Influenza A Virus RNA-Dependent RNA Polymerase: Analysis of RNA Synthesis In Vitro

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Influenza A virus RNA-dependent RNA polymerase, purified from virion ribonucleoprotein particles and from which endogenous genomic RNA (vRNA) has been depleted by treatment with micrococcal nuclease, was used to study transcription initiation, elongation, and termination in vitro. Templates that contained either minus- or plus-sense influenza virus nucleoprotein minigenes with conserved 5' and 3' termini and the uridylylate tract were constructed. The dinucleotide ApG and alfalfa mosaic virus RNA4 (AIMV4) were used as primers. ApG primed the synthesis of full-length positive-strand or cRNA products and shorter transcripts, depending upon the molar ratio between the nucleoprotein and the vRNA template. Sequence analysis of the ends of these transcripts demonstrated that the 5' termini of both transcripts and the 3' terminus of the full-length product were complementary to the 3' and 5' termini of the vRNA template, respectively, whereas the 3' terminus of the incomplete product corresponded to a sequence located 40 bases downstream from the 5' terminus of the template and was about 20 nucleotides downstream from the uridylylate tract, which is the putative signal for polyadenylation. Binding of the cap structure of AIMV4 by the polymerase activated RNA synthesis by ligation-elongation of small genomic RNA fragments which were likely derived from a genome segment protected by the polymerase from micrococcal nuclease digestion. The sequence of these fragments mapped to a region 14 to 28 nucleotides upstream of the 3' terminus of the viral genome. Polymerase subunit involvement in transcription initiation with ApG or AIMV4 was characterized by studying the effect of purified polyclonal antisubunit immunoglobulins of the G class (IgGs) in transcription assays. These results showed that anti-PB2 IgG inhibited transcription initiation in both ApG- and AIMV4-primed reactions, whereas anti-PB1 antibodies also blocked transcription initiated with AIMV4. The differences observed in product size, product sequence, and differential inhibition by antisubunit IgGs are discussed. These observations would support the notion that the influenza virus RNA-dependent RNA polymerase undergoes a conformational change after the binding of the cap structure of host cell heterogeneous nuclear RNA by PB2, which then usually leads to endonucleolytic cleavage of the capped primer 13 nucleotides downstream from the cap.

The influenza A virus genome is composed of eight segments of single-stranded RNA with negative polarity. In the virion particle, the segments are associated with the nucleoprotein (NP), forming a ribonucleoprotein (RNP) structure, which also associates with three virus-encoded polypeptides that constitute the RNA-dependent RNA polymerase. Transcription and replication of the viral genome occur in the nucleus of the infected cell and involve the synthesis of mRNA and cRNA from the negative-sense viral genome, genomic RNA (vRNA), and viral progeny RNAs from the cRNA molecules (14, 20). The RNA polymerase initiates mRNA synthesis at the 3' termini of the genome segments with a 10- to 13-nucleotide capped RNA fragment which is derived from the 5' termini of the host heterogeneous nuclear RNAs and which acts as a primer (2, 3, 19). RNA elongation then proceeds to a stretch of uridylylate residues which is 17 to 22 nucleotides from the 5' terminus of the vRNA and which serves as a transcription termination and polyadenylation signal (9, 22, 23, 31). Initiation of viral RNA replication at the precise 3' termini of vRNA or cRNA is primer independent and results in the synthesis of complete plus and minus RNA molecules (10, 30,

40). The mechanisms involved in the switching from transcription to replication are not understood, although several hypotheses have been postulated to account for the multiple enzymatic functions of the influenza virus polymerase (14). After primary transcription, new virus-encoded proteins are synthesized, which are able by themselves or in association with cellular factors to initiate transcription-replication of the viral genome (cRNA and vRNA synthesis). The three polymerase subunits and NP have been shown to be sufficient to carry out all the enzymatic functions necessary for replication of the genome (6, 13, 16, 25, 26). However, this result does not rule out the possible involvement of cellular factors in the regulation of polymerase functions. The biochemical functions of the polymerase have been studied by using RNP cores derived from detergent-disrupted virus and RNPs from nuclear extracts of infected cells. Virion-derived RNPs are able to transcribe endogenous templates when either a dinucleotide, adenylyl-(3'-5')-guanosine (ApG), or capped mRNA is used as the primer (18, 25). Nuclear extracts from infected cells also synthesize polyadenylated mRNA in the presence of capped RNA primers or full-length and polyadenylated transcripts in ApG-primed reactions (1, 37). Nuclear extracts of infected cells harvested at 6 h postinfection synthesize cRNA in the absence of exogenous primer as well as negative-sense vRNA from a cRNA template (35).

