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Sequence-Specific Binding of the Influenza Virus RNA Polymerase to Sequences Located at the 5′ Ends of the Viral RNAs

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The enzymatic activity of recombinant influenza virus RNA polymerase is strictly dependent on the addition of a template RNA containing 5′ and 3′ viral sequences. Here we report the analysis of the binding specificity and physical characterization of the complex by using gel shift, modification interference, and density gradient techniques. The 13S complex binds specifically to short synthetic RNAs that mimic the partially double stranded panhandle structures found at the termini of both viral RNA and cRNA. The polymerase will also bind independently to the single-stranded 5′ or 3′ ends of viral RNA. It binds most strongly to specific sequences within the 5′ end but is unable to bind these sequences in the context of a completely double stranded structure. Modification interference analysis identified the short sequence motifs at the 5′ ends of the viral RNA and cRNA templates that are critical for binding.

The influenza virus RNA polymerase is a multicomponent complex composed of three virally encoded proteins termed PB1, PB2, and PA (25). The polymerase plays two distinct transcriptional roles in the life cycle of the virus (16). First, it is responsible for the synthesis of viral mRNA from the negative-sense genomic viral RNA (vRNA). An unusual feature of this process is the utilization of the 5′ termini of host cell mRNAs as primers for the initiation of transcription (33, 34). The 5′ and 3′ ends of the vRNA template have partial inverted complementarity and thus can form a panhandle structure (8, 20, 35, 43). This structure in combination with a run of five to seven uridine residues located approximately 15 to 17 nucleotides from the 5′ end serves as a transcription termination and polyadenylation signal (15, 26, 27, 36). The second role of the polymerase is for viral genome replication. The 5′-terminal base of vRNA and the complementary cRNA is an ATP, which implies that initiation of vRNA and cRNA synthesis does not require a primer (17, 50). The complete transcription of the vRNA template into cRNA demands that the termination and polyadenylation signal be ignored (17). One function of the viral nucleoprotein (NP) may be to suppress this signal by preventing premature termination (2, 39). One of the most perplexing questions about influenza virus replication is what are the factors that determine which role the polymerase complex is going to play.

Several in vitro systems have been established to study the transcriptional activity of the polymerase (18, 21, 22, 24, 28, 29, 37). The most intensively studied of these systems derive their polymerase from purified virion cores which are processed in a variety of ways to deplete them of their endogenous RNAs (18, 31, 37). Results from these systems have focused attention on the conserved sequences located at the 3′ end of vRNA. However, recently an in vitro system using influenza polymerase expressed by recombinant vaccinia viruses (Vac 3P) demonstrated that active polymerase requires sequences located at the 5′ and 3′ ends of the viral genome (14). This system has now been used to study the interaction of the viral polymerase with its template RNA. Here we report the use of an in vitro complex formation assay to determine the sequence and structural requirements for the specific binding of the polymerase to vRNA- and cRNA-like template RNAs.

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MATERIALS AND METHODS

Plasmid constructs. Plasmids pPH-V, pV5′, pV3′, pM-wt, pPHd5′, and pV-i$U_{10}U_8C_5A_3$ have been described previously (14, 31). pC5′V3′ was constructed by ligating the complementary oligonucleotides 5′AGCTTAATACGACTCACTATAA GCAAAAGCAGGGTGTTTTTTCA3′ and 5′GATCTGAAA AA AC A CC CT GC TT TT GC TT AT A G TGA GT CG TA T TAA3′ between the *Hin*dIII and *Bgl*II sites of the 3.0-kb fragment of plasmid pPHd5′. Plasmid pV5′C3′ was constructed by replacing the *Bgl*II-*Pst*I fragment of pPH-V with the corresponding 47-bp fragment from plasmid pV-iU_{10} $U_8C_5A_3$ (31).

Virus and cell culture. Recombinant vaccinia viruses that individually express the three influenza virus polymerase proteins (Vac-PB1, Vac-PB2, and Vac-PA [40]) or express the T7 RNA polymerase (VTF7-3 [13]) were propagated on HeLa cell monolayers and titrated on Vero cells, using standard procedures (10). Influenza virus A/WSN/33 was propagated in MDBK cells as previously described (44).

Antibodies. Polyclonal rabbit antisera raised against the individual P proteins expressed by using recombinant baculoviruses were a kind gift from D. Summers. The antiserum specific for the C-terminal peptide of PB1 was provided by R. Krug (9). Monoclonal antibody 10.2, reported for the first time here, was obtained from a mouse inoculated with baculovirus-expressed PB1 and binds to an epitope located between Asn-44 and Ile-69. The rabbit antipeptide antiserum specific for the PB2 protein was raised against the C-terminal peptide CSILTDSETATKRIR, and the rabbit antiserum specific for the PA protein was raised against the C-terminal peptide CLRDNLEPGTFDLG.

Preparation of virus-infected cell extracts. HeLa cells in monolayers were infected with influenza virus A/WSN/33 (multiplicity of infection [MOI] of ~10 per cell) or recombinant vaccinia viruses which express either PB1, PB2, and PA (MOI of 3:3:3 per cell) or the T7 RNA polymerase (MOI of 10

TABLE 1. Primary sequences of the RNA probes used in this work

Probe	Sequence[a]
PHV	5′AGUAGAAACAAGGGUGUUUUUUCAGAUCUAUUAAACUUCACCCUGCUUUUGCU3′
PHC	5′AGCAAAAGCAGGGUGAAGUUUAAAUGAUAUGAAAAAACACCCUUGUUUCUACU3′
V5′C3′	5′AGUAGAAACAAGGGUGUUUUUUCAGAUCUAUUAAACUUCACCCUUGUUUCUACU3′
C5′V3′	5′AGCAAAAGCAGGGUGUUUUUUCAGAUCUAUUAAACUUCACCCUGCUUUUGCU3′
V5′	5′AGUAGAAACAAGGGUGUUUUUUCAGAUCCCCGGGUACCGAGCUCGAAUU3′
V3′	5′GGGAAUUCGAGCUCGGUACCGGGGAUCUUAUUAAACUUCACCCUGCUUUUGCU3′

[a] Underlined sequences correspond to the terminal sequences of vRNA. Overlined sequences correspond to the terminal sequences of cRNA.

per cell). The influenza virus-infected cells were harvested at 2 to 18 h postinfection. The vaccinia virus-infected cells were harvested 16 h postinfection. Nuclear extracts were prepared as previously described (11, 30). Extracts were stored at −70°C at a concentration of 10 mg/ml and were stable for several months.

