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Differential Activation of the Influenza Virus Polymerase via Template RNA Binding

CHRISTOPHER CIANCI, LAURENCE TILEY, AND MARK KRYSTAL*

Department of Virology, Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, Connecticut 06492

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Primary transcripts synthesized by the influenza virus polymerase contain the capped 5′ ends of eukaryotic mRNAs. These sequences are derived from host mRNA and scavenged by the viral polymerase as a prerequisite to transcription. The first step in this reaction is the specific binding of the viral polymerase to the cap structure of the host RNA. The role that template RNA plays in this RNA binding reaction was examined in quantitative capped mRNA binding and endonuclease assays. Capped RNA binding was shown to be a template-dependent property of the influenza virus polymerase. Addition of only the 5′ end of viral RNA stimulates capped mRNA binding by the viral polymerase, but endonuclease activity requires the addition of the 3′ end. The addition of template RNA corresponding to the positive-sense complementary RNA replicative intermediate was also able to stimulate capped mRNA binding but was not able to efficiently activate the viral endonuclease. Thus, regulation of endonuclease activity by the influenza virus polymerase can be dependent on template RNA binding.

Influenza virus is a segmented negative-strand RNA virus, with each segment packaged into a viral nucleocapsid. The viral polymerase, along with the ribonucleoprotein, is required for initiating infection. The polymerase complex comprises three protein subunits, PB1, PB2, and PA, and is responsible for both mRNA transcription and replication. By a strategy unique to the orthomyxo- and bunyaviruses, mRNA synthesis is primed with the capped 5′ termini of host cell mRNAs (5, 6, 17). The first step in this process is the binding of the polymerase complex to a host cell mRNA, presumably through specific binding to the cap structure (3, 4, 26). High-affinity binding to the mRNA cap requires an RNA tail of at least four nucleotides (8). This scavenging of host mRNA is mediated by the PB2 subunit (4, 7, 27). After binding, the polymerase endonucleolytically cleaves the mRNA to a short oligomer, typically 10 to 15 bases long. This short capped RNA serves as a primer for viral mRNA synthesis (6, 8, 21). At some later point, the polymerase switches from a transcription mode to a replication mode. For influenza virus RNA replication to occur, primer-independent transcription of viral RNA (vRNA) coupled with readthrough of the poly(A) site is required, resulting in complementary RNA (cRNA) template production. cRNA can then be directly transcribed into vRNA by a similar mechanism (16, 22). The switch from primer-dependent mRNA transcription to primer-independent replication is poorly understood, but an increase in the pool of nucleoprotein is believed to play a role (2).

We recently reported an in vitro system employing influenza virus polymerase expressed by recombinant vaccinia viruses (12, 25). In this system, formation of an active polymerase complex requires the coexpression of the three polymerase proteins. The parameters of ribonucleoprotein assembly and polymerase activation were measured through the use of virus-specific endonuclease and template RNA-binding assays. The polymerase can bind to either 5′- or 3′-terminal viral sequences separately, but binding to the 5′ sequences is more specific and of higher affinity. Endonuclease activity, however, is dependent upon the polymerase's binding of template RNA containing both the 5′ and 3′ ends of the vRNA. This suggests that activation of endonuclease activity occurs through a two-step reaction, first through specific binding to the 5′ end sequence and then through interaction with the 3′ end. Intriguingly, the binding of polymerase to template RNA containing both the 5′ and 3′ termini of the cRNA replicative intermediate could not significantly stimulate cleavage (12). Thus, template-specific differences in endonuclease activity exist. This distinction could be due to a direct effect of the viral sequences on the endonuclease reaction per se or to differences at an earlier step, such as the binding of the polymerase to capped mRNA.

In order to specifically examine the polymerase-mRNA binding reaction, it must be isolated from the other steps of transcription. In the absence of added ribonucleoside triphosphates (rNTPs) and Mg^{2+}, the capped RNA binding reaction is uncoupled from the subsequent endonucleolytic and elongation steps of transcription (16). Previously, capped mRNA binding requirements were examined by using influenza virus polymerase-mRNA complexes collected by centrifugation through glycerol cushions (8, 26). Cross-linking experiments have also been used, and these were able to identify PB2 as the protein responsible for cap binding (3, 4, 26, 27). Here, we describe an immunoprecipitation assay that uses an anti-PB2 antibody to monitor binding of the polymerase to a synthetic mRNA labeled only in the cap structure. UV cross-linking was also employed for direct examination of cap-protein interactions of recombinant vaccinia virus-expressed influenza virus polymerase in the presence of various template RNAs. The results indicate that in the absence of a specific template RNA, the recombinant polymerase is unable to bind capped RNAs. vRNA- or cRNA-like templates were able to stimulate cap binding activity to equivalent levels. Interestingly, 5′ vRNA sequences alone also stimulated cap binding equally well. However, as reported previously, only templates containing 5′ and 3′ vRNA sequences permitted the subsequent cleavage of