It has been suggested that the free pool of NP in the nucleus of the infected cell serves as an antitermination factor during cRNA synthesis and is essential for elongation in vRNA syn-

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thesis (1, 11, 12, 35). However, this soluble NP has not been shown directly to be involved in the switch of the polymerase from a primer-dependent to a primer-independent mode of RNA synthesis. A more defined biochemical system might be of value in establishing the difference between transcription and replication initiation and the factor(s) involved in this regulation.

To better understand the function of the polymerase in RNA synthesis and the role played by NP in its regulation, we used virion-derived polymerase from which endogenous template has been depleted by micrococcal nuclease digestion (33, 34) for transcription reactions with an RNA template containing all the putative regulatory signals of the influenza virus genome, with ApG or capped alpha mosaic virus RNA4 (AIMV4) being used as primers. We determined the sequences at the 3' and 5' termini of the transcription products in order to define the initiation and termination of transcription. We evaluated the effect of added baculovirus-expressed purified NP on transcription reactions primed with ApG or capped RNA molecules. To further characterize the role of the polymerase subunits in RNA synthesis, we investigated the effect of purified immunoglobulins of the G class (IgGs) against polymerase subunits in transcription reactions. These results suggested that depending upon the primer utilized, the influenza virus polymerase assumes different conformations which may influence the initiation, elongation, and termination of transcription.

MATERIALS AND METHODS

Virus production and preparation of micrococcal nuclease-treated RNPs (MT-RNPs). Influenza A virus WSN was grown in the allantoic cavities of 10-day-old embryonated chicken eggs. Allantoic fluid containing virus was harvested 48 h postinoculation and clarified by centrifugation at $1,000 \times g$, and then there was a second centrifugation at $80,000 \times g$ for 90 min at 4°C . The virus pellets were resuspended in a small volume of TNE (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA) and further purified by centrifugation at $100,000 \times g$ for 8 h on a 20 to 45% (wt/vol) potassium tartrate gradient in TNE. Virus bands were collected by syringe aspiration through a needle hole on the side of the centrifuge tube, diluted sixfold with TNE, and pelleted at $100,000 \times g$ for 30 min at 4°C . Virus was resuspended in TNE buffer and stored at -70°C until use.

MT-RNPs were prepared by incubating purified influenza virus with an equal volume of disrupting buffer (80 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] [pH 7.4], 1% Triton N-101, 2 mM dithiothreitol, 0.2% lysolcithin, 300 mM NaCl) for 1 h at 30°C . The disrupted virus was overlaid on a 25% glycerol cushion (40 mM HEPES [pH 7.4], 0.25% Triton N-101, 25% glycerol, 150 mM NaCl) and centrifuged for 3 h at $200,000 \times g$ at 4°C . The protein content of fractions taken from the top to the bottom of the tube was determined by silver staining and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing the RNPs were pooled and incubated at 37°C for 30 min with 100 U of micrococcal nuclease per ml in the presence of 1 mM CaCl_2 . After digestion of the RNP RNA, the nuclease was inactivated by adding EGTA [ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid] to a final concentration of 2 mM. The NP-polymerase complexes (150 μl) were applied to the top of (600 μl) a 10 to 30% (wt/vol) sucrose step gradient (in 40 mM HEPES [pH 7.4], 150 mM NaCl, and 1 mM EGTA) in a Beckman centrifuge tube (5 by 41 mm; volume, 0.8 ml) and centrifuged for 4 h at $210,000 \times g$. Fractions were analyzed on SDS-PAGE gels which had been silver stained. Fractions containing NP and the three polymerase subunits were dialyzed against HEPES buffer (40 mM HEPES [pH 7.4], 100 mM NaCl, and 1 mM EGTA) for 3 h and frozen at -70°C until use.

RNA template synthesis. Plasmid pBR322 carrying a full-length copy of the NP gene (genome segment 5) of influenza A virus (Udorn strain) was digested with restriction enzymes *Dra*III and *Sac*I, which cleaved the NP gene at positions 32, 505, and 1443. Digested DNA was separated by electrophoresis in a 1% agarose gel. Plasmid DNA containing the influenza virus 3' and 5' termini (4,515 bp) was excised from the gel and purified with Gene Clean (Bio 101). The protruding 3' termini created by the restriction enzymes were removed by T4 DNA polymerase 3'-5' exonuclease activity (32). Following ligation with T4 DNA ligase, the truncated NP gene was amplified by PCR with two sets of primers. The first pair of primers contained the following sequences: 5'-AG CAAAAGCAGGGTTAATAATCAC-3' and 5'-TAATACGACTCACTATA AGTAGAGAAACAAGGGTATTT-3', which corresponded to the 3' and 5' termini of the influenza virus NP gene. The sequences of the second pair of primers were as follows: 5'-AGTAGAAACAAGGGTATTTTCT-3' and 5'-TAA

