

Recombinant Influenza Virus Polymerase: Requirement of both 5' and 3' Viral Ends for Endonuclease Activity

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Influenza virus polymerase complexes that were expressed in the absence of genomic viral RNA and nucleoprotein were examined for endonuclease activity and transcriptase ability in vitro. Nuclear extracts of cells that express influenza virus polymerase through recombinant vaccinia virus infection did not display specific endonuclease activity in vitro. This polymerase presumably represents an early form of enzyme present in infected cells prior to ribonucleoprotein assembly. Upon addition of a virus-like model RNA template, containing the partially complementary sequence found at the ends of viral RNA, endonuclease activity is stimulated in a concentration-dependent and sequence-specific manner. Once stimulated, the polymerase is able to elongate from the added viral template. Thus, addition of viral template is required for polymerase activity, while the presence of nucleoprotein is not required for limited transcription. Also, full activation of this recombinant viral polymerase is dependent on the presence of both the 3' and 5' ends of the viral genome, as model RNA containing either end alone could not effectively trigger the endonuclease.

The RNA-dependent RNA polymerase of influenza virus is made up of a complex of three viral proteins (PB1, PB2, and PA), associated with the eight nucleoprotein (NP) encapsidated viral gene segments. The initial function of the polymerase complex is the transcription of virus-specific mRNAs. This is accomplished via three distinct activities. First, the PB2 subunit has been shown to bind specifically to the cap structure of host-encoded cellular mRNAs (6, 7, 46, 47). These mRNAs are then cleaved 9 to 15 bases downstream of the cap structure (8–10, 24, 35–37). These short capped oligonucleotide are used as primers for the transcription of viral mRNAs, so that these messages contain heterologous host-derived sequences at their 5' ends (3, 11, 13, 14, 25, 41). At some point during the replication cycle, the polymerase switches to act as a replicase, whereby viral RNA (vRNA) is faithfully synthesized to a full-length positive-strand cRNA in a primer-independent manner (17, 49). The cRNA is then used as a template for the synthesis of more vRNA (23, 26, 30, 45). The mechanism of the switch from transcription to replication activities and the detailed properties of each form of the enzyme are not well understood, although the NP has been implicated as a controlling element (4, 40). In addition, it has been shown that the only viral proteins necessary for transcription and replication are the three polymerase proteins and the NP (21).

Recent molecular analyses of the polymerase-ribonucleoprotein (RNP) purified from virus or infected cells have begun to elucidate the promoter requirements for transcription. Each of the eight viral genome segments contains 12 and 13 conserved nucleotides (nt) at their 3' and 5' ends, respectively. These conserved sequences, along with an extra two to three segment-specific bases at the 5' and 3' ends of vRNA (13, 37, 41, 43), display inverted partial complementarity and have been shown to form panhandle structures in viral RNP and in infected cells (18, 20). This partially double-stranded terminal sequence was originally thought to be important in polymerase function and/or other facets of viral replication. However, it

was recently shown by two independent in vitro reconstitution systems that only the viral 3' end is required for transcriptase activity (33, 38, 39, 48). In one system, polymerase-NP mixtures devoid of RNA, after purification on CsCl-glycerol gradients, were used with added template vRNA (33, 48). It was observed that mutations at nt 2 and 11 had the greatest effect on transcriptase activity, with a number of other bases also contributing to transcriptase specificity (34). Other, more limited studies using viral cores treated with micrococcal nuclease gave similar results, identifying nt 1 to 4 and 9 to 11 as critical for transcription (39). It should be noted that both systems used polymerase isolated from intact virions, which are already packaged into RNP.

In this study, functional recombinant viral polymerase has been obtained through the use of vaccinia virus vectors expressing the three polymerase proteins (42). When coexpressed with the viral NP, these proteins can replicate and transcribe synthetic RNP in vivo (21, 22). In the study presented here, this form of recombinant polymerase was isolated and tested for its endonuclease and transcriptase properties in vitro. It was found that endonuclease stimulation required the addition of template RNA. Also, in contrast to the previously published reports of studies using polymerase purified from virus, stimulation of this recombinant polymerase is dependent on the addition of virus-like RNA containing both the 5' and 3' terminal sequences from viral gene segments. Endonuclease activity was also dependent on the coexpression of the three polymerase proteins and could not be reconstituted from combinations of the separately expressed proteins. In addition, neither endonuclease nor limited transcriptase activity was dependent on the presence of viral NP.

