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Monoclonal Antibodies against Influenza Virus PB2 and NP Polypeptides Interfere with the Initiation Step of Viral mRNA Synthesis In Vitro

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Two panels of monoclonal antibodies (MAbs) specific for the influenza A virus PA and PB2 polypeptides have been obtained from mice immunized with denatured proteins produced in *Escherichia coli*. All MAbs (13 specific for the PA polypeptide and 8 specific for the PB2 protein) reacted to the corresponding influenza virus protein in Western blotting (immunoblotting), immunoprecipitation, and immunofluorescence assays. To gain information about the roles of the nucleoprotein (NP) and PB2 and PA proteins during viral mRNA synthesis, the 21 anti-P antibodies and 3 anti-NP antibodies (J. A. López, M. Guillen, A. Sánchez-Fauquier, and J. A. Melero, J. Virol. Methods 13:255–264, 1986) were purified and tested for their ability to inhibit the transcriptase activity associated with viral cores purified from virions. Four of the antibodies (one anti-PB2 and the three anti-NP MAbs) inhibited transcription by more than 50% compared with unrelated control antibodies. The inhibitory effect was not due to a nonspecific effect of the antibody preparations, because these MAbs did not inhibit transcription when tested on influenza B virus nucleocapsids, which are not recognized by the antibodies. To determine whether the antibodies were acting on an early transcription step, transcription reactions were carried out in the presence of globin mRNA (a mixture of α- and β-globin chains) and only one labeled nucleoside triphosphate (either GTP or CTP). The results obtained showed that MAbs to the PB2 and NP polypeptides interfered with the initiation step of mRNA-primed transcription. The implications of these results regarding initiation of viral mRNA synthesis are discussed.

The genome of influenza A virus consists of eight segments of negative-sense, single-stranded RNA molecules. In the viral particle, the RNA segments are complexed with the major viral structural protein, the nucleoprotein (NP), to form ribonucleoprotein (RNP) complexes (26, 28). Three virus-encoded polypeptides (PB1, PB2, and PA) (P proteins) (18), which constitute the viral polymerase, are associated with each RNP. Biochemical and genetic studies have established that the NP and the three P proteins are involved in the synthesis of the three classes of virus-specific RNA molecules detected in infected cells, i.e., (i) mRNA molecules which are capped and polyadenylated, (ii) virion RNA molecules found in the viral particle (vRNA molecules), and (iii) cRNA molecules which serve as templates for the synthesis of vRNA molecules (reviewed in references 29 and 32). Moreover, it has recently been shown that PB2, PB1, PA, and NP are the minimum set of viral proteins required for expression of influenza virus-like RNAs in vivo (11, 20, 25, 34).

Influenza virus transcriptase activity has been detected in disrupted virions (4), purified viral cores (43), and extracts prepared from infected cells (2, 13, 47). Viral transcriptase activity is highly stimulated by dinucleotides (ApG and GpC) (33, 39, 43) and by RNAs containing a type 1 cap structure at the 5′ end (6–8, 21, 24, 30, 38). Dinucleotide-primed transcripts initiate exactly at the 3′ end of the vRNA template and contain, at the 5′ end, the dinucleotide added to the reaction (39). In contrast, transcripts obtained in the presence of capped RNAs are 10 to 15 nucleotides longer than the dinucleotide-primed products. These additional nucleotides are generated by a virus-encoded endonucleolytic activity that cleaves the capped mRNAs 10 to 15 nucleotides from their 5′ ends, and these fragments are then used as primers for mRNA synthesis (6, 21, 37, 38, 42). It has been shown that viral cores incubated in the presence of capped RNAs and a single nucleoside triphosphate (NTP) are able to cleave the mRNA and link one nucleotide residue to the mRNA-derived fragment (9, 21, 24, 37), and it has been suggested that the incorporation of the first residue is directed by either the second or third residue (C or G, respectively) of the viral RNA template since either G or C residue, but neither A nor U, can be the first nucleotide incorporated under those in vitro assay conditions (24, 30).