* Corresponding author. Mailing address: Department of Virology, Bristol-Myers Squibb Pharmaceutical Research Institute, 5 Research Parkway, Wallingford, CT 06492. Phone: (203) 284-7974. Fax: (203) 284-6088. Electronic mail address: MARK R. KRYSTAL@CCMAIL.BMS.COM.

the mRNA. These results demonstrate that cap binding is a template-dependent property and suggest that differences between vRNA and cRNA sequences may regulate the endonucleolytic activity of the polymerase.

MATERIALS AND METHODS

Viruses and cell culture. A/WSN/33 influenza virus was grown in MDBK cells as described before (24). Influenza virus A/PR/8/34 (H1N1) was grown for 48 h in the allantoic fluid of 10-day-old chicken eggs and harvested as described before (1). Virus was collected from pooled allantoic fluid and purified by sucrose gradient centrifugation. Viruses were pelleted and resuspended in NTE (100 mM NaCl, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA).

Individual recombinant vaccinia viruses expressing influenza virus polymerase proteins PB1, PB2, and PA (23) or T7 RNA polymerase (VTF7-3 [10]) were grown in HeLa S3 cells and titrated on Vero cells by standard procedures (9).

Polymerase stocks. Influenza virus polymerase cores were prepared from sucrose-purified A/PR/8/34 virus as described before (21). Briefly, virus was disrupted by incubation in a lysis buffer containing 1% Triton N-101 and 1% lysolecithin at 30°C for 15 min. The viral lysate was layered onto a discontinuous 30 to 50 to 70% glycerol gradient and centrifuged at 25,000 × *g* for 3 h. Polymerase-containing fractions were identified by in vitro transcription activity and pooled.

Infected-cell nuclear extracts were prepared by infecting HeLa cells with A/WSN/33 influenza virus at a multiplicity of infection of 10 to 20 or with recombinant vaccinia viruses at a multiplicity of infection of 3. Infected cells were harvested at 16 h postinfection, and nuclear extracts were prepared as described before (11, 18).

Plasmids and reagents. Plasmids for the vRNA model template, containing both 5′ and 3′ sequences of wild-type vRNA (pPH-V), the 5′ vRNA alone (pV5′), or the 3′ vRNA alone (pV3′) and the cRNA model template, containing both 5′ and 3′ ends of wild-type cRNA (pM-wt), have been described before (12, 19). PB2-specific rabbit antiserum was raised against the C-terminal sequence CSILTDSETATKRIR (25).

RNA transcriptions and mRNA capping. High-yield T7 RNA polymerase transcriptions of model templates were accomplished in a 200-μl cocktail with commercial transcription buffer (New England Biolabs, Beverly, Mass.): 10 mM dithiothreitol (DTT); 1 mM each ATP, CTP, GTP and UTP; 5 μg of DNA; 100 U of RNasin (Promega, Madison, Wis.); and 500 U of T7 RNA polymerase (New England Biolabs). Transcripts were purified by polyacrylamide gel electrophoresis (PAGE) with 8 M urea, passively eluted, and ethanol precipitated.

The synthetic capped mRNA was derived from the 67-nucleotide T7 transcript from *Sma*I-digested pGEM7Z(f+) (Promega). It was capped and methylated in vitro with 2.5 U of guanylyltransferase (Gibco/BRL, Gaithersburg, Md.) in a reaction mix containing 25 mM Tris-HCl (pH 7.5), 10 mM DTT, 5 mM $MgCl_2$, 100 μg of yeast tRNA per ml, 100 U of RNasin, 100 μM *s*-adenosylmethionine, 10 μM GTP, [α-^{32}P]GTP, and 3 μl of crude vaccinia virus capping enzyme per 25 μl of cocktail (14). The capping reactions were carried out at 37°C for 1 h. Capped mRNAs were then phenol extracted, filtered through G-25 Sephadex spin columns, and ethanol precipitated.