In vitro synthesis, labeling, and purification of RNAs. Plasmids were linearized with either *Mbo*II (pPH-V, pM-wt, pC5′V3′, pV5′C3′, and pV3′) or *Eco*RI (pV5′) and transcribed by using T7 RNA polymerase to generate RNAs designated PHV, PHC, C5′V3′, V5′C3′, V3′, and V5′ respectively (Table 1). The transcripts were purified by electrophoresis through 10% polyacrylamide gels containing 8 M urea. The RNA bands were located by UV shadowing, excised, and recovered by passive elution into 1 mM EDTA. The RNAs were then ethanol precipitated and quantitated by optical density at 260 nm. Radiolabeled RNAs were prepared either by the incorporation of [α-^{32}P]GTP during transcription (or [α-^{32}P]UTP for probe V3′ because of the inappropriate distribution of G residues), by phosphorylation of the 5′ end with polynucleotide kinase and [γ-^{32}P]ATP (3,000 Ci/mmol), or by 3′ end labeling with T4 RNA ligase and [5′-^{32}P]pCp (3,000 Ci/mmol). A DNA oligonucleotide with the same sequence as PHV was phosphorylated at its 5′ end as described above. 5′- and 3′-end-labeled probes were repurified as described above.

Gel shift assays. RNA probes were diluted in 50 mM NaCl–0.25 mg of bovine serum albumin (BSA) per ml, denatured by heating to 90°C for 2 min, and then incubated at 37°C for 10 min prior to use. Ten microliters of binding reaction mixtures contained 1×10^4 to 2×10^4 cpm of RNA probe and 2.5 μg of nuclear extract. The final buffer components were 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.6), 0.5 mM EGTA, 20 μM EDTA, 2 mM $MgCl_2$, 7.5 mM NaCl, 110 mM KCl, 1.2 mM dithiothreitol, 27.5 μg of RNase-free BSA per ml, 1,000 U of placental RNase inhibitor per ml, and 12% glycerol. The binding reaction mixtures were incubated at room temperature for 30 min, and reactions were terminated by the addition of heparin to a final concentration of 5 mg/ml. For the competition experiments, the probe RNA was mixed with the competitor RNA before the nuclear extract was added to the reaction. For supershift experiments, the binding reaction was allowed to proceed for 10 min before the addition of 0.5 μl of antibody and then terminated with heparin 20 min later. The complexes were resolved by native gel electrophoresis (4% 79:1 acrylamide/bis acrylamide, 50 mM Tris-HCl [pH 8.4], 50 mM glycine, 3% glycerol; 12 V/cm) and visualized by autoradiography.

Modification interference assays. 5′- or 3′-end-labeled PHV and PHC RNAs were subjected to limited chemical modification under denaturing conditions, using diethyl pyrocarbonate or anhydrous hydrazine as described previously (4, 45). A portion of each probe was set aside for later analysis (later referred to as preshifted RNAs). Approximately 4×10^5 cpm of the modified RNAs was incubated with 80 μg of Vac 3P nuclear extract, sufficient to shift ~25 to 40% of the RNA into the specific complex when analyzed by native gel electrophoresis. The bound and free RNAs were excised from the gel, electroeluted with a Hoefer GE 200 gel elutor, phenol extracted, and then ethanol precipitated. These purified RNAs and the preshifted RNAs that had been set aside were then cleaved specifically at the modified bases by treatment with aniline acetate (32). The amount of each RNA recovered was determined by Cerenkov counting, and then 4,000 cpm of each RNA was analyzed by electrophoresis through 8 M urea–20% polyacrylamide sequencing gels.

Sucrose gradient centrifugation. Twenty-microliter binding reaction mixtures containing 5×10^5 cpm of probe RNA and 20 μg of nuclear extract were prepared as described above. After the addition of heparin (to 5 mg/ml), the reaction volumes were increased to 100 μl with 5% sucrose containing 2.5 mg of catalase per ml. The samples were then centrifuged through 10 to 20% (wt/vol) sucrose gradients at 37,000 rpm for 18 h at 3°C, using a Beckman SW50.1 rotor. Sucrose solutions contained 10 mM HEPES (pH 7.6), 0.5 mM EGTA, 2 mM $MgCl_2$, and 100 mM KCl. Fractions were collected dropwise from the bottom of the tube. Ten-microliter aliquots of each fraction were analyzed in a scintillation counter to determine the migration of the probe RNA. Catalase activity was determined by the peroxide decomposition assay (3).