MATERIALS AND METHODS

Viruses and cells. Influenza virus A/WSN/33 was grown and propagated in MDBK cells as described previously (44). Individual recombinant vaccinia virus vectors expressing the influenza virus PB2, PB1, or PA protein (42) were grown in HeLa cells and titrated in Vero cells.

Construction of plasmid DNAs. Plasmid pPH-V, when digested with *Mbo*II and transcribed in vitro by T7 polymerase,

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encodes a 53-base RNA transcript containing the termini of gene segment 8 of influenza virus A/WSN/33 with a short spacer sequence in the middle. This plasmid is similar to plasmid pV-wt described earlier (33) except that a *Bgl*II site was engineered within the short spacer sequence (see Fig. 5A). Plasmid pV-3' was constructed by isolating the *Bgl*II-*Pst*I fragment (the *Pst*I site is in the polylinker region) of pPH-V and ligating the fragment into the *Bam*HI-*Pst*I window of plasmid pTZ18U (Pharmacia). Plasmid pV-5' was constructed by digesting pPH-V with *Bgl*II and *Bam*HI (the *Bam*HI site is in the polylinker region of the plasmid) and religating, thus removing the 3' viral sequences.

Preparation of recombinant polymerase. HeLa cells were triply infected with vaccinia virus vectors individually encoding the three influenza virus polymerase proteins or with WSN virus at a multiplicity of infection of 3 for each virus. After 16 h for influenza virus or 20 to 24 h for vaccinia virus infection, cells were washed with phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+} and then trypsinized. Cells were washed two to three times with PBS, and extracts were made via an ammonium sulfate precipitation protocol (15, 32). In some cases, the nuclear extracts were run on a CsCl-glycerol gradient (27) and the polymerase-containing fractions were identified through Western blot (immunoblot) analysis. Viral polymerase devoid of vRNA was purified on CsCl-glycerol gradients as described previously (18, 33).

Preparation of RNA substrates by in vitro transcription. RNA transcripts which were used as cap donor were prepared from *Sma*I-digested pGEM-7Zf(+) DNA (Promega Corp., Madison, Wis.) by in vitro runoff transcription (12). This 67-nt transcript was converted to a ^{32}P -radiolabeled cap-1 ($\text{m}^7\text{G}^{32}\text{pppGmAAUACUCAAGCUAUN}_{42}$) structure by concurrent capping and methylation reactions (25 μl) containing approximately 5 pmol of RNA, 2.5 U of mRNA guanylyltransferase-(guanine-7)-methyltransferase-5'-triphosphatase enzyme complex (28, 29, 31) from vaccinia virus (GIBCO/BRL, Gaithersburg, Md.), and 3 μl of the carboxymethyl-Sephadex fraction of vaccinia virus 2'-*O*-methyltransferase (1, 2) in 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5)-2.5 mM MgCl_2 -8 mM dithiothreitol-10 μM GTP-0.1 mM *S*-adenosylmethionine-5 μg of RNase-free carrier *Escherichia coli* tRNA-20 U of recombinant RNAsin (Promega)-50 μCi of [α - ^{32}P]GTP (200 Ci/mmol). After 1 h of incubation at 37°C, the RNAs were phenol-chloroform extracted, spin chromatographed (Sephadex G-25 column), and ethanol precipitated. Capped transcripts were further purified by electrophoresis in 15% polyacrylamide gels containing 7 M urea. The capped 67-nt transcript was eluted by soaking gel slices overnight at 37°C in 0.75 M ammonium acetate containing 0.1 mM EDTA, 0.1% sodium dodecyl sulfate, (SDS), and 10 μg of *E. coli* tRNA (RNase free) per ml and then recovered by ethanol precipitation (38). Transcripts to be used as vRNA templates were prepared from *Mbo*II-digested plasmid pPH-V or pV-3' or *Eco*RI-digested pV-5', using T7 RNA polymerase as specified by the manufacturer (Promega). Transcripts were run on gels and identified by UV shadowing and eluted overnight at 4°C in water. The eluted RNA was extracted with phenol-chloroform, ethanol precipitated, and quantitated spectrophotometrically.