Some of the P protein roles during the process of viral mRNA synthesis have been elucidated. It has been shown that the PB2 protein binds the cap 1 structure of host cellular mRNAs used as a primer for influenza virus transcription (5, 9, 50, 51) and that the PB1 polypeptide is involved in the elongation of the growing mRNA chain (9, 19, 51). No role in transcription has yet been assigned to the PA polypeptide, although this protein appears to be associated and move together with the other two P proteins during mRNA synthesis (9).

The functions of NP during virus-specific RNA synthesis are unclear. On one hand, the NP protein plays a major structural role in maintaining the RNP structure because the RNA can be displaced from purified viral nucleocapsids by polyvinyl sulfate or removed by treatment with RNase without disrupting the RNP structure (26, 40). On the other hand, it is known that NP is required for RNA replication, since temperature-sensitive mutants in the NP gene show defects in RNA synthesis (reviewed in reference 32), antibodies to NP inhibit

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the transcriptase activity associated with disrupted virions (52), and nucleocapsids in which most of the NP has been removed are unable to synthesize template-sized RNA transcripts (17, 23). Furthermore, there is a population of NP molecules in the infected cell which is not associated with nucleocapsids and is required for antitermination during mRNA synthesis and for elongation in vRNA synthesis (3, 47).

One of the major goals in influenza virus research is the characterization, at the molecular level, of the transcriptase-replicase complex(es) of the virus. This goal includes the delineation of the functionally relevant domains in each protein of the complex and those involved in the interaction between the different subunits. One way to address these issues is to produce monoclonal antibodies (MAbs) against the individual subunits and to use them as probes in structural and functional analyses. In this study we describe the preparation of two panels of MAbs specific for the influenza virus PA (13 MAbs) and PB2 (8 MAbs) polypeptides. These 21 antibodies along with 3 anti-NP antibodies previously described (31) were examined for their ability to inhibit the RNA transcriptase activity associated with purified viral cores in in vitro assays. The results obtained demonstrate that one of the anti-PB2 antibodies and the three anti-NP antibodies specifically inhibited the RNA polymerase activity. Moreover, we show that anti-PB2 and anti-NP antibodies interfere with the initiation phase of mRNA synthesis. This suggests that not only PB2 but also the NP polypeptide is involved in this step of RNA synthesis.

MATERIALS AND METHODS

Bacterial hosts and plasmids. The sources of PA and PB2 genes were plasmids pUPA and pUPB2, respectively (12). These plasmids contain a cDNA copy of the corresponding P gene of influenza virus A/Victoria/3/75 cloned in the polylinker of pUC18 vector. Plasmid pAR3040 was used as a vector for expression of the PA and PB2 polypeptides in *Escherichia coli* (44). This plasmid contains a T7 promoter, plus the protein initiation site of gene 10 (overlapping an *Nde*I restriction site) followed by a DNA sequence which acts as an efficient termination signal for the T7 RNA polymerase. Plasmids pARA-13 (which contains the full-length PA gene) and pARB2-8N (which includes a DNA fragment encoding the amino-terminal 402 amino acids of the PB2 gene) were obtained by subcloning the corresponding fragments into the *Nde*I site of vector pAR3040 (10). Analyses of the proteins induced by plasmids pARA-13 and pARB2-8N were carried out in *E. coli* BL21 (DE3)pLysS cells (49).