FIG. 1. (A) A deleted NP gene (152 nt long) of vRNA sense was used as a template in a transcription reaction mixture containing RNP-derived polymerase from which endogenous influenza virus RNA had been depleted by treatment of isolated RNPs with micrococcal nuclease, as described in Materials and Methods. Radiolabeled RNA transcripts from the reaction were resolved in a 6% polyacrylamide-7 M urea gel and visualized by autoradiography. Lane M, 152-nt RNA marker; lane 1, transcription products of MT-RNPs (0.6 μg) in the presence of 0.4 mM ApG; lane 2, the same as lane 1 but without ApG; lane 3, 1 μg of truncated vRNA incubated with 0.6 μg of MT-RNPs in a transcription reaction mixture primed with 0.4 mM ApG; lane 4, a transcription reaction the same as that in lane 3 but primed with 1 μM AIMV4. (B) A cRNA template (152 nt long) was assayed in a transcription reaction as described above. Lane M, RNA marker; lane 1, transcription products of MT-RNPs (0.6 μg) in the presence of 0.4 mM ApG; lane 2, the same as lane 1 but without ApG. In lanes 3, 4, and 5, 1 μg of truncated cRNA was incubated with 0.6 μg of MT-RNPs in a transcription reaction mixture containing ApG, AIMV4, or a 12-nt-long oligoribonucleotide (Oligoribo) complementary to the 3' terminus of the cRNA.

TACGACTCACTATAAGCAAAGCAGGGTTA-3', corresponding to the 5' and 3' termini of segment, 5, respectively. The underlined portions of the primers indicate the sequence of the T7 promoter that was incorporated into the final PCR product. The bases in boldface indicate the mutations introduced into the T7 promoter, which would direct transcription initiation with a sequence corresponding to the 5' terminus of the influenza virus genome. The truncated NP

FIG. 2. The effect of template concentration on RNA synthesis was evaluated with transcription reaction mixtures containing (0.5 μ g) MT-RNPs with 0.4 mM ApG as primer. Lane M, 152-nt RNA marker; lanes 1 through 5, increasing amounts of template vRNA (0.05 to 1.6 μ g).

gene was amplified by PCR under standard conditions, using one or the other set of primers. Amplified DNA was resolved in a 1.5% agarose gel, excised from the gel, and purified by using the Qiaex DNA extraction protocol (Qiagen). DNA molecules with the T7 promoter located at the 3' terminus or the 5' terminus of the influenza virus sequence were used as templates in a standard transcription reaction with T7 RNA polymerase. Regardless of whether the T7 promoter was positioned at the 5' or 3' terminus of the influenza virus sequence, T7 polymerase would generate a 152-nucleotide (nt)-long RNA transcript with vRNA or cRNA polarity. The sequence of the vRNA corresponded to the first 28 and last 124 nt of the NP gene, and the cRNA was complementary to this sequence. These transcripts were then used as templates for in vitro analysis of polymerase activity. Using a similar approach, we also amplified a truncated DNA derived from RNA segment 7 (300 nt), which served as a template with T7 polymerase and generated longer RNA templates for polymerase assays.

NP expression and purification. Monolayer cultures of Sf9 cells were infected with an NP-baculovirus recombinant (8) at a multiplicity of infection of 5 PFU per cell. Subsequently, infected cultures were incubated at 28°C for 4 days. After 4 days, the cells were harvested by centrifugation at $200 \times g$ for 20 min at 4°C. The cell pellet was resuspended in 100 mM NaCl–10 mM Tris-HCl (pH 7.4) containing 1% Nonidet P-40, and the cells were disrupted in a Dounce homogenizer. The lysed cells were centrifuged at $800 \times g$ for 15 min, and the resultant nuclear pellet was resuspended in 0.1 M phosphate buffer, pH 7.4, containing 0.8 M NaCl, 0.4% Triton N-101, 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride. Nuclei were disrupted by consecutive sonication, and NP was brought into solution by rocking for 60 min at 4°C. The nuclear extract was clarified by centrifugation. The supernatant was diluted with 0.1 M phosphate buffer, pH 7.4, to bring the NaCl concentration to 0.3 M and adsorbed to an anti-NP immunofluorescence column for 6 h at 4°C. The column was washed extensively with 0.05 M Tris-HCl (pH 7.4), 0.5 M NaCl, 0.2% Triton X-100, and 10% glycerol; adsorbed material was eluted with 3 M potassium thiocyanate in 0.05 M Tris-HCl, pH 8.8. The protein content of the different fractions was assayed by the protein determination procedure of Heil and Zillig (8) and by SDS-PAGE analysis. The fractions which contained NP were pooled and dialyzed overnight against 0.05 M Tris-HCl (pH 7.6), 0.1 M NaCl, and 0.5 mM dithiothreitol in the presence of 1% lysophosphatidylcholine in the dialysis tube. The purified NP protein was further analyzed by Western blotting (immunoblotting), with a polyclonal rabbit antiserum against virion NP being used (8).