Capped RNA binding and endonuclease assays. For a typical 20-μl reaction mix, 10 to 30 nM (25×10^3 to 100×10^3 cpm) capped pGEM probe was used with 0.5 μg of influenza virus cores or 5.0 μg of nuclear extract. Reaction buffer also contained 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 100 mM NaCl, 2 mM DTT, 100 μg of yeast tRNA per ml, and 20 U of RNasin (Promega). After incubation for 15 min at 31°C, RNase T_1 was added to digest the RNA tail portion of the probe. Then 5 μl of anti-PB2 peptide serum was added, and incubation was continued on ice for 15 min. For immunoprecipitation analysis, 20 μl of protein G (Pharmacia) was added, and the sample was rocked for 20 min. After three washes with 0.05 M Tris (pH 7.8)–150 mM NaCl–0.02% Nonidet P-40 and one wash with phosphate-buffered saline (PBS), the protein G was resuspended in 500 μl of H_2O, combined with 4 ml of Opti-Fluor scintillation cocktail, and assayed in an LKB Rack-Beta counter. For UV cross-linking experiments, the samples were irradiated with UV light (15 min with a Sylvania germicidal 8-W lamp at a distance of 5 cm) prior to T_1 digestion. Labeled proteins were analyzed by autoradiography after electrophoresis on 10 to 20% gradient gels (Bio-Rad).

Endonuclease assays were performed with the same substrate and assay conditions as the capped RNA binding assays except for the addition of 5 mM $MgCl_2$. Samples were incubated at 31°C for 45 min, reactions were terminated with the addition of formamide stop solution, and the mixes were analyzed on 20% polyacrylamide–urea gels.

RESULTS

Cap binding assay with virus cores. A quantitative cap binding assay was used to examine the initial steps in primary transcription. An RNA transcript was capped with [α-^{32}P]GTP and vaccinia virus enzymes. This substrate was incubated with purified virus cores and subjected to UV cross-linking, and the RNA tail was then digested with RNase T_1. Analysis by SDS-PAGE (Fig. 1A, lane 1) displays a single protein band of approximately 90 kDa, migrating slightly more slowly than the viral PB2 protein (not shown). Data from two laboratories have previously shown that this cross-linked protein is PB2 (3, 4, 26, 27). This was confirmed by immunoprecipitation experiments with peptide antiserum to the PB2 protein (see Fig. 2). The slightly slower migration of this band compared with authentic PB2 protein is presumably due to the addition of the cross-linked ribonucleotides, which should add ~3 kDa to the molecular mass of the protein. The specificity of this assay is illustrated in Fig. 1A, as addition of a nonradioactive competitor cap-containing mRNA (rabbit globin mRNA) quantitatively competes with the radioactive substrate, while addition of uncapped 5S rRNA causes no inhibition (Fig. 1A). Quantitation of the labeled PB2 protein (Fig. 1B) indicates that maximal inhibition occurs at a globin mRNA concentration of 0.12 μM, which is a fivefold excess over the substrate. No