Analysis of plasmid-encoded proteins. Experiments were essentially done as described previously (49). Bacterial cultures were grown until the A_{600} reached 0.6 at 37°C in Luria-Bertani medium containing ampicillin (50 μg/ml) and chloramphenicol (25 μg/ml). Then, IPTG (isopropyl-β-D-thiogalactopyranoside) (5 mM) was added and the cultures were incubated for 150 min. Subsequently, the cells were chilled, pelleted, and resuspended in buffer A (100 mM NaCl–50 mM Tris · HCl [pH 8]–1 mM EDTA–5% glycerol) (1% of the original culture volume). Cell lysates were prepared by three consecutive freeze-thawing cycles, and soluble and insoluble proteins were separated into pellet and supernatant fractions by centrifugation at 10,000 × *g* for 10 min. The insoluble pellet was resuspended in buffer B (buffer A containing 0.1% sodium deoxycholate) and treated with DNase I (10 μg/ml) in the presence of 10 mM $MgCl_2$ for 4 h at 4°C. Then pellet and supernatant fractions were prepared as described above, and the pellet was washed in buffer D (buffer B containing an additional 0.4 M NaCl). The proteins present in the pellet fractions were resolved in a sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gel and visualized by Coomassie brilliant blue staining.

Purification of denatured PA and PB2 polypeptides. Proteins insoluble in buffer D were resuspended in SDS sample buffer and resolved by preparative SDS-polyacrylamide gel electrophoresis (PAGE). The running buffer contained Coomassie blue (0.1 g/liter) to allow visualization of stained polypeptides during electrophoresis. A gel slice containing the corresponding P polypeptide was cut, macerated, and incubated in 0.1 M NH_4HCO_3–0.01% SDS for 16 h at 4°C with shaking. Gel fragments were removed by centrifugation, and the supernatant containing the eluted P polypeptide was lyophilized and resuspended in water.

Immunization and preparation of hybridoma cell lines. BALB/c mice were injected intraperitoneally with 50 μg of recombinant protein mixed with complete Freund adjuvant. Two booster immunizations (50 μg of protein mixed with incomplete Freund adjuvant) were given at 3-week intervals. Two final injections, 50 μg of protein in phosphate-buffered saline (PBS), were given 4 and 2 days before the isolation of splenocytes. Hybridoma cells were obtained by standard procedures (27) using Sp2-0 myeloma cells (48). Hybridoma cells producing specific antibodies were identified by enzyme-linked immunosorbent assay (ELISA) using PA and PB2 proteins purified from bacteria as antigens. All positive hybridomas were subcloned at least twice before expansion. The anti-PA antibodies were designated PA/2, PA/8, PA/9, PA/10, PA/11, PA/12, PA/13, PA/14, PA/15, PA/16, PA/17, PA/18, and PA/20; the anti-PB2 antibodies were designated PB2/1, PB2/5, PB2/8, PB2/12, PB2/20, PB2/22, PB2/25, and PB2/28.

Purification of MAbs. The procedure used to purify the antibodies has been described elsewhere (15). Briefly, ascitic fluids containing antibody were adjusted to 1.5 M NaCl–0.75 M glycine (pH 8.9) and passed through a protein A–Sepharose CL-4B column by the fast protein liquid chromatography system. After extensive washing of the column with the same buffer, the antibodies were eluted with 0.1 M sodium citrate (pH 3), and the recovered fractions were immediately neutralized with 3 M Tris · HCl (pH 7.5). The antibody-containing fractions were identified by ELISA, pooled, dialyzed against PBS, and concentrated by centrifugation in a Centricon 30 microconcentrator (Amicon).

Immunoblot (Western blot) and immunoprecipitation assays. Immunoblot analysis was done as described previously (15). For immunoprecipitation assays, MDCK cell cultures were infected at a multiplicity of infection of 5 and labeled from 7 to 8 h postinfection with 500 μCi of [^{35}S]methionine. Cells were collected in PBS, pelleted, and lysed in a buffer containing 10 mM Tris · HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 0.5% sodium deoxycholate. Extracts were clarified by centrifugation at 10,000 × *g* for 5 min, adjusted to 1% SDS, and boiled for 3 min to dissociate P protein complexes (14). After boiling, the SDS concentration of the extracts was reduced to 0.1% by addition of an appropriate volume of the buffer described above. Immunoprecipitation assays were performed as previously described (41) using protein A-Sepharose beads. The antibody-bound proteins were resolved in an SDS–7.5% polyacrylamide gel containing 4 M urea.