RESULTS

Sequence-specific binding of influenza virus polymerase to model viral RNA templates. The in vitro enzymatic activity of vaccinia virus-expressed recombinant influenza virus RNA polymerase (Vac 3P) shows a strict requirement for a virus-specific template RNA (14). This has also been reported for certain preparations of polymerase from influenza virions (19). In particular, the cap-snatching, endonucleolytic activity requires an RNA template capable of mimicking the partially double stranded panhandle structure formed by the 5′ and 3′ ends of the virion RNAs. This implies that the Vac 3P polymerase possesses a sequence-specific RNA binding activity. To study this activity, a short, 5′-^{32}P-end-labeled, synthetic RNA containing the 5′ and 3′ sequences of vRNA (PHV; Table 1) was incubated with either a Vac 3P extract or a control extract from cells infected with a vaccinia virus recombinant expressing the T7 RNA polymerase. Analysis of the

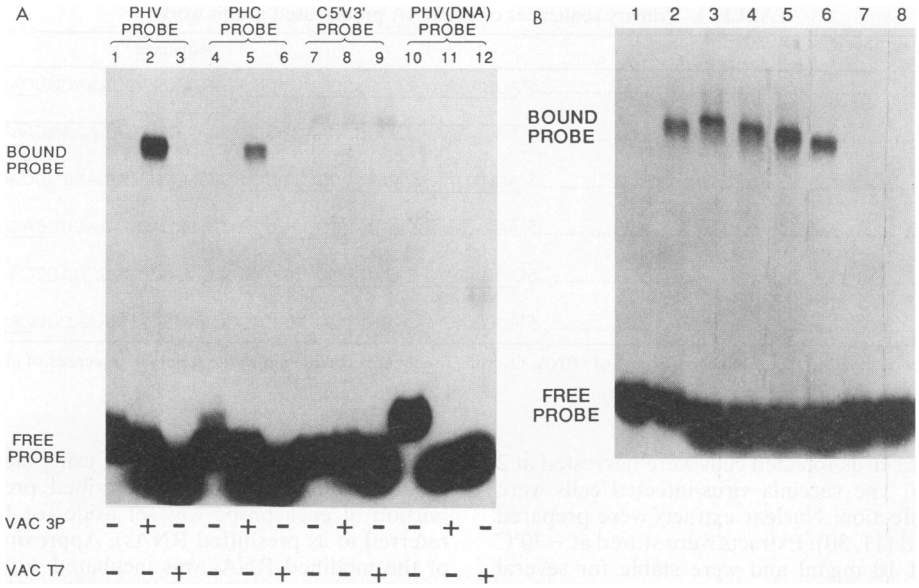

FIG. 1. Binding of recombinant influenza virus RNA polymerase to synthetic template RNAs. (A) RNAs that mimic the terminal panhandle structures of vRNA (PHV; lanes 1 to 3), cRNA (PHC; lanes 4 to 6), a hybrid of 5′ cRNA and 3′ vRNA (C5′V3′; lanes 7 to 9), and a DNA version of vRNA [PHV(DNA); lanes 10 to 12] were synthesized in vitro and 5′ end labeled by using T4 polynucleotide kinase and [γ-^{32}P]ATP. Approximately 25 fmol (3×10^4 cpm) of each probe was incubated with 2.5 μg of nuclear extract from vaccinia virus-infected HeLa cells expressing the influenza virus polymerase (Vac 3P; lanes 2, 5, 8, and 11) or expressing the T7 RNA polymerase (Vac T7; lanes 3, 6, 9, and 12). Probes incubated with extract dilution buffer alone are shown in lanes 1, 4, 7, and 10. Protein-RNA complexes were visualized by electrophoresis through a nondenaturing 4% polyacrylamide gel. (B) Binding of the PHV RNA probe to extracts prepared from influenza virus-infected cells. Lane 1, no protein; lane 2, Vac 3P; lanes 3 to 6, influenza virus-infected cell extracts prepared 2, 4, 6, and 18 h postinfection, respectively; lane 7, Vac PB1-plus-PB2 extract; lane 8, Vac T7.

reaction products by nondenaturing gel electrophoresis demonstrated the formation of a high-molecular-weight complex that was unique to the Vac 3P extract (Fig. 1A; compare lanes 2 and 3). A DNA probe of precisely the same sequence did not form a complex, confirming the specificity for an RNA substrate (lane 11). A similar high-molecular-weight complex was observed when a synthetic RNA containing the 5′ and 3′ sequences of cRNA (PHC; Table 1) was used (lanes 4 and 5). The apparent difference in the level of binding to the cRNA probe was due to the lower specific activity of the probe rather than a difference in affinity. Subsequent experiments using probes of identical specific activity did not show this effect (data not shown). A hybrid RNA containing the 5′ sequences from cRNA and the 3′ sequences from vRNA (V3′C5′; Table 1) was not bound by the polymerase (lanes 8 and 9). The duplex region of this RNA is predicted to be completely double stranded, with none of the bulges present in either the vRNA- or cRNA-specific molecules.

The ability of the recombinant polymerase to distinguish between these closely related probes strongly indicates that the interaction is both sequence and structure specific. Sequence specificity was confirmed directly, by mixing increasing amounts of unlabeled specific (vRNA) or nonspecific (tRNA) competitor with the vRNA probe before adding it to the binding reaction. As shown in Fig. 2, the specific RNA competed quantitatively with the probe RNA, whereas the nonspecific RNA competed minimally even at high molar excess.

Nuclear extracts from influenza virus-infected HeLa cells and purified viral cores were also tested for binding activity in this assay. Extracts made anywhere from 2 to 18 h postinfection exhibited strong binding activity with the PHV probe (Fig. 1B, lanes 3 to 6). However, purified viral cores did not exhibit any binding activity detectable with this assay (data not shown).

