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Promoter Analysis of Influenza Virus RNA Polymerase

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Influenza virus polymerase, which was prepared depleted of viral RNA, was used to copy small RNA templates prepared from plasmid-encoded sequences. Template constructions containing only the 3′ end of genomic RNA were shown to be efficiently copied, indicating that the promoter lay solely within the 15-nucleotide 3′ terminus. Sequences not specific for the influenza virus termini were not copied, and, surprisingly, RNAs containing termini identical to those from plus-sense cRNA were copied at low levels. The specificity for recognition of the virus sense promoter was further defined by site-specific mutagenesis. It was also found that increased levels of viral protein were required in order to catalyze both the cap endonuclease-primed and primer-free RNA synthesis from these model templates, as well as from genomic-length RNAs. This finding indicates that the reconstituted system has catalytic properties very similar to those of native viral ribonucleoprotein complexes.

The RNA-dependent RNA polymerases of animal viruses have been extensively studied with regard to many aspects of protein structure and reaction conditions. However, the elements of the template RNA which promote optimal expression by the polymerase could be studied only by inference, using existing viral RNA sequences. This promoter analysis is of interest since it is unknown how a viral polymerase recognizes specific viral RNAs from among the many host-encoded RNAs found in an infected cell. In addition, the inhibition of recognition by these polymerases may be a new approach toward developing antiviral compounds, since RNA-dependent RNA polymerase activity is not known to exist in mammalian cells.

Animal viruses containing plus-sense genome RNA can be replicated when plasmid-derived RNA is introduced into cells by transfection (13, 17). In poliovirus, the purified polymerase will replicate a genome RNA in in vitro reactions, and when this preparation is transfected into cells it is infectious (13). However, the template elements which serve as a transcription promoter for the poliovirus-encoded polymerase are unknown, since even RNA homopolymers can be copied (29). SP6 transcripts have also been used to produce model defective interfering RNAs for the Sindbis virus genome. When the RNA is introduced into infected cells, it is replicated and packaged. The RNA sequences which were responsible for both recognition by the Sindbis virus polymerase and packaging of the genome into virus particles were shown to be within 162 nucleotides (nt) of the 5′ terminus and 19 nt of the 3′ terminus of the genome (17). For brome mosaic virus (BMV), a positive-strand RNA plant virus, SP6 transcripts have been used to identify the promoter as a 134-nt tRNA-like 3′ terminus (6). Polymerase recognition and synthesis were shown to be dependent on both sequence and secondary structural features (5).

The negative-sense RNA viruses have been refractory to study of the sequence requirements of the replicase. The purified polymerase of vesicular stomatitis virus is active in transcription only when virus-derived ribonucleoprotein (RNP) complexes are included as a template (4, 7, 19). RNPs have been reconstituted from naked RNA of vesicular stomatitis virus defective interfering particles by using infected cell extracts as the protein source. These RNPs were then replicated when added back to infected cells (18). With regard to influenza viruses, it was recently reported that naked RNA purified from virus was used to reconstitute RNPs. The viral nucleocapsid protein (NP) and polymerase proteins were gel purified and renatured on the viral RNA, using thioredoxin (26). However, these authors did not show that the activity of the preparation was specific for influenza virus RNA, nor did they analyze the signals which promote transcription (26).

During the course of influenza virus infection, the polymerase catalyzes three distinct transcription activities. These include the synthesis of (i) subgenomic mRNA, which contains a 5′ cap and a 3′ poly(A) tail; (ii) a full-length plus strand (cRNA) copied from the genome RNA; and (iii) genomic viral RNA (vRNA) synthesized from the full-length cRNA (reviewed in references 12 and 16). Viral proteins PB2, PB1, and PA are thought to catalyze all influenza virus-specific RNA synthesis in the presence of excess NP (12, 16). These polymerase functions have been studied by using RNP cores derived from detergent-disrupted virus and RNPs from the nuclear extracts of infected cells. Transcription from the RNPs derived from disrupted virus occurs when either dinucleotide adenylyl-(3′-5′)-guanosine (ApG) or capped mRNAs are used as primers. The plus-sense mRNA products have terminated synthesis 17 to 20 nt upstream of the 5′ terminus of the RNA template and have been processed by the addition of poly(A) tails. These products cannot serve as templates for the virus sense genome since they lack terminal sequences (8). RNPs derived from nuclear extracts of infected cells also synthesize polyadenylated mRNA in the presence of capped RNA primers. However, if ApG is used under these conditions, both types of RNAs, polyadenylated mRNA and full-length cRNA, can be observed (1, 27). Recently, it was shown that replicative synthesis of cRNA could occur in the absence of exogenous primer if the nuclear extract was harvested at certain times postinfection. In these same preparations, the synthesis of negative-sense vRNA from a cRNA template was also observed (24). The synthesis of full-length cRNA

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was shown to be dependent on the presence of NP which was free in solution (1, 24). These findings led to the suggestion that the regulatory control between mRNA and cRNA synthesis by the RNP complex is based in part on the requirement for an excess of soluble NP (1).

Another line of investigation has focused on the preparation of polymerase-RNA complexes derived from RNPs from detergent-disrupted virus. When the RNP complex is centrifuged through a CsCl-glycerol gradient, the RNA can be found associated with the three polymerase (P) proteins at the bottom of the gradient. Near the top of the gradient, free NP can be found (10, 14). These upper fractions have been shown to contain small amounts of polymerase proteins (this report). The purified polymerase-RNA complex (bottom of gradient) is active in initiating ApG-primed synthesis of RNA but fails to elongate to more than 12 to 19 nt. When fractions from the top of the gradient containing the NP are added back to the polymerase-RNA complex, elongation can ensue (9). These data suggest that the NP is needed for elongation but that initiation can occur in the absence of NP.

It has been shown that the genomic RNA of influenza viruses is in a circular conformation via base pairing of the termini to form a panhandle of 15 to 16 nt (10, 11). Since the viral polymerase was found bound to the panhandle, this led to the suggestion that a panhandle structure is required for recognition by the viral polymerase (10). Therefore, it was hypothesized in these two reports that the promoter for the viral RNA polymerase was the double-stranded RNA in panhandle conformation.

In this study, we prepared, from the upper fractions of the CsCl-glycerol gradient centrifugation, polymerase which was depleted of genomic RNA. This polymerase was able to copy short model templates which were derived from transcription of appropriate plasmid DNA with bacteriophage T7 RNA polymerase in a sequence-specific manner. The termini of this model RNA are identical to the 3′ 15 nt and the 5′ 22 nt conserved in segment 8 from all influenza A virus vRNAs. By manipulating the plasmid in order to prepare different RNAs to serve as templates, we demonstrated that recognition of and synthesis from this model RNA were specific for the promoter at the 3′-terminal sequence and did not require the panhandle. In addition, site-specific mutagenesis identified nucleotide positions responsible for the viral polymerase, favoring synthesis from genomic-sense templates over complementary-sense RNA. Conditions were also found under which cap endonuclease-primed RNA synthesis could be observed, using model RNAs. In addition, the reconstituted system permitted virus-specific synthesis from genome-length RNAs, derived either from plasmids or from RNA purified from virus through phenol extraction.

MATERIALS AND METHODS

Purification of the viral RNA polymerase. RNP cores were prepared from whole virus by standard methods (20, 22). Two to three milligrams of virus was disrupted by incubation in 1.5% Triton N-101–10 mg of lysolecithin per ml–100 mM Tris hydrochloride (pH 8.0)–100 mM KCl–5 mM $MgCl_2$–5% glycerol–1.5 mM dithiothreitol. The sample was fractionated by centrifugation on a 30 to 70% glycerol (wt/vol) step gradient in the presence of 50 mM Tris hydrochloride (pH 7.8) and 150 mM NaCl. The core preparation was centrifuged at 45,000 rpm in an SW50.1 rotor for 4 h at 4°C. Fractions enriched in RNP were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of protein samples from each fraction and staining with silver. The core fractions were then subjected to a second gradient centrifugation, as described by Honda et al. (10). The second gradient had steps of 0.5 ml of 3.0 M CsCl and 45% (wt/vol) glycerol, 1.75 ml of 2.5 M CsCl and 40% glycerol, 1.25 ml of 2.0 M CsCl and 35% glycerol, and 1.0 ml of 1.5 M CsCl and 30% glycerol. All steps were buffered with 50 mM Tris hydrochloride (pH 7.6) and 100 mM NaCl. A 0.5-ml volume of RNP cores was layered on top, and the sample was centrifuged at 45,000 rpm in an SW50.1 rotor for 25 h at 4°C. Polymerase fractions were again identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the protein samples and silver staining. Active polymerase fractions were generally found in the region of the gradient correlating with 1.5 to 2.0 M CsCl. These fractions were pooled and then dialyzed against 50 mM Tris hydrochloride (pH 7.6)–100 mM NaCl–10 mM $MgCl_2$ and concentrated in Centricon-10 tubes (Amicon Corp.), or fractions were dialyzed in bags against 50 mM Tris hydrochloride (pH 7.6)–100 mM NaCl–10 mM $MgCl_2$–2 mM dithiothreitol–50% glycerol.