Purification of viral cores. Influenza viruses A/Victoria/3/75 and B/Singapore/222/79 were grown in embryonated eggs, and viral cores were prepared as described previously (37, 43). Briefly, purified virus (2 to 4 mg) was centrifuged through a 30% sucrose cushion in TNE (10 mM Tris · HCl [pH 8], 100 mM NaCl, 1 mM EDTA), and the pellet was incubated at 30°C

for 10 min in 1 ml of a buffer containing 100 mM Tris · HCl (pH 8), 100 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 5% glycerol, 1.5% Triton N-101, and 1% lysolecithin. The mixture was then loaded onto a gradient which had four steps (1 ml each) of 33, 40, 60, and 70% glycerol (in 50 mM Tris · HCl [pH 7.8]–150 mM NaCl) and centrifuged at 45,000 rpm in an SW50.1 rotor. Fractions were then collected from the top, and those fractions containing viral cores lacking matrix protein (which sedimented to about the middle of the gradient) were pooled and stored frozen at −80°C.

RNA polymerase assay. Transcriptase activity of purified nucleocapsids was assayed as described for A/PR/8/34 virus (36). The effects of the MAbs on in vitro transcriptase activity were tested as follows. Each of the purified MAbs (10 μl) (see figure legends for concentrations) was preincubated for 30 min on ice with 1 to 3 μg of purified nucleocapsids in 18 μl of a reaction mixture containing 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.9), 50 mM NaCl, 5 mM $MgCl_2$, 0.05% Nonidet P-40, 1 mM dithiothreitol, and 1 U of human placental ribonuclease inhibitor per μl. The transcription reaction was initiated upon addition of a 7-μl sample containing the four NTPs (including [α-^{32}P]CTP) and a primer (either dinucleotide ApG [Pharmacia] or globin mRNA [Bethesda Research Laboratories]) diluted in the same buffer described above, and the mixture was incubated for 60 min at 30°C. Three aliquots were taken, one of them at 0 min and duplicate samples at 60 min. The amount of radioactivity incorporated was determined after trichloroacetic acid precipitation and Cerenkov counting. Total radioactivity incorporated in the reaction was calculated after subtracting background values (incorporation at 0 min) and taking into account the volume of the aliquot precipitated with trichloroacetic acid. The final concentrations of reagents in the reaction were as follows: MAb, 0.16 to 4 μg/μl; ApG, 0.4 mM; globin mRNA, 0.5 μg; ATP, UTP, and GTP, 500 μM each; and CTP, 30 to 40 μM (20 Ci/mmol).

Assays for the initiation of viral RNA transcription and for limited elongation of initiated products. Reaction conditions to test the effects of MAbs on initiation of mRNA synthesis were as described in the previous paragraph except that globin mRNA was always used as primer, and a single NTP (either [α-^{32}P]CTP or [α-^{32}P]GTP) (2.5 μM, 160 Ci/mmol) was included in the reaction. After 10 min of incubation at 30°C, the reaction mixture was separated into two aliquots, and one of them was treated with proteinase K and phenol extracted as described in the next paragraph. To determine whether the initiated products could be elongated, the other aliquot was supplemented with 200 μM ATP, GTP, and CTP and the incubation was continued for 30 more min before proteinase K treatment and phenol extraction.

Analyses of RNA products by electrophoresis. The reactions were terminated by addition of a buffer containing 100 mM Tris · HCl (pH 7.5), 150 mM NaCl, 12.5 mM EDTA, 1% SDS, and proteinase K (100 μg/ml). After incubation for 15 min at 37°C, the samples were extracted with phenol-chloroform, and the RNA products were recovered after ethanol precipitation in the presence of 2 μg of yeast tRNA. The reaction products were analyzed by electrophoresis in polyacrylamide gels containing 7 M urea and by autoradiography.