Endonuclease assay and transcription. The ^{32}P -capped RNA (67 nt) was incubated with the nuclear extract of the three polymerase proteins (designated 3P Vac extract) for 1 h at 31°C in a reaction volume of 10 μl . The reaction mixture consisted of 50 mM HEPES (7.5), 0.25% (vol/vol) Triton N101, 100 mM KCl, 5 mM MgCl_2 , 1 mM dithiothreitol, 5 μg of *E. coli* tRNA, and 20 U of recombinant RNAsin (Promega) (5,

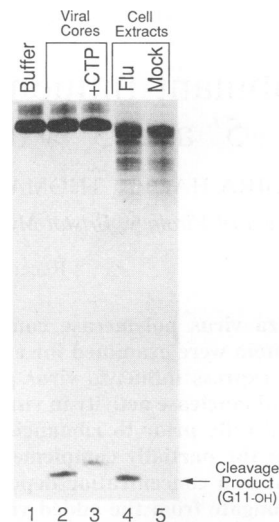


FIG. 1. Endonuclease activity in infected cell extracts. The ^{32}P -capped pGEM substrate was added to various extracts and analyzed on a 20% polyacrylamide-7 M urea gel. Lanes 1, buffer control; 2, purified viral cores; 3, purified viral cores with added CTP; 4, influenza virus (Flu)-infected HeLa cell extract; 5, mock-infected HeLa cell nuclear extract. The specific cleavage product (G11-OH) is indicated.

36). Reactions were terminated by the addition of 10 μl of 80% formamide-10% glycerol-2.5 mM EDTA-0.01% xylene cyanol. Reaction products were heated for 3 min at 95°C and separated on a denaturing 20% polyacrylamide-7 M urea gel. For reactions that elongate the primer fragments, one or more of the ribonucleoside triphosphates (rNTPs) were included during the cleavage reaction. The extract for elongation reactions was first sedimented on a CsCl-glycerol gradient as described previously (27), and the polymerase-containing fractions were identified by Western analysis. The 100- μl transcription reactions were terminated by addition of SDS to 0.2%, extraction twice with phenol-chloroform and once with chloroform, and ethanol precipitation and were loaded onto a 20% polyacrylamide-7 M urea gel. Cleavage activity was quantitated in a Betascope 603 blot analyzer (Betagen, Mountain View, Calif.).

RESULTS

Endonuclease cleavage in nuclear extracts of infected cells.

An mRNA-like substrate, uniquely labeled at the 5' cap structure, was produced in order to carry out analyses of the endonuclease activity of the influenza virus polymerase (12). The substrate used is derived from an SP6 runoff transcription reaction of *Sma*I-digested pGEM-7zf(+) plasmid DNA (Promega). The 67-base transcript is 5' capped and methylated by using [α - ^{32}P]GTP and vaccinia virus enzymes as described. Unlike other mRNA substrates which are cleaved heterogeneously by the influenza virus polymerase, this substrate is specifically cleaved by viral cores to yield a single capped endonuclease product of 11 bases (12) ($\text{m}^7\text{G}^*\text{pppGmAAUACUCAAG-OH}$; designated G11-OH; Fig. 1, lane 2). Assignment of this cleavage product was made from comparison of its migration with that of specific cleavage products of the substrate with known nucleases. Also, partial cleavage of the substrate is observed, presumably because it is added in molar excess of polymerase, which may continue to bind to the cleaved primer and therefore may not cycle. Upon addition of

single nucleotides, only CTP caused a mobility shift of 1 nt longer, suggesting that this substrate was priming opposite the third G from the 3' end of the viral genome (Fig. 1, lane 3) (12).

As a first approach toward analyzing a recombinant polymerase, nuclear extracts were prepared from influenza virus-infected cells and examined for endonuclease activity. HeLa cells were infected or mock infected with influenza virus A/WSN/33 at a multiplicity of infection of 3, extracts were prepared, and reactions were carried out with the synthetic capped 32 P-labeled substrate derived from *Sma*I-digested pGEM. A specific band corresponding to the 11-base cleavage product is observed with use of purified viral cores and the influenza virus-infected extract but not in the mock-infected cell extract (Fig. 1). The nuclear extracts contain some general nonspecific nuclease activity, as evidenced by the trailing bands below the 67-base starting material band. These bands are highly variable between extracts but are usually distinct from the specific 11-base cleavage product.