Preparation of plasmid. The plasmid design is shown (see Fig. 2). Insert DNA for the pV-wt plasmid (see Fig. 2) was prepared by using a DNA synthesizer (Applied Biosystems). The top strand was 5′-GAAGCTTAATACGACTCA CTATAAGTAGAAACAAGGGTGTTTTTTCATATCATT TAAACTTCACCCTGCTTTTGCTGAATTCATTCTTCTG CAGG-3′. The bottom strand was synthesized by primer extension, with 5′-CCTGCAGAAGAATGA-3′ as the primer. The 95-base-pair DNA was digested with *Hin*dIII and *Pst*I and purified by extraction with phenol-chloroform, ethanol precipitation, and passage over a NACS-prepack ion-exchange column (Bethesda Research Laboratories). This DNA was ligated into pUC-19 which had been digested with *Hin*dIII and *Pst*I and then used to transform *Escherichia coli* DH5-α, which had been made competent by standard protocols. Bacteria were spread on agar plates containing X-Gal (5-bromo-4-cloro-3-indoyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside), and blue colonies were found to have the plasmid containing the predicted insert since the small insert conserved the *lacZ* reading frame and did not contain a termination codon. The pM-wt plasmid was prepared by a similar strategy, except that both strands were chemically synthesized, with the upper strand having the sequence 5′-GAAGCTTAATAC GACTCACTATAAGCAAAAGCAGGGTGAAGTTTAAA TGATATGAAAAAACACCCTTGTTTCTACTGAATTCA TTCTTCTGCAGG-3′.

The pV-d5′ plasmid (see Fig. 2) was prepared by using the oligonucleotides 5′-AGCTTAATACGACTCACTATAA GATCTATTAAACTTCACCCTGCTTTTGCTGAATTCAT TCTTCTGCA-3′ and 5′-GAAGAATGAATTCAGCAAAA GCAGGGTGAAGTTTAATAGATCTTATAGTGAGTCG TATTA-3′. The DNAs were annealed and ligated into the *Hin*dIII-*Pst*I-digested pUC-19, and white colonies were found to contain the correct plasmid because this insert resulted in a frameshift in the *lacZ* gene. The point mutants were isolated following digestion of pV-d5′ with *Bgl*II and *Pst*I and ligation of the linearized plasmid with a single-stranded oligonucleotide of mixed composition. Since *Bgl*II leaves a 5′ extension and *Pst*I leaves a 3′ extension, a single oligonucleotide was all that was necessary for ligation of the insert. The host cell was then able to repair gaps caused by the lack of a complementary oligonucleotide. Oligonucleotides were designed to repair the frameshift in the *lacZ* gene so that bacteria which contained mutant plasmids were selected by their blue color.

Plasmid pHgaNS, which was used to prepare an RNA

identical to segment 8 of influenza virus A/WSN/33, was prepared by using the primers 5′-CCGAATTCTTAATAC GACTCACTATAAGTAGAAACAAGGGTG-3′ and 5′-CCT CTAGACGCTCGAGAGCAAAAGCAGGTG-3′ in a polymerase chain reaction with a cDNA clone. The product was then cloned into the *Xba*I-*Eco*RI window of pUC-19.

Preparation of RNA templates. Plasmid DNAs were digested with *Mbo*II or other appropriate endonucleases (see Fig. 2), and the linearized DNA was transcribed by using the bacteriophage T7 RNA polymerase. Runoff RNA transcripts were treated with RNase-free DNase I, and then the RNA was purified from the proteins and free nucleotides by using tip-5 ion-exchange columns (Qiagen, Inc.). Following precipitation in ethanol, purified RNAs were suspended in water, and a sample was analyzed by electrophoresis followed by silver staining of the polyacrylamide gel in order to quantitate the yield of RNA.

Influenza virus polymerase reactions. In a 25-μl total volume, about 30 ng of nucleoprotein and 200 pg total of the three polymerase proteins were mixed with 10 ng of template RNA, and the solution was made up to a final concentration of 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05% Nonidet P-40, 0.4 mM dinucleotide ApG (Pharmacia, Inc.), 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, and ≈0.6 μM [α-^{32}P]UTP (40 μCi at 3,000 Ci/mmol; New England Nuclear Corp.). The reaction mixtures were assembled on ice and then transferred to a 30°C water bath for 90 min. The reactions were terminated by the addition of 0.18 ml of ice-cold 0.3 M sodium acetate–10 mM EDTA and were then extracted with phenol-chloroform (1:1 volume ratio). Following the first extraction, 15 μg of poly(I)-poly(C) RNA was added as a carrier, and the sample was extracted again with phenol-chloroform. The samples were then extracted with ether and precipitated in ethanol. Following centrifugation, the RNA pellet was washed twice with 70% ethanol and then dried under a vacuum.

In reactions using the high concentration of polymerase, conditions were identical to those described above, except that 20 ng of template RNA was added. In reactions using genome-length RNAs, the amount of polymerase used was doubled, 50 ng of template RNA was used, and the UTP concentration was raised to 2.6 μM.

The RNA was suspended in a dye mix containing 78% formamide, 10 mM EDTA, 0.1% xylene cyanol, and 0.05% bromophenol blue. Typically, a sample from this RNA was electrophoresed on an 8% polyacrylamide gel in the absence of urea, the remainder was denatured by being heated to 100°C for 1.5 min, and then some was loaded on an 8% polyacrylamide gel containing 7.7 M urea. Gels were fixed by a two-step procedure, first in 10% acetic acid and then in 25% methanol–8% acetic acid. Gels were dried onto filter paper and then exposed to X-ray film.

When different RNAs were being tested for use as templates, the RNA preparations were always analyzed on polyacrylamide gels and stained with silver so that equal amounts of each template were used. In order to quantitate the amount of product, gels were exposed to X-ray film in the absence of an intensifying screen to improve the linearity of the densitometer readings. Autoradiographs were analyzed by using an FB910 scanning densitometer (Fisher Biotech), and peaks were evaluated by using computer software from Fisher Biotech.

Nuclease analysis of reaction products. For RNase T_1 analysis of the two principal RNA products, reaction products were analyzed by 8% polyacrylamide gel electrophoresis (without urea), and the gel was not treated with fixative. The wet gel was exposed to an X-ray film, and the appropriate gel pieces were located and excised. The gel piece was crushed in 0.3 ml containing 10 mM Tris (pH 7.5), 1 mM EDTA, 0.1% sodium dodecyl sulfate, and 1 μg of tRNA as a carrier. The RNA diffused into this solution for 3 h, and then the gel was pelleted and the supernatant was made 0.3 M in sodium acetate. The supernatant was then extracted twice in phenol-chloroform and once in ether and then precipitated in ethanol. The RNA pellet was suspended in 5 μl of formamide, denatured in boiling water for 1.5 min, and then diluted by the addition of 0.1 ml of 10 mM Tris (pH 7.5)–1 mM EDTA. RNase T_1 (50 U; Boehringer Mannheim Biochemicals) was added, and the samples were incubated for 60 min at 37°C. Control RNAs identical to V-wt and M-wt RNAs were synthesized in the presence of [α-^{32}P]UTP and similarly digested with RNase T_1. Reaction products were extracted in phenol-chloroform, precipitated in ethanol, and then analyzed on 20% polyacrylamide gels containing 7.7 M urea.

Nuclease S1 analysis of reaction products was done on transcribed RNA by first terminating the standard polymerase reaction through the addition of S1 buffer to a volume of 0.2 ml with 0.26 M NaCl–0.05 M sodium acetate (pH 4.6)–4.5 mM zinc sulfate. The sample was divided into two 0.1-ml volumes, and 100 U of S1 nuclease (Sigma Chemical Co.) was added to one tube. The samples were incubated for 60 min at 37°C. Following the incubation, EDTA (10 mM final concentration) and 15 μg of poly(I)-poly(C) RNA were added, and the sample was extracted with phenol-chloroform and precipitated in ethanol. The samples were then subjected to polyacrylamide gel electrophoresis.