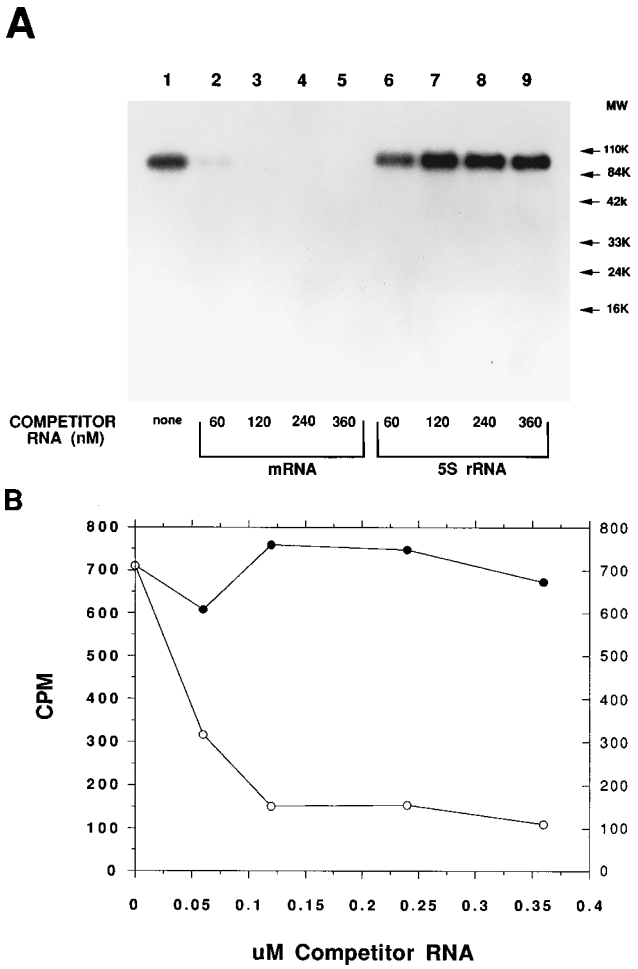

FIG. 1. (A) Specificity of cap binding reaction. ^{32}P-capped RNA substrate (0.025 μM) was UV cross-linked to influenza virus cores in the presence of increasing concentrations of RNAs. Samples were analyzed by 10 to 20% PAGE. The amount and type of RNA (either globin mRNA or 5S rRNA) are indicated under the lanes. Lane 1, no competitor RNA added. (B) Quantitation of cross-linked capped RNA-polymerase complex. The amount of labeled protein in panel A was quantitated directly through the use of a Betagen 603 Betascope. Data are plotted as ^{32}P counts in the protein band versus amount of competitor RNA added. ○, globin mRNA as competitor; ●, 5S rRNA as unlabeled competitor.

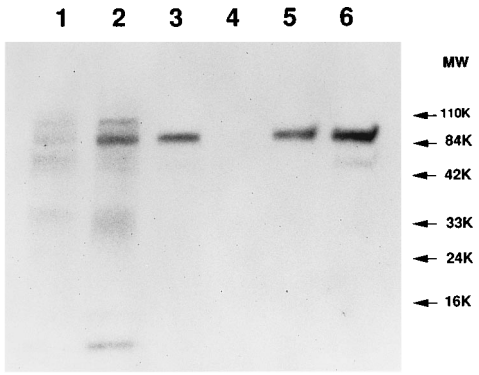

FIG. 2. Cap binding to recombinant vaccinia virus-expressed influenza virus polymerase. Either Vac-3P extract or virus cores were incubated with labeled mRNA substrate and subjected to UV cross-linking and RNase treatment prior to analysis by 10 to 20% PAGE. Lane 1, 5 μg of Vac-3P extract; lane 2, 5 μg of Vac-3P extract with 5 pmol of pPH-V RNA added; lane 3, 0.5 μg of virus cores; lanes 4, 5, and 6, immunoprecipitations of lanes 1, 2, and 3, respectively, with anti-PB2 antipeptide antiserum. The migration positions of prestained molecular weight markers are indicated (in thousands).

competition for cap binding is observed at any of the 5S rRNA concentrations tested. It should be noted that the binding cocktail contains 100 μg of yeast tRNA per ml, which also does not inhibit cap binding.

Cap binding extracts from recombinant vaccinia virus-infected cells. Recombinant polymerase expressed via vaccinia virus vectors provides a template-free source of enzyme to which synthetic RNAs can be bound. Therefore, nuclear extracts prepared from cells coinfected with three separate vaccinia viruses, each expressing one of the three influenza virus polymerase proteins (designated Vac-3P extract) were tested for capped RNA binding activity. Figure 2 shows the SDS-PAGE analysis of these extracts after UV cross-linking to the end-labeled capped mRNA probe. Lane 1 shows the protein labeling pattern of a Vac-3P extract in the absence of added RNA template. Several diffuse bands are visible, presumably corresponding to cellular and vaccinia virus cap and RNA binding proteins. The addition of a model vRNA template prior to the radiolabeled capped RNA (Fig. 2, lane 2) results in the appearance of a band comigrating with that seen when purified influenza virus cores are used (Fig. 2, lane 3). Not surprisingly, additional bands of host cell and/or vaccinia virus origin are also detected. However, upon immunoprecipitation with antibody specific for PB2, only the band corresponding to the PB2 protein is precipitated (Fig. 2, lane 5). Virtually no labeled protein is specifically precipitated in the Vac-3P sample in the absence of added RNA template (Fig. 2, lane 4). Previously, we had shown that the endonuclease activity encoded by the recombinant polymerase is greatly stimulated by the addition of this model template RNA. The present data indicate that the stimulation seen probably occurs at the earlier step of cap binding rather than the subsequent endonuclease step.