In vitro transcription reactions. Transcription reactions were carried out in a standard reaction mixture containing 50 mM HEPES (pH 7.4); 60 mM KCl; 5 mM magnesium acetate; 1 mM dithiothreitol; 4 U of RNasin (Promega, Madison, Wis.), 0.6 mM (each) ATP, GTP, and CTP; 50 μ M UTP; 25 μ Ci of [α - 32 P]UTP (3,000 Ci/mmol); 0.6 μ g of MT-RNPs; 0.5 μ g of template RNA. The reaction mixtures were incubated at 32°C for 120 min. After phenol-chloroform extraction, the samples were passed through a Sephadex G-50 column to remove free radiolabeled nucleotides, and then precipitation with 2.5 volumes of ethanol in the presence of tRNA as a carrier was performed. Precipitated material was resuspended in 6 μ l of loading buffer containing 80% formamide, 10% glycerol, 1 mM EDTA, 0.025% bromophenol blue, and xylene cyanol. Samples were loaded on a 6% polyacrylamide–7 M urea gel, which was run at 600 V until the bromophenol blue dye migrated one-half the length of the gel. Gels were dried

(unless otherwise indicated) and exposed to Kodak X-Omat AR-5 film. As indicated, lighter exposures were analyzed by scanning densitometry.

Sequencing of the termini of the transcription products. The sequences at the 5' and 3' termini of the transcription products were determined as follows. Transcripts in the gel were localized by superimposing the gel with a 5-h-exposed X-ray film. RNAs were eluted by soaking gel slices for 5 h in TNE containing 0.5 M ammonium acetate and then precipitating the RNAs with 2.5 volumes of absolute ethanol. After resuspension in 20 μ l of diethyl pyrocarbonate-treated water, the cap structure of AIMV4-primed transcripts was removed from the 5' terminus with tobacco acid pyrophosphatase (Epicentre Technology, Madison, Wis.), which exposes a free phosphate group at the 5' terminus (24). RNA decapping was carried out in a 20- μ l reaction mixture containing 50 mM sodium acetate (pH 6.0), 10 mM β -mercaptoethanol, 1 mM EDTA, and 10 U of tobacco acid pyrophosphatase enzyme. After 30 min of incubation at 37°C, the RNAs were phenol-chloroform extracted and ethanol precipitated. Subsequently, the RNA 5' and 3' termini were ligated with 50 U of T4 RNA-ligase (Pharmacia LKB Biotechnology) in a 50- μ l (final volume) ligation reaction mixture (75 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM ATP). The ligation mixture was incubated overnight at 4°C. Circularized RNAs were reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Pharmacia LKB Biotechnology) and a 17-nt DNA primer (5'-TTCATTACTCATGT CAA-3') complementary to a region 66 bases away from the 5'-3' junction. First-strand cDNA was further amplified by PCR in the presence of a second 18-nt DNA primer (5'-GGATCTTATTCTTCGGA-3') which was complementary to the first cDNA strand and back to back with the first primer. Amplified DNA was resolved by electrophoresis in a 2% agarose gel, and DNA corresponding to full-length and incomplete transcripts was excised and purified by using the QIAquick gel extraction protocol (Qiagen). Purified DNA was ligated to a cloning vector containing a 5' terminus T overhang (Invitrogen) with T4 DNA ligase at 12°C overnight. Competent *Escherichia coli* cells were transformed and plated on agarose plates containing X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) for colony selection. White colonies (plasmid plus insert) were tested by PCR with the set of primers described above. PCR reactions were

FIG. 3. (A) Baculovirus-expressed, purified NP was added in increasing amounts (indicated in milligrams above the lanes) to transcription reaction mixtures containing 0.4 mM ApG. MT-RNPs (0.6 μ g) were incubated with vRNA template (0.8 μ g) under standard conditions (lane 1). Lane M, 152-nt marker. (B) The conditions were similar to those described for panel A, except that AIMV4 was used as the primer.