RESULTS

Preparation of influenza virus RNA polymerase and template RNA. RNP cores of influenza virus A/Puerto Rico/8/34 (A/PR/8/34) were prepared by disruption of virus in lysolecithin and Triton N-101 followed by glycerol gradient centrifugation (22). Fractions containing cores were then subjected to a second centrifugation in a CsCl-glycerol step gradient (10). Fractions containing the polymerase were identified by gel electrophoresis of samples followed by silver staining. Figure 1 shows the polymerase preparation after CsCl centrifugation. Bovine serum albumin was added during dialysis to protect against protein loss.

The overall design of the plasmids used to prepare template RNAs in this study is depicted in Fig. 2. The entire insert was prepared by using oligonucleotides made in a DNA synthesizer, which were then cloned into the polylinker of pUC-19. The insert contained a truncated promoter sequence recognized by the bacteriophage T7 RNA polymerase (25), so that the first nucleotides synthesized were the terminal 22 nt of the conserved sequence from the 5′ end of the genome RNA. When the plasmid was cut with restriction endonuclease *Mbo*II (which cuts 7 bases upstream of its recognition site), the RNA which resulted from T7 RNA polymerase transcription ended with the terminal 3′ nucleotides of the influenza virus sequence. Included in the sequence was the poly(U) stretch adjacent to the 5′ end of the conserved terminus, which is thought to constitute at least part of the termination-polyadenylation signal (21). The total length of this model genomic RNA was 53 nt, since a 16-nt spacer separated the terminal conserved sequences. The model RNA which contained both termini identical to

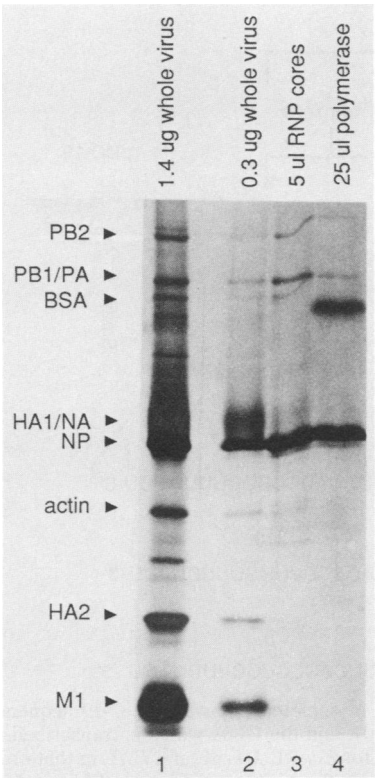

FIG. 1. Purification of the polymerase preparation. RNP cores were purified from whole virus and then subjected to CsCl-glycerol gradient centrifugation. The polymerase was purified from fractions with 1.5 to 2.0 M CsCl. Samples were then analyzed by polyacrylamide gel electrophoresis on a 7 to 14% linear gradient gel in the presence of 0.1% sodium dodecyl sulfate, followed by staining with silver. Protein samples contained 1.4 μg of whole virus (lane 1), 0.28 μg of whole virus (lane 2), 5 μl of RNP cores (lane 3), and 25 μl of RNA polymerase (lane 4). Known assignments of the proteins are indicated at the left. BSA, Bovine serum albumin; NA, neuraminidase; HA1 and HA2, hemagglutinin cleavage fragments; PB1, PB2, and PA, the three viral polymerase proteins.

those of vRNA was named V-wt. The RNA M-wt encoded the exact complementary strand of V-wt so that the termini match those of cRNA. It was hoped that V-wt and M-wt could serve as models for influenza virus-specific vRNA and cRNA, respectively.

Viral polymerase catalyzes synthesis of a full-length copy of the template. In the reaction using the influenza virus polymerase, V-wt template, and ApG primer, a product was obtained which comigrated with a 53-nt RNA on denaturing gels. RNA migrating as a doublet at a position of about 40 to 45 nt (Fig. 3A, lane 2) was also seen. This shorter product is shown below to be RNA which had terminated at a stretch of adenosines present between nt 43 and 48 in the virion sense template. In addition to the template-specific transcripts, a general background of light bands could be seen which corresponded to truncated RNA products transcribed from viral genomic RNA not removed during the CsCl-glycerol centrifugation step (data not shown). When no primer was used, there was no specific transcription product seen (Fig. 3A, lane 3). Globin mRNA, containing a terminal cap 1 structure, was inactive as the primer with initial preparations of polymerase (results not shown).

When the polymerase reaction was terminated by the addition of excess buffer favorable for nuclease S1 digestion and nuclease was added, the radioactively labeled product was resistant to digestion (Fig. 3B, lane 2). These conditions very efficiently digested the V-wt single-stranded RNA radioactively synthesized with T7 RNA polymerase (Fig. 3B, lanes 3 and 4). These nuclease S1 data confirmed that the opposite strand was indeed being synthesized in these reactions. The product of the reaction might be a double-stranded RNA, but it could not be ruled out that the product was in fact single stranded and later annealed to the template RNA in the presence of the high salt concentration used in the nuclease reaction.

The RNA products were purified by electrophoresis on an 8% gel, excised, eluted from the gel, and then digested by RNase T_1. The products were analyzed by electrophoresis, and the patterns were compared with those generated by RNase T_1 digestion of internally labeled M-wt and V-wt control probes. Although the T_1 digestion was probably partial, the full-length RNA (Fig. 3C, lane 1) had a pattern identical to that of the plus-sense RNA, M-wt (Fig. 3C, lane 3), and did not have the pattern of the V-wt RNA (Fig. 3C, lane 4). The observed pattern was essentially identical to that which is predicted from the sequence of the RNA and thus showed that the polymerase faithfully copied the V-wt template. The smaller RNA product, a doublet with most templates, was also digested with RNase T_1. Its pattern was similar to that of the full-length RNA product (Fig. 3C, lane 2), except the 14-base oligonucleotide was not present. Instead, a faint 13-base oligonucleotide was seen, thus mapping the termination of the short RNA to position 44, a site at which two uridines would be incorporated. Since the amount of smaller RNA product decreased at higher UTP concentrations and disappeared when CTP was used as a label, these bands appeared to be an artifact of low UTP concentrations in the polymerase reaction (results not shown).

Conditions for the polymerase reactions using model RNA templates. It was found that protein samples containing about 30 ng of NP and about 200 pg total of the three P proteins would react optimally with 5 to 10 ng of RNA. By using cold competitor RNA, poly(I)-poly(C), it was found that excess RNA nonspecifically inhibited transcription, possibly via nonspecific binding of the NP (data not shown) (15, 23). In the absence of nonspecific competitor, variations in the amount of template between 1 and 10 ng produced little change in the efficiency of RNA synthesis (data not shown). The NP and RNA were present at about equal molar concentrations, which were each about a thousand-fold in excess of the moles of the complex (assuming the proportions to be 1:1:1) formed by the three P proteins in the typical reaction.

Since these reconstituted RNPs were able to use ApG but not globin mRNA as a primer, we tested the model RNPs for other variables of the transcription reaction. In all other ways tested, the reconstituted RNPs behaved in solution similarly to those RNPs purified from detergent-disrupted virus. The optimum temperature for RNA synthesis was 30°C (Fig. 4A, lane 2), as has been repeatedly found for the viral polymerase (2, 27, 28). Also, the optimum salt conditions were 60 mM NaCl (Fig. 4B, lane 2), again consistent with conditions used previously by several groups (2, 10, 24). Figure 4C shows a time course experiment. The amount of RNA synthesis appeared to increase roughly linearly for the first 90 min, as was found for viral RNPs (27).