Cap binding activity was also examined in extracts of cells infected with incomplete mixtures of recombinant polymerase proteins. Extracts containing either single polymerase proteins or two of the three polymerase proteins did not exhibit cap binding activity, and attempts to reconstitute activity through simple mixtures of extracts also proved unsuccessful (data not shown). These results mirror the lack of success in trying to reconstitute activity through simple mixtures in template binding and endonuclease assays (12, 25).

Effect of template RNA species on cap binding and endonuclease activities. Previously, it was shown that even though isolated 5′ or 3′ ends of vRNA can bind to the polymerase complex, neither end alone can efficiently stimulate endonuclease activity (12, 25). Therefore, it was examined whether the RNAs containing isolated end sequences can activate cap binding when bound to the polymerase complex. We also examined the stimulatory ability of an RNA corresponding to the replicative intermediate (cRNA) in the cap binding assay, as this cRNA model template specifically binds to the polymerase complex. Saturating amounts of 5′-cap-labeled mRNA substrate were incubated with recombinant polymerase containing one of the model templates and analyzed for cap binding activity by immunoprecipitation and scintillation counting. These results are shown in Fig. 3. In the absence of a template, very little cap binding activity was detected. Added pPH-V RNA and the mixture of 5′ and 3′ end RNAs were excellent stimulators of cap binding, while the 3′-end RNA did not efficiently stimulate the cap binding activity. Surprisingly, addition of either the 5′ vRNA template or a cRNA model template (containing both 5′ and 3′ end sequences) was able to stimulate cap binding to maximum levels.

In an effort to directly compare the cap binding and endonuclease results, duplicate reaction samples were also analyzed in the endonuclease assay. Figure 4A illustrates this experiment, with the results quantitated in Fig. 4B. In Fig. 4A, lane 1, no RNA was added to the recombinant polymerase, and, as expected, there was almost no cleavage activity. Figure 4A, lane 2, contains saturating amounts of pPH-V RNA, which can stimulate cleavage activity. When the 5′ and 3′ ends are provided by two separate RNAs added in combination, even higher levels of activity are seen (Fig. 4A, lane 5). Considerably less activity is observed when RNAs containing only 5′ or 3′ ends (Fig. 4A, lanes 3 and 4, respectively) are added individually at saturating levels. The stimulation of endonuclease by the isolated 5′ end is always greater than the stimulation by the isolated 3′ end, but this represents only 15% of the signal from the combined RNAs (Fig. 4A, lanes 2 and 5). Addition of a single model template RNA containing the 5′ and 3′ ends of

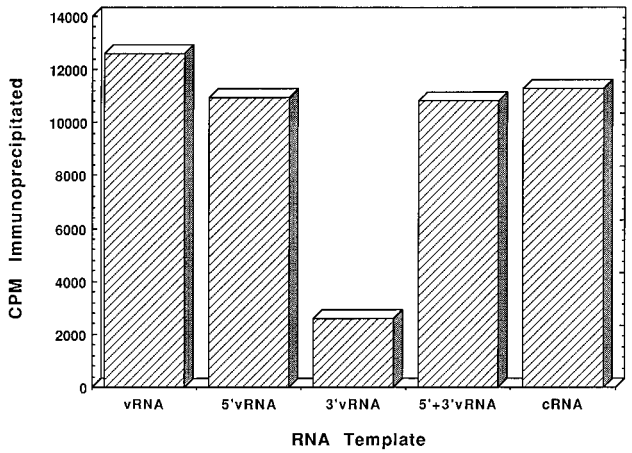

FIG. 3. Relationship of polymerase-capped RNA binding to added template RNA. Ten picomoles of RNA template was incubated with polymerase and a constant and saturating amount of labeled mRNA substrate. Half of the reaction mix was precipitated with antibody to PB2 and quantitated by scintillation counting. Data are plotted as total counts precipitated in the presence of saturating amounts of the various viral RNA templates. 5′vRNA contains the 5′ end of vRNA (pV5′); 3′vRNA contains the 3′ end of vRNA (pV3′); 5′+3′vRNA contains an equal mixture of the 5′vRNA and the 3′vRNA; cRNA is RNA containing the 5′ and 3′ ends of cRNA (pM-wt).