FIG. 4. Sequence analysis of the RNA transcripts generated by the influenza virus polymerase in ApG-primed transcription reactions. The full-length and incomplete RNA transcripts were processed as described in Materials and Methods to generate double-stranded DNA which was sequenced directly with a primer (5'-GAAGGATCTTATTCTT-3') complementary to the vRNA strand of the DNA. (A and C) The sequences of the full-length (152-nt) and incomplete transcripts at the 3'-5' junction are shown. Boldface and lightface letters indicate the 5' terminus and 3' terminus of the same transcript. (B and D) The 5' and 3' terminal sequences of the transcription products in the cRNA sense are shown. The 5' termini of both transcripts corresponded precisely with the 5' terminus of the influenza virus cRNA. The 3' terminus of the full-length transcript also matched the 3' terminus of the influenza virus cRNA. However, the 3' terminus of the incomplete transcript corresponded to a sequence located 40 bases away from the terminus of the template.

analyzed by electrophoresis in low-melting-point 2% agarose gels, and DNA bands were excised from the gel and sequenced directly with a primer complementary to one or the other DNA strand (38). Briefly, agarose slices were mixed in a microcentrifuge tube with 2 μ l of 5% Nonidet P-40, 3 μ l of Sequenase version 2.0 buffer (United States Biochemical), 1 μ l of formamide, and 1 μ l of 10 μ M sequencing primer. The tubes were put in a boiling water bath for 5 min and then transferred to a dry ice-ethanol bath. The tubes were then centrifuged at 10,000 rpm (Eppendorf microcentrifuge) for 10 min at 4°C. Twelve microliters of the resulting supernatant was then used for sequencing according to a modified sequence chain termination reaction (39).

Antisera preparation and IgG purification. Baculovirus recombinants (PB1, PB2, PA, and NP) were used to express the polymerase subunits and NP in Sf9 insect cells. Proteins for immunization were prepared as described previously (36). Rabbits were inoculated intradermally with 100 μ g of protein emulsified in an equal volume of complete Freund's adjuvant, and this inoculation was followed 2 weeks later by a subcutaneous injection of 100 μ g of protein in incomplete Freund's adjuvant. Antibody responses were determined by Western blotting against purified virion proteins, with a serum dilution of 1:200 being used. Polyclonal IgGs against the polymerase subunits and NP were purified as described previously (36).

RESULTS

Transcription reaction with MT-RNPs. RNA polymerase in MT-RNPs was assayed for RNA synthesis in the presence of 0.4 mM ApG or in the absence of any primer. The intent of these studies was to determine if small RNA fragments of the genome were protected from nuclease digestion by NP or the polymerase and therefore might serve as templates for RNA synthesis. Figure 1A, lane 1, shows ApG-primed transcription products synthesized in the absence of added template. A smear of heterogeneous products that were derived from minus-sense genome RNA templates which had been protected

from nuclease digestion was generated. No RNA synthesis occurred in the absence of the ApG primer (Fig. 1A, lane 2). When a 152-nt-long minus-sense RNA template was added to the transcription reaction mixture in the presence of 0.4 mM ApG or AIMV4, the polymerase synthesized a 107-nt RNA product in the ApG-primed reaction mixture (Fig. 1A, lane 3) and a 119-nt-long RNA product in the AIMV4-primed reaction mixture (lane 4). The difference in the sizes of the products of the ApG- and AIMV4-primed reactions was most likely due to the incorporation of the cap plus 13 nt at the 5' terminus of the transcript when the polymerase initiated transcription with AIMV4. A small amount of full-length product was detectable when ApG was used as primer with the minus-sense template (Fig. 1A, lane 3). However, when AIMV4 was used, a product slightly larger than 152 nt was synthesized, probably because of the incorporation of the capped 13-nt primer (Fig. 1A, lane 4).

The same set of transcription reactions was performed with a 152-nt-long plus-sense RNA template with precise influenza virus termini. Background RNA synthesis in the presence of ApG without the addition of the template was similar to that shown above (Fig. 1B, lane 1). The addition of the exogenous plus-sense RNA template without ApG again showed no synthesis (Fig. 1B, lane 2); neither ApG (lane 3) nor AIMV4 (lane 4) nor a 13-nt-long oligoribonucleotide complementary to the 3' terminus of the template (lane 5) promoted significant RNA synthesis. With the addition of the 13-nt oligoribonucleotide, we attempted to see if this RNA would allow RNA elongation

FIG. 5. Sequence analysis of the RNA transcripts synthesized by the polymerase in a transcription reaction primed with AIMV4. RNA transcripts were sequenced as described in Materials and Methods. Panels A and C show the sequences corresponding to the 5' and 3' termini of the full-length transcript (lightface letters). Boldface letters indicate an insertion of 11 (A) and 10 (C) nucleotides between the 5' and 3' termini of the transcripts. A linear representation of the cRNA sense of the 5' and 3' termini of the sequence is depicted in panels B and D. Boldface letters show the fragment added to the 5' terminus of the transcripts, which corresponded to a sequence 14 or 18 bases upstream of the 3' terminus of influenza virus genome segment 5.

from a 3' OH, as has been observed with other RNA-dependent RNA polymerases (14). Although the plus-sense RNA did not serve as the template for RNA synthesis, it did eliminate the synthesis of the small RNA species transcribed from the nuclease-protected genomic RNA fragments (Fig. 1B, lanes 3 through 5). Therefore, it is likely that the polymerase was able to bind to the large excess of added cRNA template but was unable to initiate transcription from this template.