Specificity of the elongation reaction. Various RNAs were tested for suitability as templates for the RNA polymerase of influenza virus. The pV-wt plasmid clone was digested with

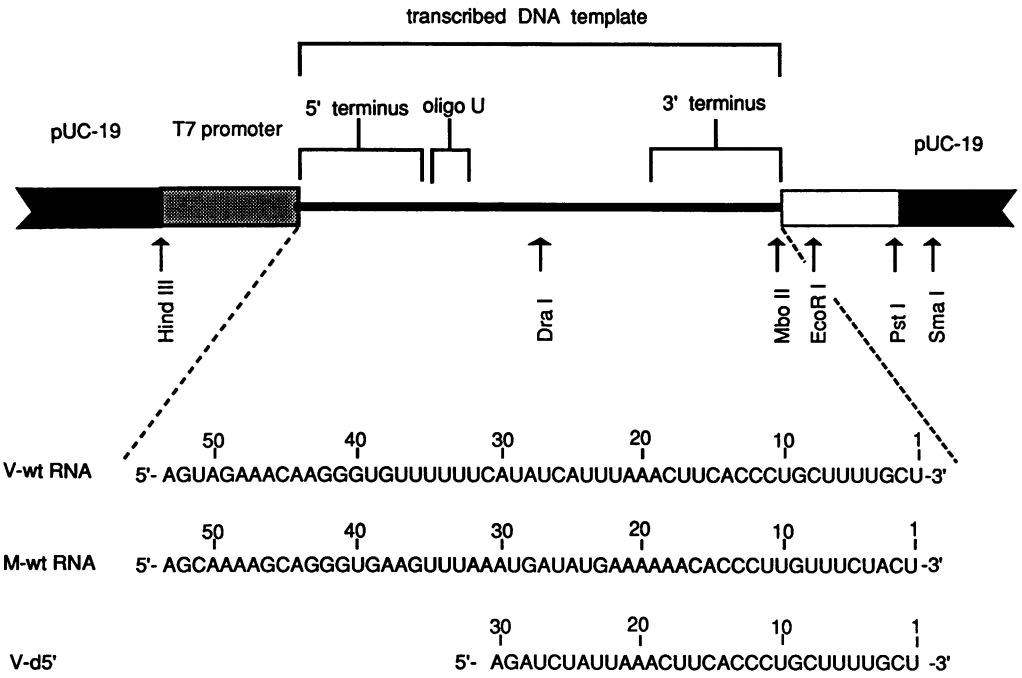

FIG. 2. Plasmid constructs used to prepare RNA templates. The plasmid design is depicted, showing pUC-19 sequences (■), the truncated promoter specifically recognized by bacteriophage T7 RNA polymerase (▩), and the DNA which is transcribed from plasmids which have been digested with *Mbo*II (——). Sequences encoding the recognition sites for *Eco*RI, *Mbo*II, and *Pst*I, in that order, are shown (▭). Sites of cleavage by restriction endonucleases are indicated. Beneath the diagram, the entire sequences of RNAs which result from synthesis by T7 RNA polymerase from *Mbo*II-digested plasmid are given. The V-wt RNA has 5′ and 3′ termini identical to those found in RNA segment 8 of influenza A viruses, separated by 16 spacer nt. The M-wt RNA represents the exact opposite strand, or message sense, of V-wt. T7 transcripts of plasmids cleaved by *Dra*I, *Eco*RI, *Pst*I, and *Sma*I result in RNAs 32, 58, 66, and 91 nt long, respectively. The sequence of V-d5′ RNA is indicated. The plasmid design is essentially the same as that used for the V-wt RNA except for the 5′ deletion and minor changes in the spacer sequence. The point mutants of V-d5′ RNAs which were studied are indicated in Table 1.

*Eco*RI, *Pst*I, or *Sma*I, and T7 polymerase was used to transcribe RNA. This resulted in RNAs identical to V-wt except for the addition of 5, 13, and 38 nt at the 3′ end. An overexposure of an autoradiograph is shown in order to demonstrate that no transcripts over background were observed in reaction mixtures which contained the following as templates: two of the RNAs identical to V-wt except they contained 13 and 38 nt of extra sequence on the 3′ terminus (Fig. 5A, lanes 1 and 2), a single-stranded DNA with a sequence identical to that of V-wt (Fig. 5A, lane 4), and an unrelated 80-nt RNA generated by transcribing the polylinker of pIBI-31 with T3 RNA polymerase (Fig. 5A, lane 5). However, the RNA (V-Eco) derived from the *Eco*RI-digested template, containing 5 extra nt on the 3′ end, could be recognized and faithfully transcribed, although at approximately one-third the efficiency of the wild-type V-wt RNA (Fig. 5B, lane 3). Also of interest was that the initiation on the V-Eco RNA by the influenza virus polymerase appeared to occur at the correct base, since the transcribed RNA was the same size as the product from the V-wt template.

Analysis of the promoter region for the viral RNA polymerase. The original construct used for these studies contained the sequences of both RNA termini of genomic RNAs which could base pair and thus form a panhandle. This was done since it was shown that the vRNA in virions and in RNPs in infected cells was in circular conformation via the 15- to 16-nt-long panhandle (10, 11). It was further shown that the viral polymerase was bound to the double-stranded structure (10), thus leading to the suggestion that the promoter for RNA synthesis was the panhandle. In order to test whether the panhandle was an absolute requirement for recognition, the following templates were used. Plasmid pV-wt was digested with *Dra*I prior to transcription by the T7 polymerase (Fig. 2). This process should result in an RNA molecule of 32 nt containing only virus-specific sequences from the 5′ end of the RNA. When this RNA was used as a template, no apparent product was produced (Fig. 5B, lane 2). Therefore, the 3′ terminus of virion RNA was required for this reaction. This finding was not surprising, since the initiation site at the 3′ end of V-wt was not present in this RNA (V-Dra). A second plasmid clone was produced which deleted the 5′-terminal sequences but kept intact the 3′ terminus. This clone, pV-d5′, when digested with *Mbo*II and used for transcription by T7 polymerase, produced a major transcript of 30 nt and minor species of 29 and 31 nt (data not shown). Surprisingly, this template was recognized and copied by the influenza virus polymerase. The product of the viral RNA polymerase reaction with V-d5′ contains multiple bands reflecting the input RNA (Fig. 6, lane 1). When the products shown in Fig. 6, lane 1, were eluted from gels and subjected to RNase T_1 analysis, the pattern expected of the transcription product of V-d5′ was observed (data not shown). Since the V-d5′ RNA template was copied, the panhandle was not required for viral polymerase binding and synthesis.

Although the 5′ terminus was not required for synthesis by the polymerase, a distinct possibility was that the V-wt RNA might be a preferred template compared with V-d5′. To examine this possibility, reaction experiments were done in which the templates were mixed. The V-wt RNA was present at 5 ng in each reaction mixture. The V-d5′ was

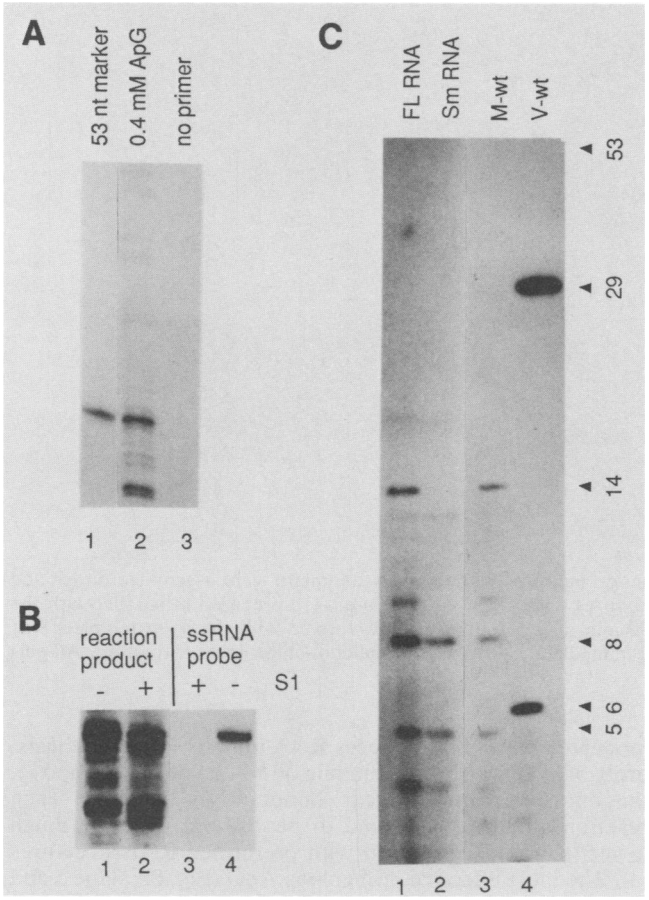

FIG. 3. Analysis of products of influenza virus polymerase. (A) Polymerase reaction mixtures containing 0.4 mM ApG (lane 2) or no primer (lane 3) were electrophoresed on 8% polyacrylamide gels containing 7.7 M urea. (B) The nascent RNA is resistant to single-stranded specific nuclease S1. After the standard polymerase reaction, the solutions were diluted in nuclease S1 buffer (lane 1), and enzyme was added (lane 2). As controls for S1 digestion conditions, radioactively labeled single-stranded V-wt RNA was treated with nuclease S1 (lane 3) or with buffer alone (lane 4). (C) RNase T1 analysis of gel-purified reaction products. The products of the viral polymerase reaction using the V-wt RNA template were subjected to electrophoresis on an 8% polyacrylamide gel. The 53-nt band and the smaller transcript were excised and eluted from the gel matrix. These RNAs were digested with RNase T_1 and analyzed by electrophoresis on a 20% polyacrylamide gel containing 7.7 M urea. For comparison, T7 transcripts of M-wt and V-wt RNAs which had been synthesized in the presence of [α-^{32}P]UTP were also analyzed with RNase T_1. The predicted radiolabeled oligonucleotides of the control RNAs are indicated. Lane 1, 53-nt full-length (FL) product; lane 2, 40- to 45-nt smaller (Sm) RNA product; lane 3, M-wt RNA labeled by incorporation of [^{32}P]UMP; lane 4, V-wt RNA labeled as in lane 3. Sizes (in nucleotides) are indicated on the right.