Analysis of recombinant polymerase. Previously, it was found that cells coinfecting with recombinant vectors expressing PB2, PB1, PA, and NP could form an active polymerase, since these cells could transcribe and replicate transfected synthetic RNPs in vivo (21, 22). Therefore, HeLa cells were coinfecting with the individual vaccinia virus vectors, and nuclear extracts were prepared (15, 32). In some cases, extracts were further purified on a CsCl-glycerol gradient (27) and fractions containing the polymerase were collected. Extracts of HeLa cells quadruply infected with the recombinant vaccinia virus vectors encoding PB2, PB1, PA, and NP, cells triply infected with the vectors encoding the three polymerase proteins, or cells infected with wild-type vaccinia virus were used as the sources of enzyme in the presence of the capped substrate. None of these extracts exhibited significant specific cleavage of the synthetic substrate (Fig. 2A, lanes 2, 4, and 6). As expression of the polymerase proteins is driven by vaccinia virus infection, the polymerase complexes present in these extracts are not complexed with vRNA. Therefore, in an attempt to rescue endonuclease activity, a synthetic virus-like vRNA (PH-V) was added to the extracts prior to reaction with the capped substrate. This 53-base RNA contains the 5' and 3' termini of gene segment 8 of influenza virus, along with internal spacer sequences (see Fig. 5A) (33). Addition of this RNA was able to rescue specific endonuclease activity in extracts containing either the three polymerase proteins or the three polymerase proteins along with the NP, but not in wild-type vaccinia virus-infected cell extracts (Fig. 2A, lanes 3, 5, and 7). The activation of endonuclease activity is linear with increasing amounts of PH-V RNA until it reaches saturation (Fig. 2B). Saturation values depended on the amount of polymerase present in the extracts, but typically approximately 1 pmol of PH-V RNA saturated the polymerase protein present in the reaction.

To examine whether this recombinant polymerase is capable of transcription in vitro, ribonucleotides were added to the incubation mixture. Shown in Fig. 3 are the results obtained with use of extracts from cells which expressed only the three polymerase proteins. Addition of single nucleotides indicated that only CTP was able to elongate the cleaved primer by 1 nt (Fig. 3, lane 8). The same product was observed when viral cores were used as the source of enzyme (Fig. 3, lane 3). The addition of ATP, CTP, and GTP to the reaction using vaccinia virus vector-expressed polymerase complex and the added vRNA elongated the G11-OH nucleotide cleavage product by 11 bases (Fig. 3, lane 7). This is the expected product resulting from termination at the position where the initial uridine

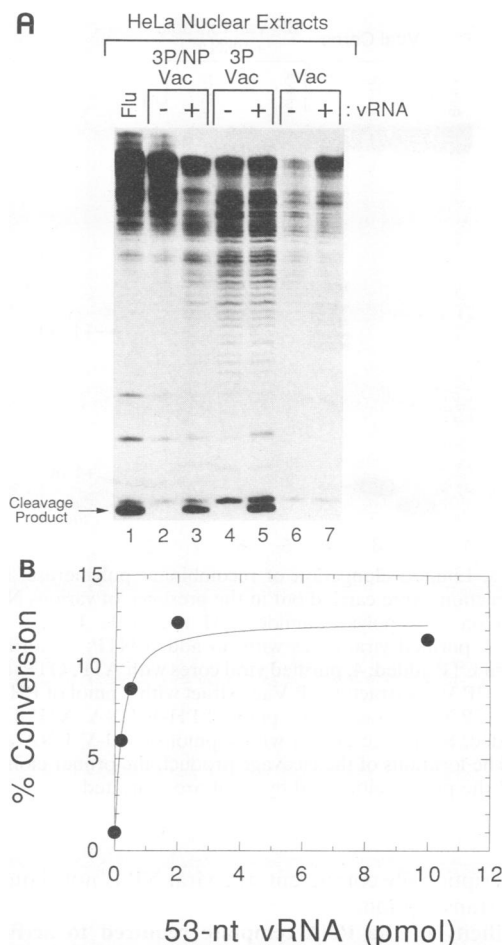


FIG. 2. Requirement of virus-like vRNA for activity. (A) Standard endonuclease reaction using the 32 P-capped pGEM substrate incubated at 31°C for 1 h. Reactions were analyzed on a 20% polyacrylamide-7 M urea gel. Lanes: 1, nuclear extract of influenza virus (Flu)-infected cells incubated with substrate; 2, nuclear extract of HeLa cells expressing PB2, PB1, PA, and NP proteins (3P/NP Vac) without added PH-V RNA; 3, 3P/NP Vac nuclear extract with added PH-V RNA; 4, 3P Vac extract; 5, 3P Vac extract with added PH-V RNA; 6, nuclear extract of HeLa cells infected with wild-type vaccinia virus; 7, nuclear extract of wild-type vaccinia virus-infected cells with added PH-V RNA. The specific polymerase-induced cleavage product is indicated. (B) Analysis of conversion of substrate to cleaved product versus amount of PH-V RNA added. Raw data were quantitated in a Betascope 603 (Betagen) and plotted. The curve is fit to a one-site binding equation.

