ribonucleoprotein A. *Nature*, **348**, 515–520.

Plotch,S.J., Bouloy,M., Ulmanen,I. and Krug,R.M. (1981) A unique cap (m^7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. *Cell*, **23**, 847–858.

Poch,O., Sauvaget,I., Delarue,M. and Tordo,N. (1989) Identification of four conserved motifs among the RNA-dependent polymerase encoding element. *EMBO J.*, **8**, 3867–3874.

Prescott,J. and Blackburn,E.H. (1997) Telomerase RNA mutations in *Saccharomyces cerevisiae* alter telomerase action and reveal nonprocessivity *in vivo* and *in vitro*. *Genes Dev.*, **11**, 528–540.

Shah,K., Wu,H. and Rana,T.M. (1994) Synthesis of uridine phosporamidite analogs: reagents for site-specific incorporation of photoreactive sites into RNA sequences. *Bioconjugation Chem.*, **5**, 508–512.

Shih,S.R. and Krug,R.M. (1996) Surprising function of the three influenza viral polymerase proteins: selective protection of viral mRNAs against the cap-snatching reaction catalyzed by the same polymerase proteins. *Virology*, **226**, 430–435.

Shih,S.R., Nemeroff,M.E. and Krug,R.M. (1995) The choice of alternative 5′ splice sites in influenza virus M1 mRNA is regulated by the viral polymerase complex. *Proc. Natl Acad. Sci. USA*, **92**, 6324–6328.

Smith,G., Levin,J.Z., Palese,P. and Moss,B. (1987) Synthesis and cellular location of the ten influenza polypeptides individually expressed by recombinant vaccinia viruses. *Virology*, **160**, 336–345.

Tavis,J.E. and Ganem,D. (1996) Evidence for activation of the hepatitis B virus polymerase by binding of its RNA template. *J. Virol.*, **70**, 5741–5750.

Tavis,J.E., Massey,B. and Gong,Y. (1998) The duck hepatitis B virus polymerase is activated by its RNA packaging signal, ε. *J. Virol.*, **72**, 5789–5796.

Tiley,L.S., Hagen,M., Matthews,J.T. and 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.

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 (m^7GpppNm) on RNAs and in initiating viral RNA transcription. *Proc. Natl Acad. Sci. USA*, **78**, 7355–7359.

Wang,Z. and Rana,T.M. (1998) RNA–protein interactions in the Tat-trans-activation response element complex determined by site-specific photo-cross-linking. *Biochemistry*, **37**, 4235–4243.

Received July 17, 1998; revised and accepted August 14, 1998