Effect of template concentration and addition of NP on transcription. In the experiments described above, the products were predominantly incomplete transcripts of the vRNA template and were probably incomplete because of strong stops upstream of the uridylyl tract. These results were obtained in RNA synthesis reactions in which the molar ratios of virion-derived NP and added template RNA were 0.5:1, much below the ratio required to completely wrap the 152-nt-long RNA template. Assuming one NP binds per 20 to 25 nt (5, 17), an NP-to-RNA molar ratio of 7:1 would be required to saturate the template. The NP concentrations in these reactions were determined by comparative silver staining as described previously (27). In order to study the effects of NP on in vitro RNA synthesis, we added increasing amounts of template RNA to transcription reaction mixtures containing MT-RNPs. The minigene vRNA template at concentrations of 0.05 to 1.6 μ g was incubated with MT-RNPs (0.6 μ g) in transcription reactions containing ApG (0.4 mM), and the products of the transcription reactions were analyzed on a 7 M urea–10% polyacrylamide sequencing gel. Figure 2, lanes 1 through 5, shows that increasing the amount of added template caused a

change in the length of the major RNA products. At lower template concentrations (Fig. 2, lanes 1 and 2), full-length transcripts as well as shorter RNA transcripts were made. The latter are likely to be background products from residual endogenous fragments which remained after micrococcal nuclease digestion (Fig. 1A and B, lanes 1) and which were completely suppressed when the added template concentration was low. The NP/RNA molar ratios in lanes 1 and 2 (Fig. 2) were 9.5:1 and 2.4:1, respectively. At higher template concentrations (0.6:1 and 0.3:1 molar ratios), the transcription products decreased in size from full-length transcripts to 107-nt-long transcripts and the amount of full-length product decreased (Fig. 2, lanes 4 and 5). Background products from residual exogenous RNA fragments also decreased as the amount of exogenous template was increased. At 0.4 μ g of RNA template (1.2:1), full-length and 107-nt transcripts were synthesized in roughly equal amounts and there were more intermediate-size RNAs (Fig. 2, lane 3). Reduction of the NP/RNA molar ratio correlated with a switch in size of the transcription products from full-length transcripts to 107-nt-long products.

A reciprocal experiment in which increasing amounts of baculovirus-expressed, purified NP were added to transcription reaction mixtures primed with ApG or AIMV4 and containing constant amounts of added RNA template was performed. The addition of baculovirus-expressed, purified NP to ApG-primed transcription reaction mixtures stimulated the synthesis of the 107-nt and larger products, including full-length products (Fig. 3A, lanes 3 through 5). The amount of NP added to the reaction mixtures (Fig. 3A, lanes 4 and 5) was sufficient to

FIG. 6. (A) Effect of purified anti-polymerase subunit IgGs on transcription reactions primed with ApG. MT-RNPs were preincubated for 30 min at 30°C with IgG (5 μ g) and were then added to a standard transcription reaction mixture. The samples were phenol extracted, and the synthesized RNAs were analyzed by electrophoresis in a 6% polyacrylamide gel containing 7 M urea. The controls as well as the IgGs tested are indicated at the top. Lane M, 152-nt marker. (B) Analyses similar to those described in the legend to panel A were carried out, with AIMV4 being substituted for ApG as the primer.

completely wrap the vRNA template (NP/template ratios of 6 to 9). Addition of purified NP to reaction mixtures primed with AIMV4 also led to a stimulation of the synthesis of both 107-nt and full-length products. However, addition of a 9.5:1 molar ratio of NP to template resulted in an inhibition of RNA synthesis. Of note is the fact that in reaction mixtures which contained equivalent amounts of template and NP, the reaction primed by ApG (Fig. 3A) synthesized less full-length RNA relative to 107-nt product and intermediate-size RNAs than did reactions primed by AIMV4 (Fig. 3B).

Sequence analysis of transcription reaction products. To determine the exact nature of the RNA products of the reactions described above, we sequenced the 5' and 3' termini of the transcription products generated by ApG- or AIMV4-primed synthesis. Individual RNA products were excised from gels, circularized, and used to generate cDNAs containing the junction between the two ends by reverse transcriptase-PCR (see Materials and Methods). Analysis of the sequences at the 3'-5' junction showed that the 5' termini of the full-length and 107-nt products were exactly complementary to the 3' terminus of the vRNA template. The 3' terminus of the full-length

product was complementary to the 5' terminus of the vRNA (Fig. 4A and C). However, the 3' terminus of the 107-nt RNA was complementary to a sequence 40 nt downstream of the 5' terminus of the vRNA template (Fig. 4). This premature termination of the smaller transcripts did not correlate with the stretch of uridylate residues which is the putative termination signal where the polymerase polyadenylates the transcript RNA. None of the reactions with the truncated NP minigene showed poly(A) addition. Analysis of the 5' terminal sequences of full-length and incomplete transcripts indicated that transcription occurred by an elongation reaction of the ApG base paired to the first two bases (UC) at the 3' terminus of the vRNA template (Fig. 4).