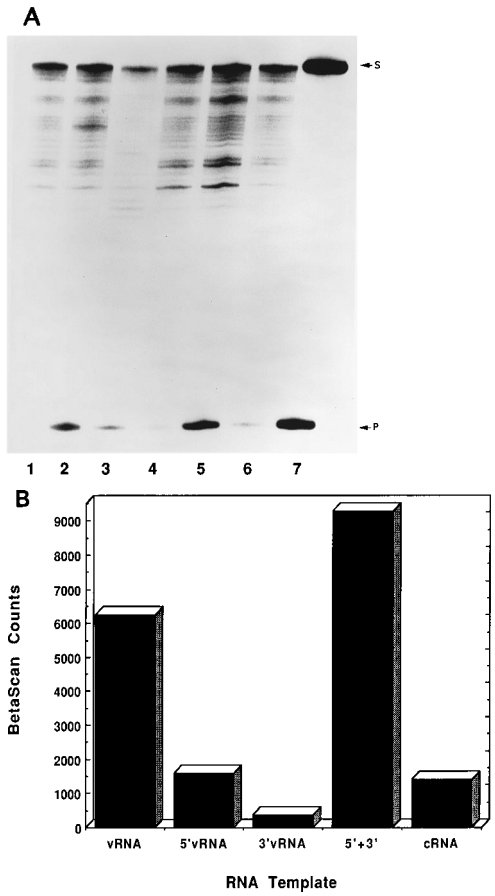

FIG. 4. (A) Effect of various RNA templates on the endonuclease. The remaining half of the reaction mixture described in the legend to Fig. 3 was quantitated for endonuclease activity. Mg^{2+} was added to the solution to stimulate the endonuclease, and cleavage products were analyzed by 7 M urea–20% PAGE. Lane 1, no template added; lane 2, pPH-V RNA added; lane 3, pV5′ RNA (5′ end of vRNA) added; lane 4, pV3′ RNA (3′ end of vRNA) added; lane 5, pV5′ and pV3′ RNAs added; lane 6, pM-wt RNA (cRNA-like template) added; lane 7, cleavage reaction done with virus cores. S, mRNA substrate; P, cleaved primer product. (B) Quantitation of cleavage activity through Betascope analysis of the data in panel A.

cRNA did not efficiently stimulate endonuclease activity (Fig. 4A, lane 6, and Fig. 4B). Thus, although isolated 5′-end RNA and cRNA were both able to activate cap binding, these RNAs have only minor stimulatory effects on endonuclease activity (Fig. 4B).

DISCUSSION

Expressing a recombinant polymerase by using vaccinia virus vectors containing the PB2, PB1, and PA genes provides an opportunity to investigate the steps involved in the assembly of the active ribonucleoprotein complex. This enzyme has never encountered any kind of viral RNA template. It was previously shown that isolated 5′ or 3′ vRNA end sequences can bind to this polymerase (25). Binding of the 5′ end was specific and of high affinity. However, binding of the 3′ end was of lower affinity and much less specific, as it could even be inhibited with excess tRNA (25). The endonucleolytic activity, however, required the addition of template RNAs containing both the 5′ and 3′ viral ends. The template dependence of the endonuclease could result from a direct stimulation of the activity itself or from the stimulation of some earlier event, such as complex assembly or cap binding. Density gradient analysis excluded the possible role of the template in the assembly of the three proteins (25).

In this study, we have looked at the effect of template sequences on the cap binding activity of the polymerase. A cap binding assay with radiolabeled capped RNA and virus cores identified a single protein band as the major viral cap binding protein. This protein was selectively precipitated by antiserum to the PB2 protein. This is in agreement with earlier work, which identified the viral PB2 protein as the major cap binding protein of the polymerase complex (3, 4, 26, 27). Extracts containing recombinant polymerase were also examined in the cap binding reactions. Specific binding to the PB2 protein was only detected after addition of the pPH-V model template RNA. This activity was only present in extracts from cells coexpressing the three polymerase proteins simultaneously and could not be reconstituted from extracts containing the individual components. This is consistent with earlier results obtained when the endonuclease reaction was used to measure polymerase activity (12). Thus, these data suggest that the preformed polymerase remains inactive until bound to its viral RNA, making the vRNA an essential cofactor for enzyme activity. How this activation occurs is not known, but one possibility is that the template RNA acts as an allosteric modulator and causes a conformational change from an inactive to an active form of the polymerase. Template sequences may also play a more direct role in cap binding and cleavage. It should be pointed out that nucleoprotein is not present in these extracts. Previously, it has been shown that nucleoprotein is not required for early steps of transcription (12, 13), although it may be required for processivity and does play a role in replication (2).