incorporation is encoded by the PH-V RNA template. In addition, species of RNA elongated by 1 or 2 nt, as well as cleaved substrate which is not elongated, are also present. These are presumably due to inefficient or abortive transcription, as has been described previously (39). When three nucleotides are added to the reaction using viral cores, a series of products corresponding to elongation of the G11-OH primer by 10, 11, 12, 16, and 17 nt is seen. These are generated due to the heterogeneity of the first virally encoded uridine in the eight vRNA gene segments (Fig. 3, lane 4). Thus, the recombinant polymerase is activated by the addition of virus-like RNA and has cleavage and transcription activities similar to those of native enzyme. In addition, since the 3P Vac extract

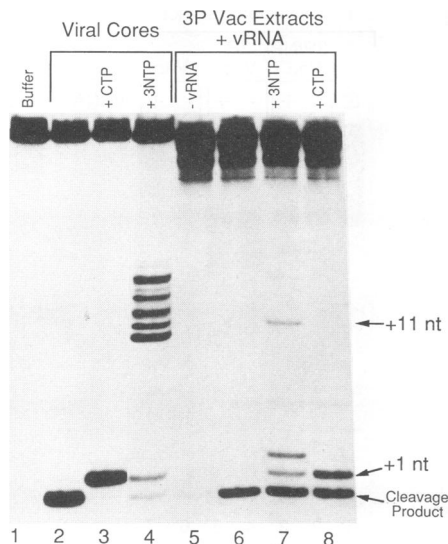


FIG. 3. Limited elongation of recombinant polymerase. Endonuclease reactions were carried out in the presence of various NTPs and analyzed on 20% polyacrylamide-7 M urea gels. Lanes: 1, buffer control; 2, purified viral cores with no added NTP; 3, purified viral cores with CTP added; 4, purified viral cores with ATP, GTP, and CTP added; 5, 3P Vac extract; 6, 3P Vac extract with 1 pmol of PH-V RNA added; 7, 3P Vac extract with 1 pmol of PH-V RNA, ATP, GTP, and CTP added; 8, 3P Vac extract with 1 pmol of PH-V RNA and CTP added. The locations of the cleavage product, the primer elongated by 1 nt and the primer elongated by 11 nt are indicated.

is transcriptionally competent, the viral NP is not required for limited transcription.

Specificity of the RNA template required to activate the endonuclease. The PH-V RNA template contains the correct termini of influenza virus RNAs. To examine whether this activation was specific for viral ends, additional RNAs were tested for the ability to activate endonuclease activity. In Fig. 4, a 56-base RNA generated through T7 transcription of *Sma*I-digested pGEM7Zf(+) was titrated into the reaction mix and examined for stimulation of endonuclease activity; 1 to 20 pmol of this nonspecific RNA was added (Fig. 4, lanes 7 to 10), compared with 0.1 to 2 pmol of the PH-V RNA (lanes 3 to 6). With this extract, densitometric analysis of the cleavage product indicated that 1 pmol of PH-V was sufficient for saturating endonuclease activity. However, no significant cleavage to the 11-nt primer product is observed with the T7-transcribed pGEM RNA, even with 20 pmol of RNA added (lane 10). A

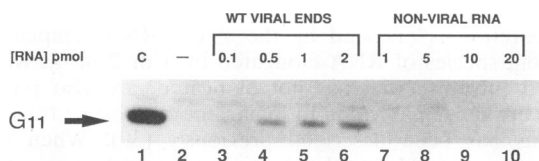


FIG. 4. Sequence specificity for virus-like RNA. Endonuclease assay using nuclear extracts of HeLa cells infected with vaccinia virus vectors encoding each of the three polymerase proteins. Lanes: 1, control reaction (C) using purified viral cores as the source of enzyme; 2, control reaction without nuclear extract; 3 to 6, addition of 0.1, 0.5, 1, and 2 pmol, respectively, wild-type of (WT) PH-V virus-like RNA; 7 to 10, addition of 1, 5, 10, and 20 pmol, respectively, of nonviral RNA derived from pGEM DNA under the T7 promoter.