Analysis of the protein components of the complex. The identity of the protein component of the complex was determined by incubating the binding reaction mixtures with antisera to the individual P proteins. Binding of an antibody

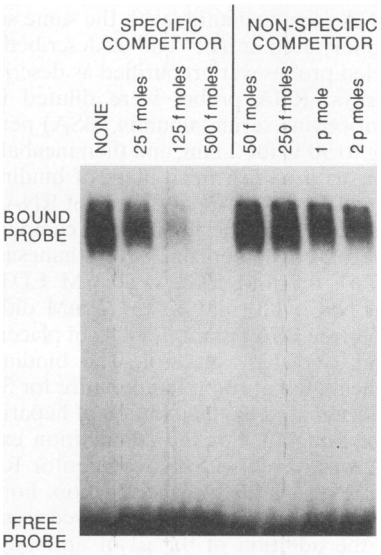

FIG. 2. Confirmation of binding specificity by titration with specific and nonspecific competitor RNAs. Approximately 30 fmol (4×10^4 cpm) of 5′-end-labeled PHV probe RNA was mixed with the indicated amounts of unlabeled specific (PHV RNA) or nonspecific (tRNA) competitor RNA. These mixtures were then incubated with 2.5 μg of Vac 3P extract and analyzed by nondenaturing electrophoresis.

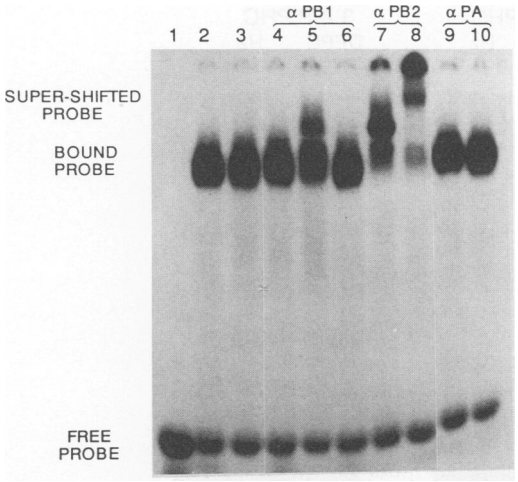

FIG. 3. Supershift analysis of the Vac 3P-PHV complex. PHV probe RNA and Vac 3P extract were combined in standard 10-μl binding reaction mixtures and incubated at 25°C for 10 min; 0.5 μl of antiserum was then added, and the incubation continued for an additional 20 min prior to analysis by nondenaturing gel electrophoresis. Lane 1, PHV probe alone; lanes 2 to 10, PHV probe-plus-Vac 3P extract. Lane 2, no antiserum; lane 3, normal rabbit serum; lanes 4 to 6, antisera specific for PB1; lanes 7 and 8, antisera specific for PB2; lanes 9 and 10, antisera specific for PA. Details for each antiserum are given in the text.

molecule to the complex causes a decrease in its electrophoretic mobility. This supershift effect was readily observed with two different antisera raised against the PB2 protein (Fig. 3, lanes 7 and 8). One antiserum was raised against a carboxy-terminal peptide, and the other was raised against a recombinant baculovirus-expressed protein. An antiserum raised against a carboxy-terminal peptide of PB1 also supershifted the complex (lane 5), indicating that PB1 is also a component of the complex. However, it should be noted that under the conditions of this assay, neither a polyclonal antiserum raised against baculovirus-expressed PB1 nor monoclonal antibody 10.2, specific for PB1, exhibited any supershifting activity (lanes 4 and 6). Both of these antisera work in Western blot (immunoblot) and immunoprecipitation assays. Apparently, only certain antibody preparations bind to the native complex with high enough affinity to cause the supershift. This may explain why the two PA-specific antisera (raised against recombinant baculovirus-expressed PA or a C-terminal peptide) were unable to supershift the complex. While this may indicate that PA is not part of the complex, it may more simply reflect the limited number of PA antisera available for testing. As shown in Fig. 1B, lane 7, PA expression is required for the formation of the complex. No binding activity is detectable in the extract prepared from cells coinfected with the PB1 and PB2 vectors alone.

The approximate size of the protein-RNA complex was determined by sucrose density gradient centrifugation using catalase as a reference standard. As shown in Fig. 4, the Vac 3P-PHV complex migrates as a discrete 13S species (approximate molecular size of 280 kDa). This species was not seen with the Vac T7 extract nor with the C5′V3′ probe. Additional experiments showed that the mobility of the PHC-bound complex was indistinguishable from that of PHV and that the template RNA is not required for the formation of the 3P complex. Analysis of the fractions from parallel sucrose gradients of Vac 3P extracts demonstrated that template-dependent endonuclease activity migrated almost identically, relative to catalase, regardless of whether template RNA was present during the centrifugation (data not shown).

Identification of the RNA sequences critical for polymerase binding. Modification interference analysis has proven to be an