absent (Fig. 7, lane 1) or was present at a 1/5 molar ratio (lane 2) or a 1/1 molar ratio (lane 3). The relative intensities of the bands from each RNA were determined by densitometry of the autoradiograph. The values were corrected for the amount of the radioactive nucleotide, UTP, which could be incorporated into each product, and the value was normalized so that the level of synthesis in each lane was set equal to one. The level of copying of V-wt decreased as V-d5′ was increased. When V-d5′ was present at a 1/5 molar ratio, the corrected level of synthesis was about one-fourth of that from V-wt (Fig. 7, lane 2). When the two templates were present in equimolar amounts, the level of synthesis from V-wt was about 60% of the total (Fig. 7, lane 3), which might be within the expected range of experimental error for equivalent levels of synthesis. Similar results were obtained when V-d5′ RNA was kept constant and the V-wt RNA was varied (data not shown). It was thus concluded that the panhandle-containing V-wt RNA was not greatly favored over the template RNA which contained only the proper 3′ terminus.

Viral polymerase does not copy RNA templates containing plus-sense termini. As described above, the influenza virus RNA polymerase performs three distinct activities during the course of an infection. Two activities involve the transcription of genome-sense RNA, and the third involves copying of the complementary-sense RNA into vRNA. We therefore constructed an RNA template which contained the 5′ and 3′ termini of the complementary sense RNA of segment 8 (Fig. 2).

When the M-wt RNA was used as the template, little synthesis was observed (Fig. 5B, lane 4). In two experiments used for quantitation, the average level of synthesis from M-wt RNA was 4% of that of V-wt. Comparing the V-wt and M-wt RNA promoters, the M-wt has only three transition changes and one point insertion within the 3′ 15 nt. These include a G to A change at position 3, a U to C change at position 5, a C to U change at position 8, and an inserted U between nt 9 and 10 (Table 1). In order to determine which of the four point differences in the 3′ termini were responsible for this specificity, many combinations were prepared and assayed for efficiency as a template (Fig. 6). These templates were derivatives of V-d5′, since they did not contain the 5′ terminus. Since small variations were seen between duplicate experiments, the averages of densitometry scans from several trials are given in Table 1. Single point changes in V-d5′ were equally well copied compared with V-d5′ itself, except for the V-A_3 RNA, which was copied at 40% efficiency (Fig. 6, lane 10; Table 1). When RNAs with two changes were tested, the activity generally dropped to very low levels (Fig. 6, lanes 3, 4, and 5). Therefore, these experiments confirmed that the specificity of the reaction for V-wt over M-wt was the result of the combination of the nucleotide changes present at the 3′ terminus of M-wt.

Cap endonuclease-primed RNA synthesis. The method of purifying the viral polymerase was modified in order to decrease the loss of protein during dialysis. Rather than using the Amicon Centricon-10 dialysis system, the enzyme was dialyzed in standard membranes, which resulted in higher concentrations of all four viral core proteins. The pattern of the protein gel of this preparation was identical to that shown in Fig. 1, lane 4, except that there was no bovine serum albumin-derived band (data not shown). It was found that 5 μl of this preparation, containing 150 ng of NP and 5 ng total of the three polymerase proteins, reacted optimally with 10 to 40 ng of model RNA template. However, the use of higher concentrations of protein increased the background, possibly because of higher levels of contaminating RNAs (virion RNAs not removed by CsCl centrifugation), yielding products of the size class around 50 to 75 nt, complicating analysis of RNA templates containing a length of 50 nt.

This high-concentration polymerase preparation was now active in cap endonuclease-primed RNA synthesis (Fig. 8A, lane 4) and also in primer-independent replication of the template RNA (Fig. 8A, lane 2). When globin mRNA was used as a primer for transcription from the 30-nt V-d5′

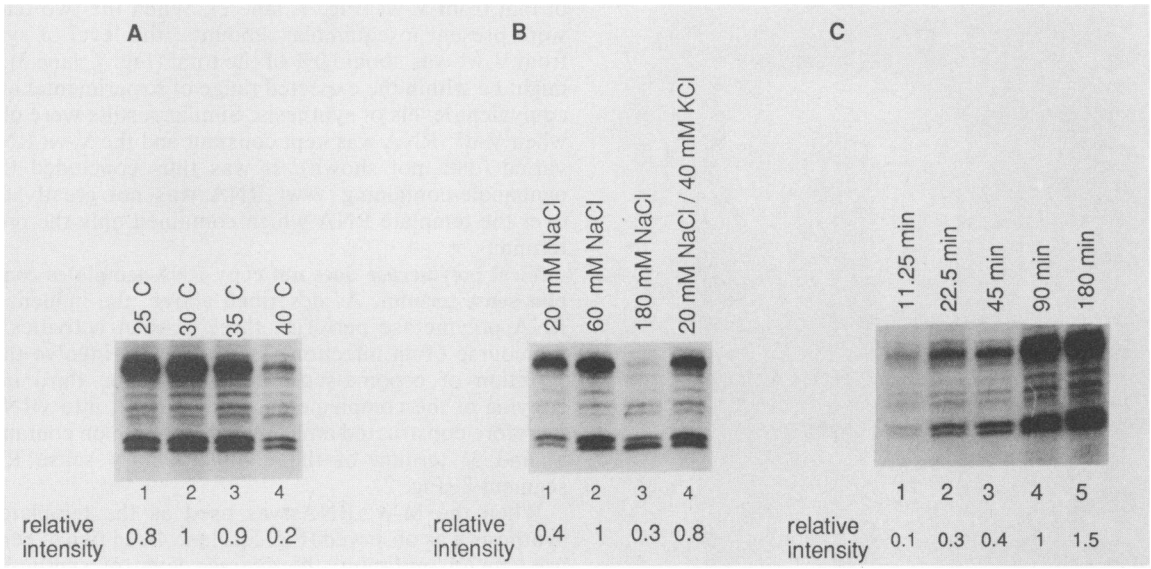

FIG. 4. Optimal reaction conditions for the viral polymerase. (A) Reaction mixtures with the V-wt template were assembled on ice and then incubated at the indicated temperatures for 90 min. (B) Reaction mixtures with the V-wt template were prepared in parallel with the indicated NaCl or KCl concentrations and were incubated at 30°C for 90 min. (C) A single reaction volume with the V-wt template was incubated at 30°C, and at the indicated times samples were removed and immediately processed by phenol-chloroform extraction. All gels contained 8% polyacrylamide with 7.7 M urea.

template, a triplet of bands of about 42 to 44 nt was apparent as a product (Fig. 8A, lane 4), consistent with cleavage of the cap structure at about 12 nt from the 5′ end of the mRNA and use of this oligonucleotide to initiate synthesis from the 30-nt model template. Since excess RNA inhibits RNA synthesis, probably via nonspecific binding of NP, as discussed above, the optimal amount of cap donor RNA added to each reaction mixture was found to be 100 ng, which is much lower than is usually used with preformed RNP structures (3). The most effective primer was ApG (Fig. 8A, lane 5 and lighter exposure in lane 6). The product migrated more slowly than that of the input template (Fig. 8A, lane 1) or the product in the absence of primer (Fig. 8A, lane 2), probably since the 5′ terminus of the ApG product is unphosphorylated. The intensity of the ApG-primed product was about 10-fold higher than that of the cap-primed product, but at 0.4 mM ApG was at a 60,000-fold molar excess of the concentration of the cap donors. Thus, although the intensity of the product band from cap priming was about 10-fold lower than that from ApG priming, the cap-primed reaction was about 6,000-fold more efficient on a molar basis. This value is similar to the approximately 4,000-fold excess efficiency