RNase T_1 analysis of reaction products. The two major labeled products obtained in the transcription initiation assay when CTP was used were eluted from a denaturing 20% polyacrylamide gel by incubating the gel fragments in water at 4°C for 16 h. The eluted RNA fragments were then mixed with 5 μg of tRNA and recovered by ethanol precipitation. The precipitate was resuspended in 5 μl of 10 mM Tris · HCl (pH 8)–1 mM EDTA and incubated with 140 U of RNase T_1 (Calbiochem) for 30 min at 37°C, and the digestion products were then analyzed by electrophoresis in a 20% polyacrylamide gel containing 7 M urea and by autoradiography. Markers included in the gel were (i) a ladder generated by incubating an RNA, labeled at its 5′ end with [γ-^{32}P]ATP, in a buffer containing 50 mM $NaHCO_3$-Na_2CO_3 (pH 9) for 8 min at 100°C and (ii) an RNA uniformly labeled with [α-^{32}P]CTP which was partially digested with RNase T_1. Both RNA transcripts were 0.6 kb in length and were derived by in vitro transcription with T7 RNA polymerase.

RESULTS

Expression of PA and PB2 polypeptides in *E. coli*. The facts that P proteins are produced in minute amounts in infected cells and that only a few molecules are present in virions made it difficult to obtain enough protein for preparing MAbs. Thus, cDNA copies of the PA and PB2 genes of influenza virus A/Victoria/3/75 (12) were cloned downstream of the T7 promoter of plasmid pAR3040 (pET-3a) (44) to make the corresponding viral protein in *E. coli*. The plasmids obtained, pARA-13 and pARB2-8N, contained a full-length copy of the PA gene and a cDNA fragment encoding the first 402 amino acids of the PB2 polypeptide, respectively (10). In both plasmids, the ATG initiation codon of the corresponding P gene was located 9 nucleotides downstream of the Shine-Dalgarno sequence present in the vector. This initiation codon would be the first in the predicted T7 mRNA transcripts, and it was thus expected that the viral proteins were initiated at their natural ATG codon.

The recombinant plasmids were then transferred to *E. coli* BL21(DE3)pLysS cells (49) for analysis of the expression of the target gene. Induction with IPTG resulted in the accumulation of polypeptides with the molecular weights expected from the cloned genes (Fig. 1, lanes 2). In both cases, virtually all the polypeptide expressed from the plasmid DNA was found in the pellet fraction of a cell lysate (lanes A) and was not solubilized in a buffer containing 0.5 M NaCl and 0.1% sodium deoxycholate (lanes D). The fact that under the latter conditions a significant fraction of the cellular proteins were solubilized facilitated the purification of the insoluble P polypeptides. Thus, the insoluble P polypeptides were excised from a preparative SDS-polyacrylamide gel, eluted, and used to immunize mice. A sample of the purified protein is shown in Fig. 1 (lanes P). Amino-terminal sequencing of the purified polypeptides confirmed that they were initiated at their natural initiation codon (data not shown).

Preparation and characterization of the anti-PA and anti-PB2 MAbs. Splenic lymphocytes from mice immunized with the corresponding recombinant polypeptide were used to produce 21 hybridoma cell lines which secreted antibodies (13 specific for the PA protein and 8 specific for the PB2 polypeptide) that recognized the viral immunogen in ELISAs (data not shown). MAb specificity was further confirmed by Western blotting and immunoprecipitation assays. In immunoblot assays, all 21 antibodies recognized the corresponding P polypeptide present in purified influenza virions (strain A/Victoria/3/75), as shown for antibodies PA/9 and PB2/28 in Fig. 2A. In addition, all 21 antibodies immunoprecipitated the corresponding P protein from influenza virus-infected cell extracts; representative results are presented in Fig. 2B. None of the MAbs showed reactivity with proteins from uninfected cells (data not shown). When the antibodies were tested by immunofluorescence, all of them stained the nuclei of influenza virus-infected cells (reference 35 and data not shown), a result