Cap binding is certainly a prerequisite for endonuclease activity. However, stimulation of cap binding does not necessarily equate with stimulation of the endonuclease. Of the RNAs tested, only those containing both the vRNA 5′ and 3′ ends stimulated both activities. Template RNA containing only the 3′ end did not efficiently promote mRNA binding by the polymerase. This may be due to the low binding affinity of the polymerase for isolated 3′ end (25). However, template RNA containing only the 5′ end of vRNA was able to stimulate cap binding to wild-type levels (wild type being defined as levels obtained with RNA containing both the 5′ and 3′ ends of vRNA), but it has a minimal effect on endonuclease activity. These data suggest a model for ribonucleoprotein assembly and the initiation of transcription, as shown in Fig. 5. The three polymerase proteins are expressed and can interact to form an inactive trimer complex (25). The polymerase complex then specifically binds to the 5′ end of vRNA, which activates cap binding. Cap binding may occur prior or subsequent to polymerase binding of the 3′ end of the vRNA. Interaction of the polymerase with the 3′-end sequence is required for stimulation of the endonuclease, which forms the cleaved primer that initiates transcription.

The properties of the viral polymerase when bound to a model cRNA-like RNA (with 5′ and 3′ end sequences) mirror those of the isolated 5′ vRNA-bound enzyme. Cap binding is stimulated to pPH-V RNA-like levels, although endonuclease is not efficiently turned on. This model template is representative of a replicative intermediate. These RNAs have little or no coding potential, and capped viral mRNAs transcribed off a cRNA template have never been observed during virus infection. Presumably, the virus has a mechanism to avoid the synthesis of such nonsense mRNAs. The ability to differentiate between vRNA and cRNA templates reported here may provide the rationale for such a mechanism. Since cRNA binding

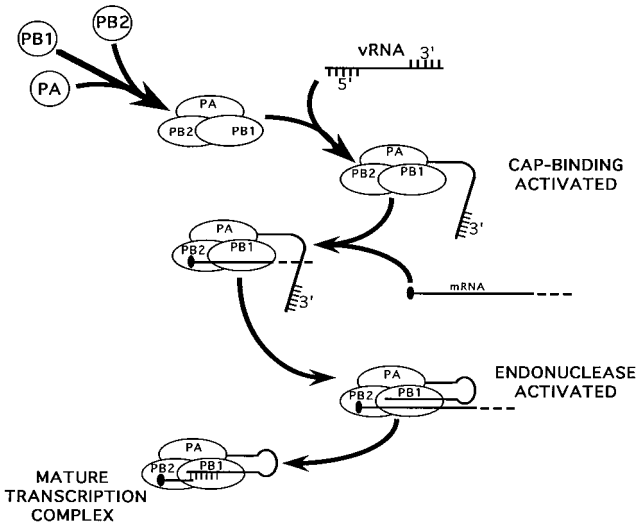

FIG. 5. Model for assembly of the mature transcription complex. Initially, polymerase proteins are expressed and interact to form an inactive trimer complex. Upon binding to the 5′ end of the vRNA, cap binding is activated, but the endonuclease is not functional until the 3′ end of vRNA has interacted with the polymerase, forming the mature transcription complex.

does not stimulate endonuclease activity, aberrant mRNA-like molecules cannot be transcribed. Why then would binding to cRNA stimulate cap binding activity? One possibility is that through binding to the cap structure, the activity of the polymerase may be greatly stimulated during replication. This could occur through allosteric stimulation of the polymerase, independent of cap priming activity. Evidence for allosteric stimulation of transcription comes from studies which showed that elongation of transcripts in vitro with ApG dinucleotide primer can be stimulated by adding excess $m^7GpppGm$ cap structures (15, 20). In that vein, we have also found that much lower concentrations of small capped oligonucleotides, too short to prime transcription, can stimulate ApG-primed transcription in vitro in a similar fashion (8a).

We have shown that binding of polymerase to various types of virus-like RNAs can have different effects on two activities of the viral polymerase, cap binding and endonuclease. This may reflect different binding modes, which in turn suggests that regulation of polymerase function is at least in part dictated by the form of RNA bound to it.

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