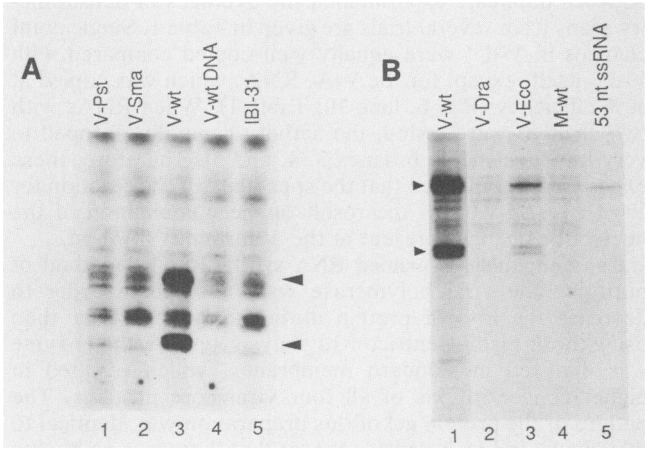

FIG. 5. Template specificity of the viral polymerase. (A) Viral polymerase reaction requires 3′-terminal promoter sequences. Different template RNAs were used in reactions under standard conditions. Lanes: 1, V-Pst RNA, which is identical to V-wt except that it has a 13-nt extension at the 3′ end; 2, V-Sma RNA, which has a 38-nt extension at the 3′ end; 3, V-wt RNA; 4, a DNA polynucleotide with a sequence identical to that of the V-wt RNA; 5, an 80-nt RNA generated by bacteriophage T3 RNA polymerase transcription of a pIBI-31 plasmid digested with *Hin*dIII. The autoradiograph was overexposed in order to emphasize the absence of specific reaction products when these templates were used. (B) A 10-ng sample of each template RNA was incubated with the viral polymerase, and the products were then subjected to electrophoresis on 8% polyacrylamide gels containing 7.7 M urea. Lanes: 1, V-wt RNA; 2, V-Dra RNA; 3, V-Eco RNA; 4, M-wt RNA. For the exact sequence differences, refer to Fig. 2 and Materials and Methods. Arrows indicate V-wt-specific transcripts of 53 and 44 nt.

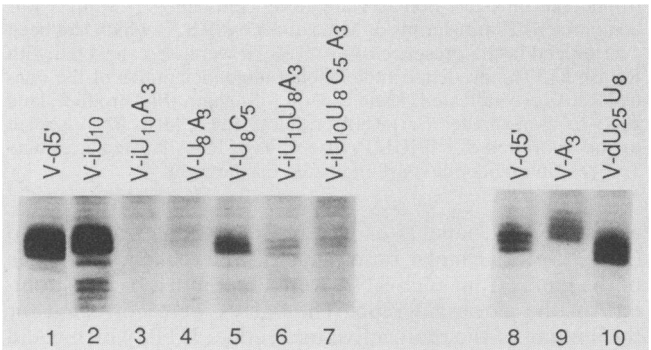

FIG. 6. Specificity of promoter sequences. RNAs which lacked the 5′ terminus and contained point mutations (Table 1) were compared with V-d5′ RNA in standard polymerase reactions. The right panel is from a separate reaction set. Quantitative comparison is outlined in Table 1.

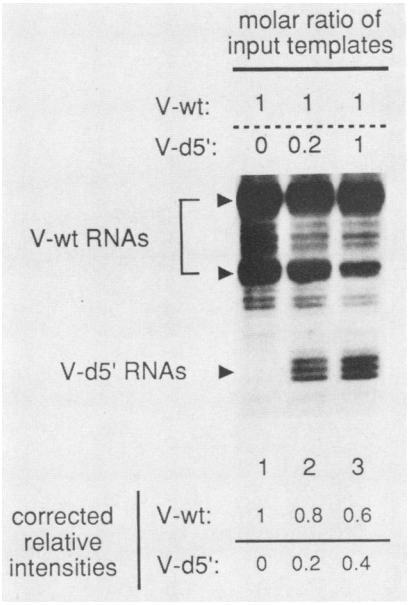

FIG. 7. RNA promoter does not require a terminal panhandle, as indicated by a polymerase reaction using two template RNAs. Each reaction mixture contained 5 ng of V-wt RNA. As a second template, the reaction mixtures contained 0 (lane 1), 0.6 (lane 2), and 3.0 (lane 3) ng of V-d5′ RNA. The resulting molar ratios are indicated. The reaction products were analyzed on an 8% polyacrylamide gel in the presence of 7.7 M urea. Following densitometry analysis of autoradiographs, the relative intensity of each peak was corrected for the amount of radioactive UMP which was incorporated in each product.

observed previously for the viral polymerase (3). It has been previously shown that cap donor RNAs containing a cap 0 structure, as in BMV RNA, are about 10-fold less active in priming the influenza virus polymerase (3). This unusual cap specificity was shared by the reconstituted RNPs studied here, as the specific product from the model RNA was greatly decreased in reaction mixtures containing BMV RNA as the cap donor. A 30-nt product was observed (Fig. 8A, lanes 2 to 4), probably because of primerless replication of the model template.

That the product RNAs were of the opposite sense of the input template V-d5′ was shown by nuclease S1 analysis (Fig. 8B). The ApG-primed (Fig. 8B, lanes 1 and 2) and the primerless (Fig. 8B, lanes 3 and 4) RNA products were essentially nuclease resistant. The product of the cap-primed reaction (Fig. 8B, lanes 5 and 6) was partially sensitive to nuclease, as about 12 nt were digested from the product. These results were most consistent with the 5′ 12 nt being of mRNA origin, as has been shown many times for influenza virus-specific mRNA synthesis.

TABLE 1. Quantitative comparison of the effect of point mutations in the promoter sequence[a]

Template[b]	3′ Sequence	Relative level of RNA synthesis
V-d5′	CACCCUGCUUUUGCU −OH	1
V-A_3	CACCCUGCUUUUACU −OH	0.4
V-C_5	CACCCUGCUUCUGCU −OH	1.0
V-$dU_{25}U_8$	CACCCUGUUUUUGCU −OH	1.0
V-U_8A_3	CACCCUGUUUUUACU −OH	0.08
V-U_8C_5	CACCCUGUUUCUGCU −OH	0.3
V-iU_{10}	CACCCUUGCUUUUGCU−OH	0.7
V-$iU_{10}A_3$	CACCCUUGCUUUUACU−OH	0.06
V-$iU_{10}U_8A_3$	CACCCUUGUUUUUACU−OH	0.2
V-$iU_{10}U_8C_5A_3$	CACCCUUGUUUCUACU−OH	0.2

[a] Sequence of V-d5′ is shown in Fig. 2. All other RNAs are identical to V-d5′, except for the indicated positions.

[b] Subscripted numbers indicate the distance from the 3′ end, and d and i refer to deleted or inserted nucleotides, respectively.

The promoter specificity of this polymerase preparation in reactions primed with ApG was found to be essentially identical to that for the lower-concentration enzyme, as shown earlier (data not shown). However, attempts thus far to perform similar analyses of promoter specificity with the primerless and cap-primed reactions have been frustrated by the comparatively high levels of background, which make quantitation difficult.

Transcription of genome-length RNA templates. A full-length 890-nt RNA identical in sequence to A/WSN/33 segment 8 was prepared by T7 RNA polymerase transcription of plasmid DNA, pHgaNS, which had been digested with restriction endonuclease *Hga*I. This RNA was copied in ApG-primed reaction mixtures containing 10 μl of the high-concentration polymerase (Fig. 9, lane 8). That the RNA was in fact a copy of the template was demonstrated by its resistance to nuclease S1 (Fig. 9, lane 9). A similar product was observed in the absence of primer (Fig. 9, lanes 2 and 3). That these product RNAs contained full-length copies of the template was confirmed by RNase T_1 analysis (data not shown). Virion RNA purified from phenol-extracted A/PR/8/34 virus was similarly copied in an ApG-primed reaction (Fig. 9, lanes 10 and 11) and in the absence of primer (Fig. 9, lanes 4 and 5). In addition, we found that when whole viral RNA was used in the reconstituted RNPs, the level of acid-precipitable counts was about 70% of that observed with native RNPs (data not shown). The viral polymerase was also able to copy these full-length RNAs when globin mRNA was used in a cap-primed reaction (data not shown).