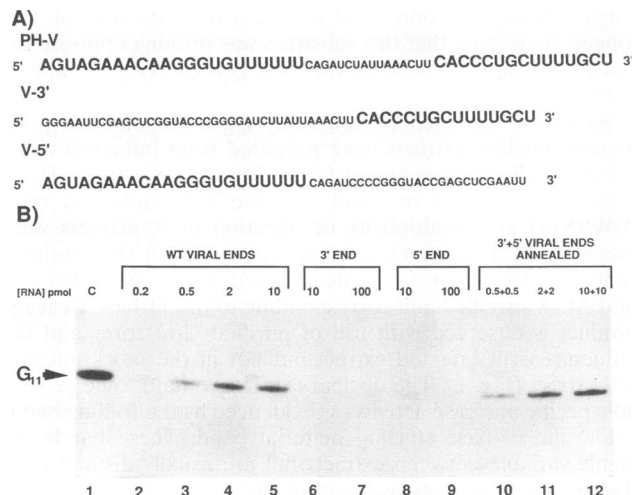


FIG. 5. Requirement for both 3' and 5' viral ends. (A) Sequence of synthetic RNA used. The virus-specific sequences are highlighted in larger typeface. (B) Endonuclease reactions with 3P Vac extract were carried out in the presence of various amounts of synthetic RNA and analyzed on 20% polyacrylamide-7 M urea gels. Lanes: 1, control reaction using purified viral cores; 2 to 5, 3P Vac extract with 0.2, 0.5, 2, and 10 pmol, respectively, of PH-V RNA added; 6 and 7, 3P Vac extract with 10 and 100 pmol, respectively, of V-3' RNA added; 8 and 9, 3P Vac extract with 10 and 100 pmol, respectively, of V-5' RNA added; 10 to 12, 3P Vac extract with 0.5, 2, and 10 pmol, respectively, of the V-3' and V-5' RNAs. G₁₁ denotes the cleavage product of the endonuclease reaction.

number of other nonviral RNAs and panhandle-containing RNAs were also tested and gave similar results (unpublished data). Therefore, activation of the viral endonuclease through added RNA is specific for viral end sequences. It should be noted that recombinant polymerase preparations will occasionally induce observable minor levels of an 11-base cleavage product in the absence of added vRNA, presumably due to nonspecific nuclease activity. However, in all cases, addition of PH-V RNA greatly stimulated the appearance of the cleavage product in a sequence-specific manner.

Requirement of 5' and 3' viral ends for activation. The PH-V RNA contains both 5' and 3' termini and should form a panhandle structure when added to the reaction (33). This type of panhandle structure is present at the termini of all eight influenza virus genes. Previous studies using polymerase isolated from intact virus have shown that the 3' terminus is all that is required to carry out endonuclease and transcriptase activities (33, 38). To determine whether the recombinant polymerase also had this property, the 42-base RNA shown in Fig. 5A was transcribed from *Mbo*II-digested plasmid pV-3', which contains the 3' terminus along with spacer sequence originating from the multiple cloning site of the transcription vector pTZ18U. Surprisingly, when this RNA was added to the endonuclease reaction, only a barely detectable level of endonuclease activity was observed at RNA levels as high as 100 pmol (Fig. 5B, lanes 6 and 7). This is stark contrast to the increasing levels of activity seen with PH-V RNA at 100-fold-lower concentrations (lanes 2 to 5). Consequently, another plasmid construct, pV-5', was used to transcribe a 49-base RNA containing the 5' terminus of the virion RNA with multiple cloning site spacer sequence at its 3' end (Fig. 5A). This RNA also did not efficiently stimulate endonuclease activity at concentrations as high as 100 pmol (Fig. 5B, lanes 8

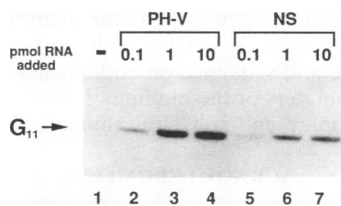


FIG. 6. Endonuclease stimulation by full-length NS vRNA. Synthetic NS vRNA was transcribed from *HgaI*-digested plasmid pHgaNS, added to the 3P Vac extract, and examined for stimulation of cleavage activity alongside PH-V RNA. Lanes: 1, no RNA added; 2 to 4, 0.1, 1, and 10 pmol, respectively, of PH-V RNA added; 5 to 7, 0.1, 1, and 10 pmol, respectively, of NS vRNA added.

and 9). As can be seen in Fig. 5A, because of the nature of the pV-5' and pV-3' constructs, the spacer sequences which are contributed from the multiple cloning site have perfect complementarity for 26 bases prior to the viral sequences. When annealed, this should be enough to drive the two RNA molecules into a duplex, with the influenza virus panhandle sequence at its end. In fact, if these RNAs containing the 3' and 5' ends are annealed and added together in the reaction, the stimulation of endonuclease activity is rescued to levels similar to that seen with the PH-V RNA, which contains the termini within a single molecule (Fig. 5B; compare lanes 10 to 12 with lanes 3 to 5). Thus, in contrast to polymerase purified from intact virions, activation of the endonuclease component of recombinant viral polymerase is dependent on the addition of both RNA termini.