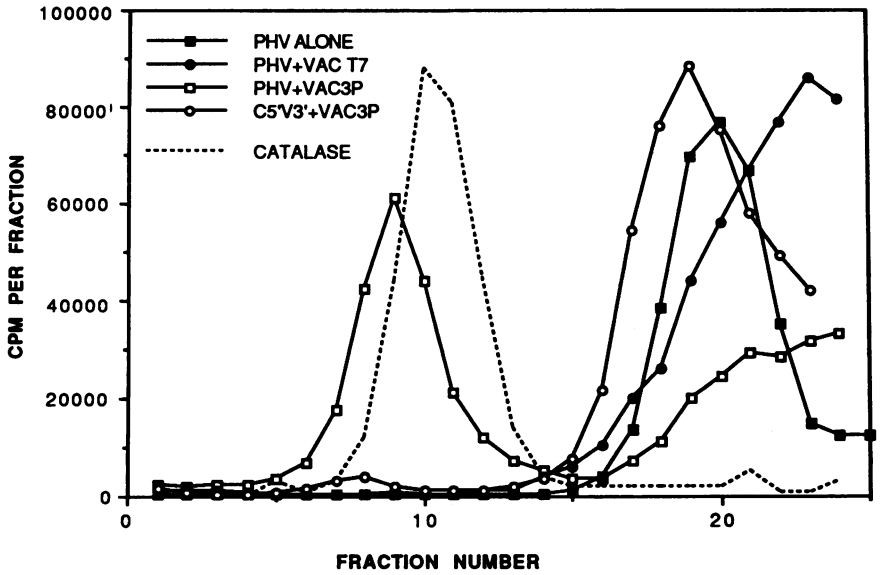

FIG. 4. Sucrose gradient centrifugation profile of the Vac 3P-RNA complex. Binding reaction mixtures containing 5×10^5 cpm of probe, 20 μg of nuclear extract, and 5 mg of heparin per ml in a volume of 20 μl were diluted to a final volume of 100 μl in 5% sucrose containing 2.5 mg of catalase per ml. The reaction mixtures were loaded onto 5-ml 10 to 20% sucrose gradients and centrifuged at 37,000 rpm for 18 h at 3°C, using a Beckman SW50.1 rotor. Fractions were collected dropwise from the bottom of the gradient, and the migration of the probe RNA was determined by scintillation counting. Catalase activity was measured by using the hydrogen peroxide decomposition assay (3). Only the catalase peak for the PHV-plus-Vac 3P gradient is shown.

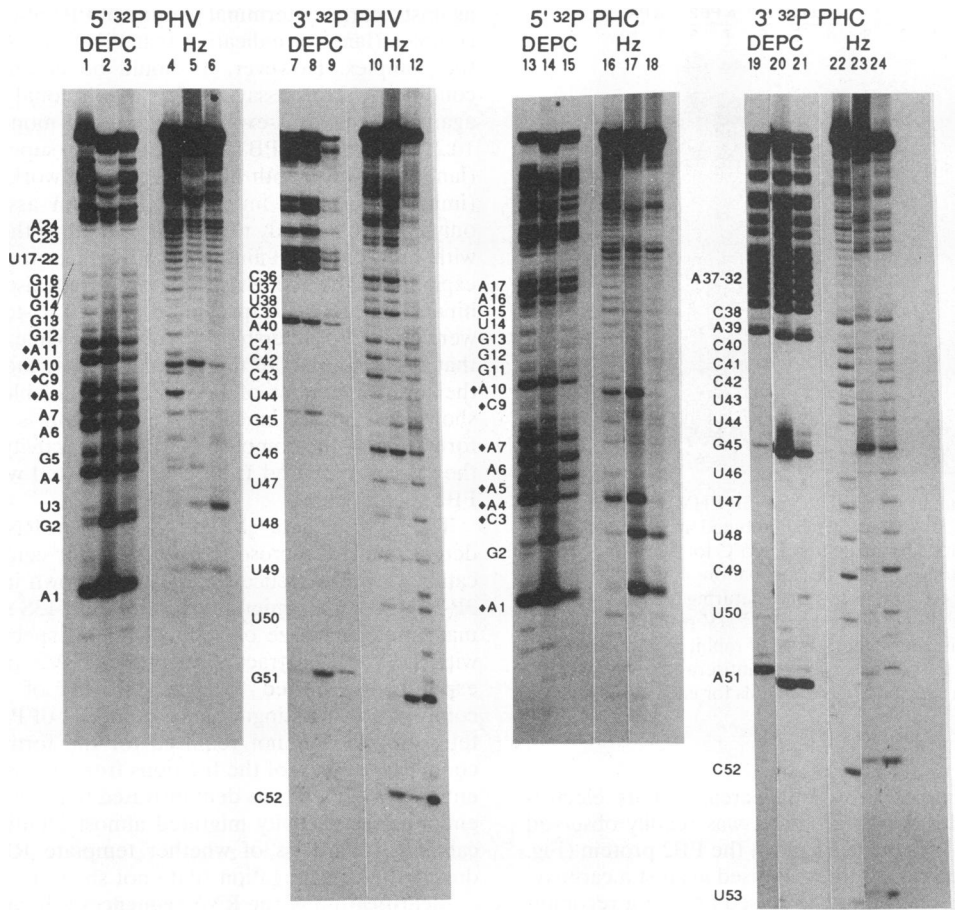

FIG. 5. Modification interference analysis of Vac 3P binding to the PHV and PHC RNA probes. 5′- or 3′-end-labeled PHV RNA (lanes 1 to 12) and PHC RNA (lanes 13 to 24) were subjected to limited chemical modification by diethyl pyrocarbonate (DEPC; A and G specific) or hydrazine (Hz; C and U specific). The modified RNAs were incubated with sufficient Vac 3P extract to bind ~30% of the probe into the complex. The residual free probe was then separated from the complexed probe by nondenaturing gel electrophoresis. The bound and free RNAs were eluted from the gel and then cleaved specifically at the modified bases by treatment with aniline. A total of 4,000 cpm of each RNA sample was then run on a 7 M urea–20% polyacrylamide sequencing gel. The sequence assignment of each band can be determined because of the base specificity of the chemical cleavages. Each group of three lanes corresponds to the three distinct populations of modified RNAs present in the preshifted probe (lanes 1, 4, 7, 10, 13, 19, and 21), the free probe (lanes 2, 5, 8, 11, 14, 17, 20, and 23), and the bound probe (lanes 3, 6, 9, 12, 15, 18, 21, and 24). Bands considered as decreased or absent from the bound populations are highlighted (◆). Only 1,000 cpm of RNA was loaded in lane 9 because of low sample recovery from the native gel.