DISCUSSION

RNA synthesis from a model template RNA. This study is the first to define promoter sequences for a polymerase of a negative-sense RNA virus, and it was found that the specificity lies in the 3′-terminal 15 nt. The RNA synthesis by the viral polymerase studied here is a model for specific recognition and elongation by the influenza virus polymerase for the following reasons. (i) The polymerase has high activity when primed with ApG, a feature unique to influenza virus polymerase. (ii) It has optimal activity at temperature and ionic conditions previously shown to be effective for the viral RNPs. (iii) The polymerase is specific for influenza virus sequences on the model RNA templates. (iv) The polymerase is active in the cap endonuclease-primed RNA synthesis which is a hallmark of the influenza virus polymerase. (v) Recognition of cap donor RNA is specific to cap 1 structures. (vi) Genomic RNA segments are specifically copied.

A terminal panhandle is not required for optimal recognition and synthesis by the viral polymerase. We have previously shown that the influenza virus segment RNAs base pair at their termini to form panhandle structures. This was achieved by two methods. A cross-linking reagent derivative of psoralen covalently bound the termini of each segment in intact virus or in RNPs from infected cells (11). The treated RNA was seen by electron microscopy to be circular, by virtue of the cross-linked termini. Similarly, the RNA ter-

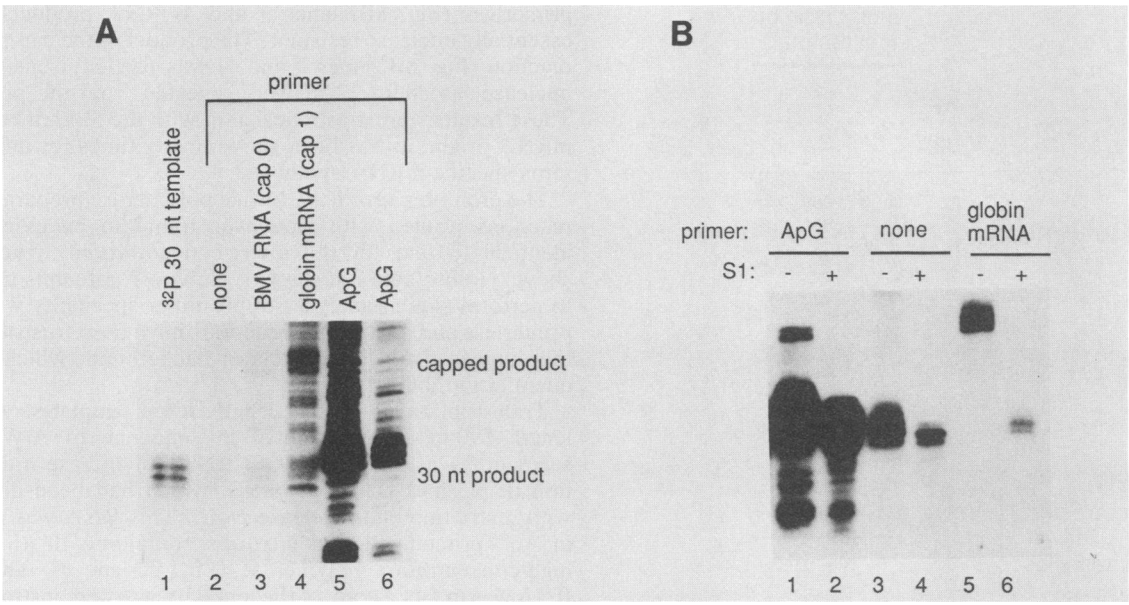

FIG. 8. High-concentration polymerase preparations active in cap endonuclease-primed and primerless RNA synthesis reactions. (A) Primer specificity of the high-concentration enzyme. Radioactively synthesized 30-nt template is in lane 1. Reaction experiments using 20 ng of V-d5′ RNA and 5 μl of viral polymerase contained the following as primer: none (lane 2), 100 ng of BMV RNA (4) containing a cap 0 structure (lane 3), 100 ng of rabbit globin mRNA containing a cap 1 structure, (lane 4), and 0.4 mM ApG (lane 5). A lighter exposure of lane 5 is shown as lane 6. (B) Nuclease S1 analysis of gel-purified RNAs. Products from reaction experiments using as a primer ApG (lanes 1 and 2), none (lanes 3 and 4), or globin mRNA (lanes 5 and 6) were electrophoresed in the absence of urea, the appropriate gel piece was excised, and the RNA was eluted. This RNA was then digested with nuclease S1 (lanes 2, 4, and 6), and the products were denatured and analyzed on an 8% polyacrylamide gel containing 7.7 M urea.

mini in RNPs were found to be sensitive to RNase V_1, which recognizes and cleaves double-stranded RNA, and the viral polymerase was found to be bound to both termini in the panhandle conformation (10). In these studies the panhandle structure of the genomic RNA was shown to exist, and it was inferred to play a role in polymerase recognition. Although the template RNAs in the present study were originally prepared to reveal panhandle-specific protein binding, it was found that the terminal panhandle had no obvious role in the polymerase reactions studied here.

Purified RNA polymerase specifically copies negative-sense templates. The viral polymerase was shown to synthesize RNA with optimal efficiency if the template had the wild-type negative-sense 3′ terminus. It was shown that RNAs of unrelated sequence were not copied and that those with extra polylinker sequences on the 3′ end were much less efficiently copied. A DNA of the correct sequence was similarly unsuitable as a template. The reaction was highly specific, since the M-wt template was replicated only at very low levels. Even though our source of polymerase was intact virus, this finding was very surprising, since it had never been suggested that the polymerase which recognizes the virus sense RNA would not efficiently copy the plus-sense strand. Studies are under way to examine the specificity of the polymerase purified from infected cells at times postinfection when the cRNA is copied into genomic templates. The present data support a model whereby the viral polymerase which copies vRNA is functionally different from that which synthesizes vRNA from cRNA by virtue of promoter recognition. It is possible that by regulated modification of the polymerase in infected cells the polymerase then becomes capable of recognizing the 3′ terminus of plus-sense RNA. By analyzing promoter mutants, we investigated the fine specificity of the reaction and found that the only single mutation which generated a significantly lower level of synthesis was that of V-A_3 RNA. Furthermore, combinations of two or more point changes in positions 3, 5, 8, and 10 greatly lowered synthesis levels.

High concentrations of polymerase are required for cap-primed RNA synthesis. The original polymerase preparation did not catalyze cap endonuclease-primed transcription under the specified conditions. However, fivefold-higher amounts of this original polymerase preparation (data not shown) or new polymerase prepared at higher concentrations were both able to catalyze this virus-specific reaction. It has been observed that although the NP selectively encapsidates influenza virus vRNA or cRNA in vivo, in vitro the NP will bind to RNA nonspecifically (15, 23). Presumably, in order for the viral template RNAs to be recognized by the viral polymerase in our in vitro reaction, they have to be encapsidated by the NP. Therefore, the addition of a capped mRNA primer would essentially compete with the template RNA for binding of NP. Since the dinucleotide ApG would not be expected to bind NP, the low-concentration polymerase was able to use only the short templates with ApG. Supporting this hypothesis is the observation that the higher-concentration polymerase preparation is inhibited through the addition of progressively higher amounts of either template RNA or any nonspecific RNA (data not shown). It should also be noted that the unusual specificity for the m7GpppXm cap 1 structure previously shown with viral RNPs was also found with the reconstituted RNPs.