The pPH-V-generated template is a short model template of 53 nt. Therefore, stimulation of the viral endonuclease by a full-length gene segment was examined. A full-length 890-base nonstructural (NS) gene segment was generated *in vitro*, using runoff T7 transcription of an *HgaI* digest of plasmid pHgaNS (33). The RNA produced from this plasmid is identical to virion RNA segment 8 of WSN virus. When added to the 3P Vac extract, this RNA did stimulate cleavage of the capped substrate, but to a lesser extent than the PH-V extract (Fig. 6; compare lanes 2 to 4 with lanes 5 to 7). This is not surprising, as secondary structure analysis of this naked RNA does not predict that the viral ends would be stable in a panhandle conformation.

Studies on viral polymerase purified from intact virions. Since previous studies using polymerase purified from intact virions showed a dependence only on the 3' terminus as the template for transcriptase activity, we next reexamined this property with regard to activation by RNA addition. Viral polymerase-NP devoid of viral RNA was purified on CsCl-glycerol gradients and tested for endonuclease activity in the presence or absence of PH-V RNA. The endonuclease was active even in the absence of added PH-V RNA, and titration of increasing amounts of the RNA into the purified viral polymerase did not stimulate endonuclease activity to a great extent (Fig. 7). In this assay, only a limiting amount of polymerase-NP protein was used (125 ng) so as to accentuate any stimulatory effect of the added PH-V RNA. Quantitation of the amount of the cleaved substrate seen in Fig. 7 through the use of a β counter indicated that stimulation of cleavage was at best 30% of levels observed in extract without added template RNA (Fig. 7; compare lanes 1 and 4).

DISCUSSION

The polymerase complex of influenza virus is encoded by over half of the viral genome and carries out all of the essential

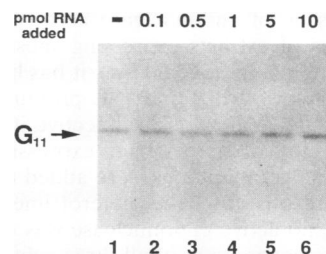


FIG. 7. Analysis of CsCl-glycerol-purified polymerase-NP. PH-V RNA was added to 125 ng of polymerase-NP, and cleavage activity was examined on a 20% polyacrylamide gel. Lanes: 1, no added RNA; 2 to 6, 0.1, 0.5, 1, 5, and 10 pmol, respectively, of RNA added. The gel was directly quantitated in a Betascope 603 blot analyzer (Betagen).

replicative functions during the virus life cycle. Much of what is known about the activities of the polymerase complex have been elucidated from *in vitro* studies of polymerase isolated from intact virus (8, 9, 24, 35, 36). Various functions of the polymerase complex have been assigned to each of the three polymerase proteins. Only recently has reconstitution of certain polymerase functions been achieved from recombinant sources. Two independent methods have been used in which polymerase from intact virus largely devoid of viral RNA has been purified and used to reconstitute activity with added recombinant RNA (18, 38). In both cases, it was found that the 3' end of the vRNA genome is sufficient to stimulate transcriptase activity. Saturation mutagenesis of the 3' end of genomic vRNA when used with polymerase-NP purified through a series of glycerol and CsCl-glycerol gradients (18, 33, 34) demonstrated that positions 2 and 11 are most important. In other experiments, limited mutagenesis of template RNA added to polymerase treated with micrococcal nuclease gave similar results, with nt 9 to 11 and 1 to 4 being the most important (39). Extension of these latter experiments also identified an RNA binding site at nt 9 to 12 of the 3' end (16).