Sequence analysis of the RNA transcripts (162 nt) synthesized in transcription reactions primed with AIMV4 showed that the polymerase initiated transcription at the 3' terminus of the vRNA template by elongating an oligonucleotide with a size of about 10 to 12 nt which was incorporated as the 5' terminus of the transcript (Fig. 5). Unexpectedly, these sequences did not correspond to the sequence at the 5' terminus of the AIMV4 RNA. Furthermore, products of transcription usually are initiated at the second base at the 3' terminus of the vRNA template, and in this case, the RNA was initiated at the first base. The sequences added to the 5' terminus were oligonucleotides with sequences identical to that 14 or 28 bases downstream of the 3' terminus of influenza virus genome segment 5 (Fig. 5). A few clones which contained sequences of other genomic segments as well as sequences corresponding to the 5' terminus of AIMV4 were identified (data not shown). We do not know why segment 5-derived fragments predominated. We surmise that these sequences had been protected from micrococcal nuclease digestion of the RNPs and were then utilized by the polymerase to initiate transcription. The capped AIMV4, however, was required for activation of the polymerase in some undetermined reaction. The 3' termini of the transcripts were also exact copies of the 5' terminus of the vRNA template, showing that the polymerase reached the end of the template without stopping at the sequence 5 uridylates or polyadenylating (Fig. 5). The 3' terminus of the shorter transcript (Fig. 3B) corresponded to a sequence located about 40 nt downstream from the 5' terminus of the template (data not shown), as was the case for the ApG-primed reaction (Fig. 4).

Effect of polyclonal antibodies on the transcription reaction.

The effects of purified monospecific polyclonal IgGs against each of the three polymerase subunits and NP were evaluated with transcription reaction mixtures containing MT-RNPs and vRNA template. MT-RNPs and individual IgGs to PB1, PB2, PA, and NP were incubated for 30 min, and the incubation was followed by *in vitro* RNA synthesis primed with ApG or AIMV4. Anti-PB2 IgG was the only antibody which caused inhibition of RNA synthesis primed with ApG (Fig. 6A, lane 4). A similar set of reactions using AIMV4 as the primer was carried out, and under these conditions, both anti-PB1 and anti-PB2 completely inhibited RNA synthesis (Fig. 6B, lanes 3 and 4). Anti-PA and NP had no effect (Fig. 6B, lanes 5 and 6).

DISCUSSION

We have carried out experiments to further characterize RNA synthesis by the influenza A virus RNA-dependent RNA polymerase. We examined transcription initiation-elongation in a reconstituted transcription system *in vitro* with MT-RNPs serving as a source of polymerase and a deleted NP gene that contained all the putative regulatory sequences of the influenza virus genome. Transcription assays with either ApG or

FIG. 7. A model for the differential activations of the influenza virus polymerase by dinucleotide ApG and the cap structure containing AIMV4. (A) (I) PB1 subunit of the influenza virus polymerase binds ApG (II), activating the enzyme to initiate transcription at the 3' terminus of the vRNA (III). This mode of RNA synthesis was inhibited by anti-PB2 IgG. (B) (I) In AIMV4-primed transcription, the PB2 subunit binds the cap structure at the 5' terminus of the RNA, bringing about a conformational change in the enzyme that leads to the endonucleolytic cleavage of the RNA 13 bases downstream from the cap. However, in the *in vitro* reaction mixtures containing MT-RNPs, an RNA fragment protected from digestion by the polymerase somehow prevents the endonucleolytic cleavage of the AIMV4 primer or the use of it. Binding of the cap-1 structure by PB2 induces conformational changes that bring to the active site of the enzyme the 3' terminus of the RNA fragment (II), which is utilized for initiation of RNA synthesis (III). This reaction was inhibited by IgGs against PB2 and PB1, supporting the model of conformation differences between polymerases engaged in RNA synthesis initiated with ApG or AIMV4.