Genome-length RNA templates are efficiently copied. Plasmid-derived RNA identical to segment 8 of the A/WSN/33 virus was specifically copied by the polymerase. In reactions using RNA extracted from virus, all eight segments were copied, although the HA gene was copied at a lower level. The background in these reactions was decreased in com-

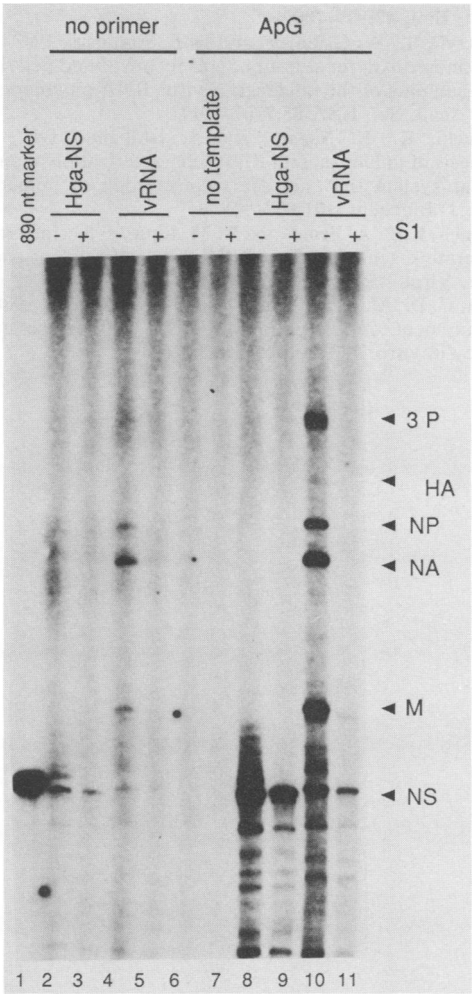

FIG. 9. Genome-length RNA synthesis from reconstituted RNPs. Products of reaction experiments using 10 μl of polymerase and, as templates, an 890-nt RNA identical to the sequence of segment 8 of virus A/WSN/33 and RNA extracted from A/PR/8/34 virus were analyzed on a 4% polyacrylamide gel containing 7.7 M urea. An 890-nt template synthesized radioactively by T7 RNA polymerase is shown (lane 1). The 890-nt plasmid-derived RNA was used as a template in lanes 2, 3, 8, and 9. RNA extracted from virus was used as a template in lanes 4, 5, 10, and 11. No template was used in lanes 6 and 7. No primer was used in lanes 2 to 5, and ApG was used as a primer in lanes 6 to 11. Reaction products were treated with nuclease S1 in lanes 3, 5, 7, 9, and 11. HA, Hemagglutinin; NA, neuraminidase; NS, nonstructural genes.

parison with those of the 30- to 53-nt templates, probably since the contaminating RNAs in the polymerase preparation were predominantly defective RNAs of small size. We are presently analyzing the genome-length transcripts to determine whether they are full-length or terminated at the poly(A) addition site. We are also endeavoring to have foreign genes transcribed in this system as a step toward rescuing the engineered gene in a virus particle.

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LITERATURE CITED

1. **Beaton, A. R., and R. M. Krug.** 1986. Transcription antitermination during influenza viral template RNA synthesis requires the nucleocapsid protein and the absence of a 5′ capped end. Proc. Natl. Acad. Sci. USA **83**:6282–6286.
2. **Bishop, D. H. L., J. F. Obijeski, and R. W. Simpson.** 1971. Transcription of the influenza ribonucleic acid genome by a virion polymerase. I. Optimal conditions for in vitro activity of the ribonucleic acid-dependent ribonucleic acid polymerase. J. Virol. **8**:66–73.
3. **Bouloy, M., S. J. Plotch, and R. M. Krug.** 1980. Both the 7-methyl and the 2′-O-methyl groups in the cap of mRNA strongly influence its ability to act as primer for influenza virus RNA transcription. Proc. Natl. Acad. Sci. USA **77**:3952–3956.
4. **De, B. P., and A. K. Banerjee.** 1985. Requirements and functions of vesicular stomatitis virus L and NS proteins in the transcription process in vitro. Biochem. Biophys. Res. Commun. **126**: 40–49.
5. **Dreher, T. W., J. J. Bujarski, and T. C. Hall.** 1984. Mutant viral RNAs synthesized *in vitro* show altered aminoacylation and replicase template activities. Nature (London) **311**:171–175.
6. **Dreher, T. W., and T. C. Hall.** 1988. Mutational analysis of the sequence and structural requirements in brome mosaic virus RNA for minus strand promoter activity. J. Mol. Biol. **201**: 31–40.
7. **Emerson, S. U., and Y.-H. Yu.** 1975. Both NS and L proteins are required for in vitro RNA synthesis by vesicular stomatitis virus. J. Virol. **15**:1348–1356.
8. **Hay, A. J., B. Lomniczi, A. R. Bellamy, and J. J. Skehel.** 1977. Transcription of the influenza virus genome. Virology **83**:337–355.
9. **Honda, A., K. Ueda, K. Nagata, and A. Ishihama.** 1987. Identification of the RNA polymerase-binding site on genome RNA of influenza virus. J. Biochem. **102**:1241–1249.
10. **Honda, A., K. Ueda, K. Nagata, and A. Ishihama.** 1988. RNA polymerase of influenza virus: role of NP on RNA chain elongation. J. Biochem. **104**:1021–1026.
11. **Hsu, M.-T., J. D. Parvin, S. Gupta, M. Krystal, and P. Palese.** 1987. Genomic RNAs of influenza viruses are held in a circular conformation in virions and in infected cells by a terminal panhandle. Proc. Natl. Acad. Sci. USA **84**:8140–8144.
12. **Ishihama, A., and K. Nagata.** 1988. Viral RNA polymerases. Crit. Rev. Biochem. **23**:27–76.
13. **Kaplan, G., J. Lubinski, A. Dasgupta, and V. R. Racaniello.** 1985. In vitro synthesis of infectious poliovirus RNA. Proc. Natl. Acad. Sci. USA **82**:8424–8428.
14. **Kato, A., K. Mizumoto, and A. Ishihama.** 1985. Purification and enzymatic properties of an RNA polymerase-RNA complex from influenza virus. Virus Res. **3**:115–127.
15. **Kingsbury, D. W., I. M. Jones, and K. G. Murti.** 1987. Assembly of influenza ribonucleoprotein in vitro using recombinant nucleoprotein. Virology **156**:396–403.
16. **Krug, R. M.** 1983. Transcription and replication of influenza viruses, p. 70–98. *In* P. Palese and D. W. Kingsbury (ed.), Genetics of influenza viruses. Springer-Verlag, New York.
17. **Levis, R., B. G. Weiss, M. Tsiang, H. Huang, and S. Schlesinger.** 1986. Deletion mapping of Sindbis virus DI RNAs derived from cDNAs defines the sequences essential for replication and packaging. Cell **44**:137–145.
18. **Mirakhur, B., and R. W. Peluso.** 1988. *In vitro* assembly of a functional nucleocapsid from the negative-stranded genome RNA of a defective interfering particle of vesicular stomatitis virus. Proc. Natl. Acad. Sci. USA **85**:7511–7515.
19. **Naito, S., and A. Ishihama.** 1976. Structure and function of RNA polymerase from vesicular stomatitis virus. J. Biol. Chem. **251**:4307–4314.
20. **Plotch, S. J., M. Bouloy, I. Ulmanen, and R. M. Krug.** 1981. A unique cap(m7GppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. Cell **23**:847–858.

21. **Robertson, J. S., M. Schubert, and R. A. Lazzarini.** 1981. Polyadenylation sites for influenza virus mRNA. J. Virol. **38:** 157–163.
22. **Rochavansky, O. M.** 1976. RNA synthesis by ribonucleoprotein-polymerase complexes isolated from influenza virus. Virology **73:**327–338.
23. **Scholtissek, C., and H. Becht.** 1971. Binding of ribonucleic acids to the RNP-antigen protein of influenza virus. J. Gen. Virol. **10:**11–16.
24. **Shapiro, G. I., and R. M. Krug.** 1988. Influenza virus RNA replication in vitro: synthesis of viral template RNAs and virion RNAs in the absence of an added primer. J. Virol. **62:**2285–2290.
25. **Studier, F. W., and J. J. Dunn.** 1983. Organization and expression of bacteriophage T7 DNA. Cold Spring Harbor Symp. Quant. Biol. **47:**999–1007.
26. **Szewczyk, B., W. G. Laver, and D. F. Summers.** 1988. Purification, thioredoxin renaturation, and reconstituted activity of the three subunits of the influenza A virus RNA polymerase. Proc. Natl. Acad. Sci. USA **85:**7907–7911.
27. **Takeuchi, K., K. Nagata, and A. Ishihama.** 1987. *In vitro* synthesis of influenza viral RNA: characterization of an isolated nuclear system that supports transcription of influenza viral RNA. J. Biochem. **101:**837–845.
28. **Ulmanen, I., B. A. Broni, and R. M. Krug.** 1983. Influenza virus temperature-sensitive cap (m7GpppNm)-dependent endonuclease. J. Virol. **45:**27–35.
29. **Ward, C. D., M. A. M. Stokes, and J. B. Flanegan.** 1988. Direct measurement of the poliovirus RNA polymerase error frequency in vitro. J. Virol. **62:**558–562.