effective tool for defining the RNA sequences involved in specific protein-RNA interactions (4, 6, 23, 45). The RNA is subjected to limited chemical modification under denaturing conditions so that on average only one base pair per RNA molecule is chemically altered (32). This generates a pool of stable RNA molecules containing randomly distributed, modified bases. When this pool is used as substrate for a binding reaction, the RNAs containing modifications within the binding site are selectively excluded from the protein-RNA complex. The binding site can thus be inferred by comparing the bound and free populations to identify which bases are excluded. 5′- and 3′-end-labeled vRNA and cRNA panhandle RNAs were chemically modified with either diethyl pyrocarbonate or hydrazine. Diethyl pyrocarbonate reacts with the N-7 atom of adenosine and to a lesser extent guanosine residues and causes the purine imidazole ring to open. Anhydrous hydrazine in the presence of 0.375 M NaCl reacts equally with the N-3 atom of cytosine and uridine and effectively removes the base. The modified RNAs were incubated with a predetermined amount of Vac 3P extract so that ~30% of the RNA was bound by the complex. The bound probe was separated from the residual free probe by nondenaturing electrophoresis. The individual bound and free RNAs were extracted from the gel and treated with aniline to specifically cleave the ribose-phosphate backbone at the site of the modification. The bound and free populations were then compared by electrophoresis through a 20% acrylamide–7 M urea sequencing gel. The results from this analysis are shown in Fig. 5 and are summarized in Fig. 6. Bands corresponding to the individual bases of the RNA can readily be assigned because of the sequence specificity of the chemical modifications. Nonspecific cleavages resulting from endogenous nucleases present in the Vac 3P extract can be identified by referring to the cleavage patterns of the preshifted RNA that corresponds to the initial pool of modified RNAs that were not exposed to the extract (Fig. 5, lanes 1, 4, 7, 10, 13, 16, 19, and 22). Densitometric analysis of the bands in the bound, free, and preshifted control lanes for PHV identified four residues (A-8, C-9, A-10,

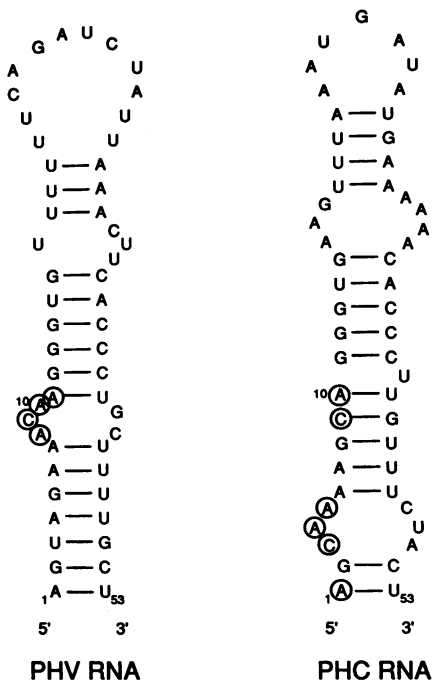

FIG. 6. Locations of the polymerase binding sites identified by modification interference analysis. Panhandle structures that the PHV and PHC RNAs can potentially adopt are shown, with the positions of the modification-sensitive bases highlighted (○). Base pairing of positions 1 and 2 with positions 53 and 52 is not predicted by computer analysis but is included to emphasize the possible structural similarities between the PHV and PHC binding sites.

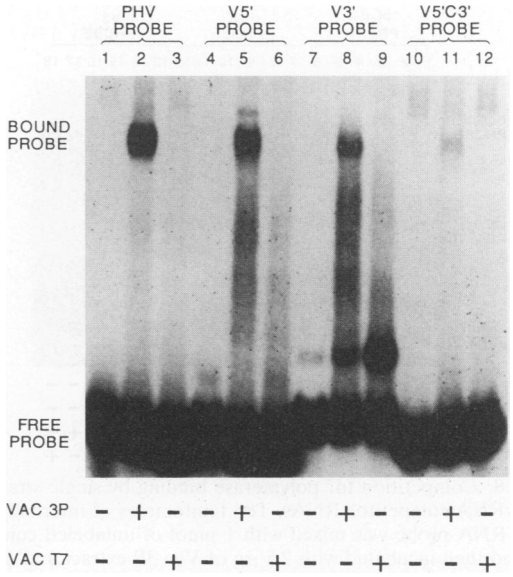

FIG. 7. Binding of the Vac 3P polymerase to single-stranded 5′- or 3′-terminal sequences of vRNA. Internally radiolabeled RNA probes corresponding to the terminal panhandle structure of vRNA (PHV; lanes 1 to 3), the 5′ end of vRNA (V5′; lanes 4 to 6), or the 3′ end of vRNA (V3′; lanes 7 to 9) and a hybrid panhandle composed of the 5′ end of vRNA fused to the 3′ end of cRNA (V5′C3′; lanes 10 to 12) were synthesized in vitro. Ten femtomoles of each probe was incubated with extract dilution buffer (lanes 1, 4, 7, and 10), Vac 3P extract (lanes 2, 5, 8, and 11), or Vac T7 extract (lanes 3, 6, 9, and 12) and analyzed by nondenaturing electrophoresis.

and A-11) that were decreased by 70 to 80% in the bound fraction. All four are located in a proposed bulge region of the 5′ side of the panhandle (Fig. 6). The fact that none of the bands were completely eliminated from the bound population implies that a single-base modification of the probe is not sufficient to completely obliterate binding. This could indicate the presence of a second binding site of lower affinity. On the basis of previous observations (12), the most likely candidate would be at the 3′ end. However, we were unable to reproducibly detect any critical nucleotides at the 3′ end of the PHV panhandle by using this technique. Analysis of the PHC RNA identified six to eight residues as important (A-1, C-3, A-4, A-5, C-9, A-10, and to a lesser extent A-7 and G-15). Of these, C-3 and C-9 were completely excluded from the bound population, indicating that they are absolutely required for binding. Once again, this analysis did not identify any 3′ end sequences involved in binding.