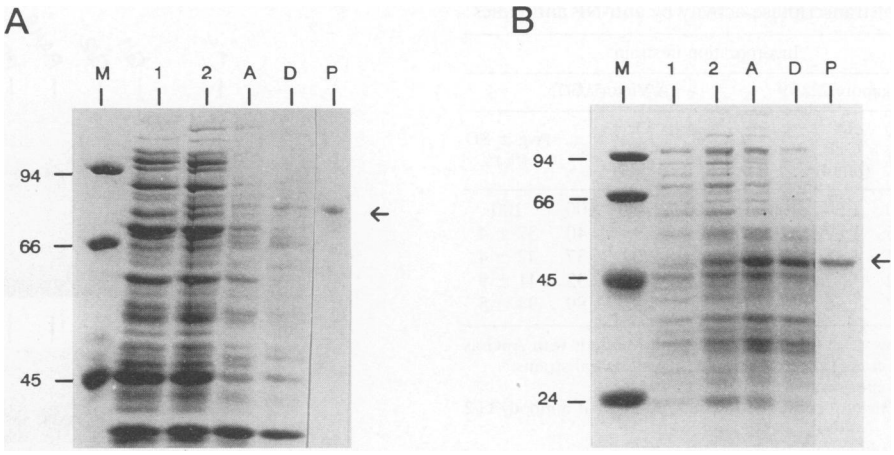

FIG. 1. Accumulation of PA and PB2 polypeptides in *E. coli* cells analyzed by SDS-PAGE and Coomassie blue staining. Analyses of the proteins expressed in *E. coli* BL21(DE3)pLysS bacteria carrying either plasmid pARA-13 (A) or plasmid pARB2-8N (B). Total cell proteins prepared from bacteria carrying the recombinant plasmid immediately before (lanes 1) or 150 min after (lanes 2) induction with IPTG are shown. Induced cells were lysed in a buffer containing 0.1 M NaCl (buffer A) (see Materials and Methods for details) and fractionated into supernatant and pellet fractions (lanes A) by low-speed centrifugation. The pellet fraction was then consecutively washed, by centrifugation, in buffers B (buffer A containing 0.1% sodium deoxycholate) and D (buffer B containing in addition 0.4 M NaCl); the pellet after the latter wash is shown in lanes D. Lanes P, a sample of the purified P polypeptide eluted from a preparative SDS-polyacrylamide gel; lanes M, molecular weight markers (positions indicated in thousands). Induced polypeptides are indicated by arrows.

which agrees with the reported nuclear location of the PA and PB2 polypeptides in infected cells (1, 22).

MAbs to the NP and PB2 polypeptides inhibit transcriptase activity associated with influenza virus purified cores. It was of interest to investigate whether the MAbs would affect the RNA synthesis activity associated with influenza virus purified nucleocapsids. For this purpose, the 13 anti-PA and the 8 anti-PB2 MAbs were purified from ascitic fluids by chromatography on protein A-Sepharose columns. Five other MAbs, three specific for the influenza virus NP (31) (MAbs M58/p51/G, M58/p44/E, and M58/p3/G, which hereafter will be referred to as NP/51, NP/44, and NP/3, respectively) and two against the G glycoprotein of respiratory syncytial virus (MAbs G/1 and G/2), were also purified (32a). A fixed volume of each of the 26 antibodies (5 to 41 μg of protein) was then preincubated with cores of influenza virus A/Victoria/3/75 before the transcription reaction was initiated by addition of the four NTPs (one of them labeled with ^{32}P) and either ApG or globin mRNA as a primer. The reactions were then allowed to proceed for 1 h, and the amount of radioactivity incorporated into RNA was measured after trichloroacetic acid precipitation. In the absence of any antibody the amount of radioactivity incorporated was 20 to 30% higher than in the presence of control antibodies G/1 and G/2, an effect which can be due to the extra amount of protein present in the transcription reactions done in the presence of antibodies. Thus, the amount of RNA synthesized in the presence of anti-A/Victoria/3/75 antibodies is here compared with that obtained in the presence of antibodies G/1 and G/2, which was considered to be 100%. Only 4 of the 24 anti-A/Victoria/3/75 antibodies (MAbs PB2/28, NP/3, NP/44, and NP/51) inhibited transcription as compared with control antibodies G/1 and G/2. The results obtained in a representative experiment with the three anti-NP antibodies and one of the noninhibitory antibodies (PA/11) are shown in Table 1. The anti-NP antibodies reduced the amount of RNA synthesized by more than 60% when either ApG (Table 1) or globin mRNA (not shown) was used as a primer. This inhibition was not due to a nonspecific effect or RNase contamination of the antibody preparations, since under the same assay conditions the antibodies did not inhibit the transcriptase activity of influenza virus B/Singapore/222/79