AIMV4 resulted in the stimulation of the synthesis of several species of RNA transcripts which included full-length vRNAs and 107-nt-long transcripts as the predominant species (Fig. 2). The relative amounts of these products depended upon which primer was used in the reaction and upon the ratio of NP protein to minigene template present (Fig. 2 and 3). The observation that the primers affected the size of the transcripts strongly suggested that transcription by the influenza virus RNA polymerase was somehow differently influenced by these two primers during elongation and termination, perhaps because of a change in the conformation of the polymerase. This hypothesis was supported by the finding that monospecific antibodies against the polymerase subunits showed a differential inhibition of transcription when they were added to the reaction mixtures containing the two primers. That is, anti-PB2 inhibited the reactions primed by ApG, but anti-PB1 and anti-PB2 inhibited those primed by AIMV4 (Fig. 6).

In Fig. 1A, we showed that the transcription reaction mixture to which MT-RNPs were added as the source of influenza virus RNA polymerase contained oligonucleotides representing segments of the RNP vRNA which had been protected from nuclease digestion by the polymerase and/or NP. These small vRNAs could serve as templates for transcription if a primer was provided (Fig. 1A). If the deleted NP minigene was

added as a template, synthesis from these small vRNA fragments was abolished, probably reflecting the greater affinity of the polymerase for the conserved 3' and 5' termini of this template (4, 7, 21, 22, 34, 38).

When the 3' and 5' termini of the two major RNA products of transcription reaction mixtures containing ApG or AIMV4 were sequenced, we obtained a surprising result which again reflected the fact that the polymerase functioned differently depending on the primer added to the reaction (Fig. 4 and 5). Sequence analysis of the 107-nt product from the ApG-primed reaction showed that the 5' terminus was a precise copy of the 3' terminus of the vRNA template and that the 3' terminus, the site of termination, corresponded to a site 40 nt downstream of the 5' terminus of the template. This site did not correlate with the uridylylate tract, the putative signal for termination and polyadenylation located 17 to 20 bases away from the 5' terminus of vRNAs. Furthermore, transcription reaction mixtures containing a deleted NP minigene with a size of 300 nt also resulted in the synthesis of a premature termination product whose 3' terminus was located about 20 nt downstream from the U tract (data not shown). Computer folding analysis of the 152- and 300-nt template RNAs did not predict any major secondary structure at these termination sites (data not shown). Therefore, we have no explanation why the polymer-

ase terminates at these sites 40 nt downstream from the 5' termini of these templates. Sequence analysis of the termini of the full-length transcripts from reactions primed with ApG at higher ratios of NP template showed that they were complete copies of the vRNA template (Fig. 4A and B). The sequence of products synthesized in reaction mixtures containing AIMV4 showed that there was a small RNA segment incorporated into the 5' terminus of the transcripts which did not arise from the 5' terminus of the AIMV4 primer. Rather, these segments corresponded to a sequence located 14 to 28 nt upstream of the 3' terminus of viral genome segment 5. This unexpected finding suggested that this sequence was bound to the polymerase in the viral RNPs and was protected from digestion by micrococcal nuclease. Surprisingly, these segments were used to initiate transcription, but only in the presence of AIMV4 as the primer. This result might suggest that when the polymerase bound a capped primer, it was somehow activated (15) to initiate synthesis and used the RNA bound at another site on the enzyme.

In all the in vitro reactions, the polymerase did not polyadenylate the transcripts. This lack of poly(A) addition, which had been observed by others in similarly reconstructed systems (28, 33, 34), sharply contrasts with the fact that detergent-permeabilized virion or core particles were able to add poly(A) to transcripts synthesized in vitro (29, 31). One might speculate that for the polymerase to carry out polyadenylation, it needs a factor(s) which has been lost during RNP purification or that the polymerase requires not only signal sequences within the template but also a correct structure not found in the in vitro system.

All of the above experiments suggested that the polymerase undergoes a conformational change(s) during transcription initiation-elongation. Figure 7 illustrates a model that could explain the differential stimulation of transcription initiation by ApG and AIMV4. In this model, ApG activates the polymerase by binding to the PB1 subunit, which then is able to initiate transcription at the 3' terminus of the vRNA template incorporating ApG into the 5' terminus of the transcription product. We do not know if activation of the polymerase releases the protected genomic RNA fragment. On the other hand, binding of the cap structure at the 5' terminus of AIMV4 by the PB2 subunit causes a conformational change in the polymerase which usually leads to the endonucleolytic cleavage of the capped primer RNA 13 bases downstream from the cap. However, because the site normally involved in the ligation of the capped primer and elongation was occupied by the protected oligonucleotide, the enzyme that had been activated by binding the capped AIMV4 initiates synthesis with the protected oligonucleotide. We do not know at this time whether or not the capped 5' terminus of AIMV4 is cleaved during this reaction. It has recently been reported by Cianci et al. (4) that the influenza virus polymerase was stimulated to bind capped mRNA by the addition of the 5' terminus of vRNA but that endonuclease activity required the addition of the 3' terminus. This result would support the notion that interaction with capped mRNA and with template does activate enzyme function, perhaps through conformational changes.

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