The polymerase binds to single-stranded 5′ and 3′ vRNAs. The results from the modification analysis indicated that the most critical sequences for polymerase binding are all located in the 5′ half of the panhandle structure. Radiolabeled probes of identical specific activity were prepared for the vRNA and hybrid vRNA-cRNA panhandles and also for RNAs containing only 5′- or 3′-end sequences (Table 1). The ability of each of these probes to be bound specifically by the Vac 3P extract was then tested by gel shift analysis (Fig. 7). As predicted from the interference analysis, the probe containing only the 5′-end sequences formed a specific complex almost as well as the vRNA panhandle probe (Fig. 7; compare lanes 1 to 3 with lanes 4 to 6), indicating that 3′ sequences are not required for the formation of this complex. The probe containing only 3′-end sequences also bound specifically to the polymerase (lanes 7 to 9), confirming the previous report (12) of the presence of a separate polymerase binding site at the 3′ end of the RNA. Intriguingly, the hybrid panhandle structures bound very poorly. In fact, the hybrid sequence containing the 5′ end of cRNA and the 3′ end of vRNA did not exhibit any binding whatsoever (Fig. 1A, lanes 7 to 9), while the hybrid RNA containing the 5′ end of vRNA and the 3′ end of cRNA bound approximately 1/10 as well as the PHV RNA (Fig. 7, lanes 10 to 12). The handles of these RNAs would be expected to be perfectly double stranded. The increased stability of the duplex and the lack of RNA bulges may therefore prevent the polymerase from gaining access to its binding site(s).

The 5′ end contains the dominant binding site. Competition experiments were conducted to confirm the specificity of the single-stranded binding activities and to determine whether the 5′ and 3′ RNAs bound to distinct sites. Labeled PHV, V5′, and V3′ RNAs were mixed with an excess of unlabeled PHV, V5′, V3′, or tRNA competitor RNA and assayed by gel shift analysis. As shown previously, unlabeled PHV RNA effectively abolished binding to the PHV probe (Fig. 8, lanes 2 and 3). Interestingly, the V5′ sequence also abolished PHV binding to the polymerase, while excess V3′ or tRNA had little effect on binding (lanes 4 to 6). When V5′ was used as the probe, excess PHV and V5′ RNAs were again able to compete effectively for binding (lanes 7 to 10). The overall level of binding to the V5′ probe was not altered by the addition of an excess of unlabeled V3′ or tRNA. However, the V3′ RNA did cause some qualitative changes in the complex, which now migrated as two distinct bands. The mobility of the free probe was also decreased, indicating that the V5′ probe RNA had annealed to the complementary V3′ RNA. This would form a double-

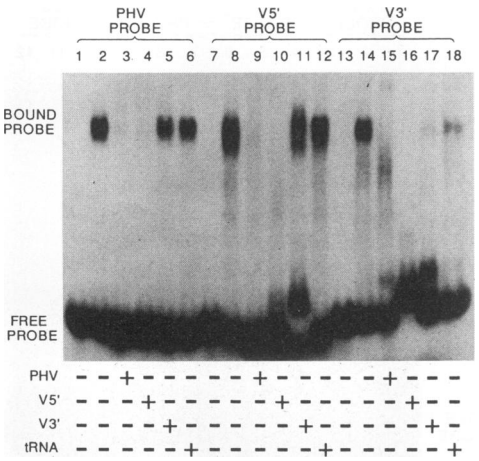

FIG. 8. Competition for polymerase binding by single-stranded 5′ and 3′ vRNA competitor RNAs. Ten femtomoles of internally radiolabeled RNA probe was mixed with 1 pmol of unlabeled competitor RNA and then incubated with 2.5 μg of Vac 3P extract and analyzed by nondenaturing gel electrophoresis. Lanes 1 to 6, PHV probe; lanes 7 to 12, V5′ probe; lanes 13 to 18, V3′ probe. Lanes 2, 8, and 14, no competitor; lanes 3, 9, and 15, PHV competitor; lanes 4, 10, and 16, V5′ competitor; lanes 5, 11, and 17, V3′ competitor; lanes 6, 12, and 18, tRNA competitor. Lanes 1, 7, and 13, free probe controls.

stranded structure analogous to the PHV RNA but approximately twice the size as a result of the additional plasmid-derived sequences (Table 1). Presumably the two complexes represent the polymerase bound to either the single- or double-stranded probe. A different pattern of competition was observed in the reciprocal experiment using V3′ as the probe (lanes 13 to 18). In this case, PHV, V5′, and V3′ all competed for binding to the polymerase. Binding was also significantly decreased by tRNA. Once again, the complementary competitor drove the probe RNA into a double-stranded conformation analogous to PHV (Fig. 8, lane 16). However, in the presence of an excess of V5′ RNA, this RNA did not bind. These data suggest that the binding reaction is driven primarily by the 5′ sequence and that binding to the 3′ end is a much weaker interaction.

DISCUSSION

In this paper, we report the physical characterization and template specificity of the influenza virus polymerase complex expressed in a recombinant vaccinia virus system. The in vitro systems that have predominantly been used in the past for analyzing the template specificity of the influenza virus RNA polymerase have utilized polymerase from purified viral cores. Before these extracts can be used to study a synthetic template, the endogenous viral template must be removed. This is usually achieved by dissociating the RNA with high salt or by degrading it with micrococcal nuclease (18, 37). However, it is extremely difficult to remove all traces of the vRNA or vRNA ends by these methods, and some properties of these preparations may be attributable to these residual RNAs. Crude preparations of viral cores and the micrococcal nuclease-treated polymerases efficiently cleave capped RNA substrates in the absence of synthetic RNA template and can transcribe synthetic templates containing vRNA 3′-end sequences (5, 14, 30, 38). The properties of the cesium salt-purified polymerases vary depending on the salt but can require a synthetic 3′ end for the stimulation of both endonuclease and transcriptase activities (18, 19). Interestingly, both forms of the polymerase lose the ability to polyadenylate their transcript RNAs. The results from these two systems are largely in agreement, identifying bases within regions 1 to 4 and 9 to 12 of the 3′ end as critical for binding (12) and transcriptase activity.