The expression of active polymerase in the absence of NP protein and virion RNA should provide a powerful means of studying polymerase function and RNP assembly. Previously we had shown that vaccinia virus vectors designed to express the PB2, PB1, and PA proteins, when coinfecting into cells with a vaccinia virus vector expressing NP, forms an active complex which can replicate and transcribe viral RNP (21, 22). Thus, this recombinant vaccinia virus expression system was used to investigate endonuclease and transcriptase activity of the influenza virus polymerase complex in the absence of NP. Nuclear extracts of triply infected HeLa cells were prepared, and virus-specific endonuclease activity was monitored by using a synthetic ^{32}P -capped mRNA. This substrate is precisely cleaved into a single 11-nt fragment by the viral polymerase (12). Little or no endonuclease activity was detected in extracts from infected cells coexpressing the three polymerase proteins alone. However, upon addition of a short 53-base model template containing both the 3' and 5' ends of viral genome, endonuclease function within this extract was stimulated. Cleavage activity in the extract titrated linearly with increasing amounts of PH-V RNA until saturation was achieved (Fig. 2B). As endonuclease activity was not induced upon addition of nonspecific RNAs (Fig. 3), these data signify that rescue of cleavage activity is a specific function of this virus-like RNA interacting with free polymerase complexes.

Extracts in which either one or two of the polymerase proteins was expressed did not exhibit any specific cleavage activity in the presence of the PH-V RNA (unpublished data).

Furthermore, rescue of endonuclease activity was attempted by using mixtures of extracts expressing subsets of the PB2, PB1, and PA proteins. In Sendai virus, it has been shown that separate complexes of NP-P and P-L proteins, when mixed together, can rescue replication of defective interfering particles (19). When any of the extracts expressing one or two polymerase protein combinations were added to each other in an attempt to reconstitute the heterotrimeric polymerase complex *in vitro*, no active endonuclease was detected. These data suggest that coexpression of all three subunits is required for correct assembly of polymerase complexes (unpublished data).

Once stimulated, the polymerase extract was also able to transcribe from the added synthetic template. Addition of ATP, GTP, and CTP resulted in the appearance of a fragment elongated by 11 nt, which is the expected size of a fragment elongated up to the block at the first encoded UTP after initiation at the third G of the model vRNA. Since NP is not present in the extract, this result conclusively demonstrates that NP is not required for limited elongation by the polymerase. However, the level of transcription observed in these extracts is very low, as the majority of cleaved primer is either elongated by one or two bases or not elongated at all (Fig. 4, lane 7). This could indicate that NP may play a role in the processivity of the polymerase, as had been suggested by earlier studies (18). Since full-length genomic RNA did not efficiently activate the polymerase, it cannot yet be determined whether NP is required for transcription of long templates. Presumably, the full-length NS gene segment does not rescue cleavage activity as well as the short model template does because the secondary structure that the naked RNA forms does not efficiently form a panhandle structure.

The polymerase formed in recombinant vaccinia virus-infected cells is clearly different from the polymerase which has been analyzed from intact virus. The recombinant polymerase has never been exposed to either vRNA or the NP protein and consequently may represent a form of polymerase present prior to RNP assembly. Two major distinctions were found in comparison of the recombinant polymerase and enzyme purified from RNPs. One difference is the requirement of template RNA for endonuclease activation and subsequent transcription. CsCl-glycerol gradient-purified cores exhibited high levels of endonuclease activity even in the absence of added template, and addition of template only slightly stimulated activity (Fig. 7). This may be due to the presence endogenous vRNA which was not removed by the centrifugation protocol. A second distinction concerned the requirement of both viral ends for activation. Addition of either the 3' or 5' end separately induced only a slight increase in endonuclease activity, with the 5' end always being slightly better at stimulation (unpublished data). This is particularly interesting since adding the 5' end alone does not contribute the correct 3' template that the polymerase can use for cleavage and needs for subsequent transcription. However, addition of the 5' and 3' ends as separate RNAs was able to rescue full stimulation. It is not clear whether these viral ends act as separate molecules to activate the polymerase or whether they act in a cooperative fashion through base pairing to form the panhandle conformation, which activates the polymerase. Stimulation is also not dependent solely on the need for a nonspecific panhandle sequence, as a 53-base related RNA which contains exact inverse complementarity for the terminal 15 bases is unable to activate the endonuclease (unpublished data). These data suggest a model whereby the vRNA may act as a scaffold to bring the polymerase proteins in the proper conformation for activity. Continued use of this recombinant polymerase

should provide an *in vitro* system for analyzing these early steps in the formation of RNP. The importance of the panhandle structure and sequence on endonuclease activation, as well as the parameters of the binding of the template RNA to the protein complex, can now be examined.

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