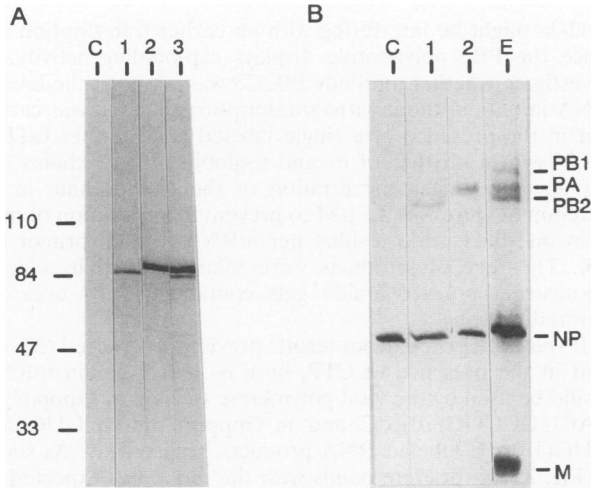

FIG. 2. Specificity of anti-P MAbs. (A) Immunoblot of the proteins present in purified virions of influenza virus A/Victoria/3/75 probed with antibody PB2/28 (lane 1), antibody PA/9 (lane 2), a mixture of antibodies PA/9 and PB2/28 (lane 3), and a negative control MAb (lane C). Molecular weights of prestained markers are indicated on the left in thousands. (B) Immunoprecipitation of ^{35}S-labeled cell extracts prepared from MDCK cells infected with influenza virus A/Victoria/3/75. Cell lysates were pretreated with 1% SDS, boiled for 3 min, and then adjusted by dilution to 0.1% SDS before addition of MAbs. Samples of treated extracts were immunoprecipitated with MAb PB2/28 (lane 1), MAb PA/9 (lane 2), or a negative control MAb (lane C), and antibody-bound proteins were resolved in an SDS–7.5% polyacrylamide gel containing 4 M urea. Lane E, cell extract utilized in the immunoprecipitation experiment. The positions of influenza virus proteins are indicated at the right.

TABLE 1. Inhibition of transcriptase activity by anti-NP antibodies[a]

MAb	Antibody concn (μg/μl)	Incorporation in strain: B/Singapore/222/79 Of ^{32}P (cpm)[b]	Of CTP (pmol)	%[c]	A/Victoria/3/75 Of ^{32}P (cpm)[b]	Of CTP (pmol)	%[c]	Avg ± SD (%)[d]
G/2	0.26	47,270	1.39	100	187,000	5.51	100	100
NP/51	0.18	56,805	1.67	120	75,619	2.23	40	37 ± 4
NP/44	0.45	49,664	1.46	105	69,454	2.04	37	42 ± 4
NP/3	0.45	51,592	1.52	109	61,047	1.80	32	41 ± 9
PA/11	0.45	53,092	1.56	112	167,033	4.92	89	94 ± 5

[a] Assay conditions were as described in Materials and Methods with ApG as a primer and purified viral cores isolated from the indicated viral strains.
[b] Background values (75 cpm) were subtracted in all cases.
[c] The amount of radioactivity incorporated in the presence of antibody G/2 was taken as 100%.
[d] Obtained in two to four independent assays.

cores (Table 1), which are not recognized by any of the 26 antibodies used in this report (data not shown).