The expression of active enzyme in mammalian cells through the use of recombinant vectors provides an alternative means to study the properties of the viral polymerase. In this case, the polymerase is not exposed to either viral RNA or the NP protein and therefore may provide an early and native form of the polymerase, guaranteed to be free of viral template RNA. Previously, we have shown that in order for this polymerase to cleave mRNA in vitro, a synthetic vRNA-like template must be added. This short RNA sequence must contain both the 5′ and 3′ ends of viral RNA (14). The results presented here describe data which examine an earlier step in this activation, the binding of the vRNA to the polymerase complex. This is a sequence-specific reaction which results in formation of an approximately 280-kDa complex, as determined by sucrose gradient centrifugation. This is approximately the size of a single 1:1:1 PB1-PB2-PA complex. The addition of antibodies to PB2 or PB1 confirmed their presence in this complex, as the complexes are supershifted in native gels (Fig. 3). Curiously, two distinct antibody preparations specific for the PA protein had no effect in this supershift assay. However, several antibodies specific for the other polymerase proteins also failed to supershift the complex. This finding indicates that even antibodies that work well in Western blot and immunoprecipitation assays do not necessarily work in the supershift assay. The limited number of PA antibodies that were available for testing prevents any firm conclusion on the presence of PA in the complex. However, it is clear that all three P proteins must be coexpressed in order to form a functional complex. Extracts prepared from cells that express the P proteins singly or in pairwise combinations are not active and cannot be reconstituted by simply adding the missing component (data not shown).

A similar complex can be detected in extracts from influenza virus-infected cells (Fig. 1B). Influenza virus-infected cells contain a pool of non-ribonucleoprotein (RNP) associated polymerase complexes (9), and presumably it is these complexes which are binding the added PHV template. However, we have been unable to demonstrate binding activity for viral cores (data not shown). We interpret this as indicating that all of the binding sites of the polymerase are already occupied by the RNP present in the viral cores. This also suggests that the association of the polymerase with the RNP is so tight that the off rate is too low to allow detection of free polymerase in this assay.

Modification interference assays were performed to identify the nucleotides within the vRNA and cRNA panhandles that are required for binding to the polymerase. Interestingly, all of the critical bases were located in the 5′ side of the panhandles (Fig. 6). For PHV RNA, a contiguous stretch of four bases from nucleotides 9 to 12 (ACAA) was identified as vital for polymerase binding. This represents the proposed bulge region of the panhandle structure and is opposite the sequence within the 3′ terminus previously proposed to be critical for transcription (38). The binding site of PHC RNA has some features in common with that of PHV but also has some significant differences. In experiments with PHC RNA, six nucleotide positions at the 5′ end were identified as important for binding to the viral polymerase. These include base 1 (A) and bases 9 and 10 (CA), which are presumed to be in a base-paired conformation (Fig. 6), as well as bases 3 to 5 (CAA), which represent a bulged region in the panhandle. Bulged regions of

RNA duplex structures have been shown to be important for the binding of regulatory proteins in many other systems (1, 7, 47, 49). Sequence-specific interactions with completely double stranded RNAs are rare, presumably because the rather uniform face of the minor groove and the depth of the major groove of A-form helices mask the distinguishing features of the sequence (41, 42). This most likely explains the results obtained with the hybrid panhandle RNAs which contain all of the sequences necessary for binding, but they are in a structural context which prevents recognition by the polymerase. Not surprisingly, these RNAs that bind so poorly also fail to stimulate the endonucleolytic activity of the polymerase (13a).

Although clearly important for polymerase activity (14), the panhandle structure is not absolutely required for polymerase binding. RNA probes containing only the 5′ or 3′ sequences of the vRNA panhandle also bound specifically to the polymerase. By far the strongest interaction was with the 5′-end probe, as demonstrated by the competition experiment (Fig. 8) and the data from the modification interference analysis (Fig. 5). Interestingly, when the polymerase bound to a single-stranded 5′ end, it was prevented from binding to another RNA if that RNA was in a panhandle conformation (Fig. 8, lanes 4 and 16) but could bind another single-stranded RNA containing only 3′-end sequences (Fig. 8, lane 11).

The observation that the polymerase binds tightly to the 5′ end of its template RNA raises the interesting question of whether the polymerase remains associated with the 5′ end after the initiation of transcription, and if so, what happens when the polymerase tries to transcribe the 5′ sequences that it is bound to. This situation is somewhat similar to that of Qβ minus-strand synthesis, in which case the polymerase transcribes from the 3′ end of the plus strand while still bound to the internal M site (46, 48). In this case, the binding to the internal site does not pose any steric constraint on the ability of the polymerase to transcribe through its binding site. However, this may not be so for the influenza virus polymerase. The juxtaposition of the 5′ end to the run of uridines (26, 27, 35) suggests a potential role in polyadenylation. Perhaps the stuttering addition of poly(A) occurs when the polymerase tries unsuccessfully to transcribe the 5′ end that it is bound to. Replication of a vRNA template would be carried out by a polymerase that was not bound to a 5′ end or perhaps even by one bound to the 5′ end of a different template.

The different binding specificities observed with the vRNA and cRNA panhandles suggest that different binding modes exist for the influenza virus polymerase, which may reflect different functional states of the polymerase (i.e., transcription versus replication). The additional interactions identified for the cRNA panhandle may provide a means for the polymerase to distinguish between its vRNA and cRNA templates, which might explain why mRNA synthesis in vivo occurs only on a vRNA template. The observations that the polymerase binds tightly to the 5′ end of the vRNA and that different bases are important for binding to the vRNA or cRNA sequences may therefore be important clues to unraveling the determinants for replication, mRNA transcription, and polyadenylation.

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