MAb PB2/28, at a final concentration of 0.4 μg/μl, repeatedly inhibited transcription by only 20%. To determine whether the inhibitory effect of antibody PB2/28 could be increased, this MAb was tested again at a final concentration of 4 μg/μl. The results obtained in one representative experiment, using globin mRNA as a primer, along with a summary of the average inhibition observed in four independent assays, are shown in Table 2. Antibodies PB2/28 and NP/3 showed a strong inhibitory effect on the transcriptase activity of A/Victoria/3/75 influenza virus cores, reducing the amount of RNA synthesized by 52% (average of four experiments) and 79% (average of four experiments), respectively. However, antibody PA/16 (Table 2) and MAbs PB2/12 and PB2/22 (not shown) caused no significant inhibition under the same assay conditions. The effect of MAbs NP/3 and PB2/28 was specific because they exerted no inhibitory effect on B/Singapore/222/79 influenza virus cores.

Effects of MAbs on initiation of influenza viral RNA transcription. We wished to determine at which step inhibition of RNA synthesis was occurring. It was anticipated, on the basis of a previous report (52), that anti-NP antibodies were inhibiting RNA chain elongation, and it was speculated that MAb

TABLE 2. Inhibition of transcriptase activity by antibodies PB2/28 and NP/3[a]

MAb	Incorporation in strain: B/Singapore/222/79 Of ^{32}P (cpm)[b]	Of CTP (pmol)	%[c]	A/Victoria/3/75 Of ^{32}P (cpm)[b]	Of CTP (pmol)	%[c]	Avg ± SD (%)[d]
G/1	12,208	0.19	100	94,736	1.46	100	100
PB2/28	15,120	0.23	123	45,892	0.71	48	48 ± 1
NP/3	13,116	0.20	107	18,518	0.28	19	21 ± 2
PA/16	ND			88,398	1.37	93	93 ± 1

[a] Assay conditions were as described in Materials and Methods with globin mRNA as a primer and purified viral cores isolated from the indicated viral strain. The final concentration of each of the antibodies was 4 μg/μl.
[b] Background values (320 cpm) were subtracted in all cases. ND, not determined.
[c] The amount of radioactivity incorporated in the presence of antibody G/1 was taken as 100%.
[d] Obtained in two (for antibody PA/16) and four (for the rest of the antibodies) independent assays.

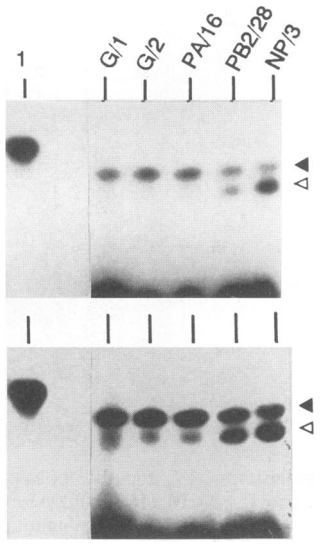

FIG. 3. Effects of MAbs NP/3 and PB2/28 on initiation of viral RNA transcription. Purified viral cores of influenza virus A/Victoria/3/75 were preincubated with the antibodies indicated at the top, at a final concentration of 4 μg/μl. After 30 min of incubation, transcription reactions were initiated by addition of globin mRNA and [α-^{32}P]CTP as indicated in Materials and Methods. After 10 min of incubation at 30°C, the samples were phenol extracted and the reaction products were analyzed by electrophoresis in a 12% polyacrylamide gel containing 7 M urea. Lane 1, a 19-mer oligodeoxynucleotide labeled at its 5′ end with [γ-^{32}P]ATP. The lower panel is a longer exposure of the same gel.

PB2/28 might be interfering with an earlier transcription step since the PB2 polypeptide displays cap-binding activity. To investigate whether antibody PB2/28 was acting at the level of RNA initiation, the in vitro transcription reaction was carried out in the presence of a single labeled NTP (either GTP or CTP) with a mixture of α- and β-globin mRNA chains as a primer. The final concentration of the triphosphate in the reaction mixture was 2.5 μM to prevent incorporation of more than one nucleotide residue per mRNA-derived primer (21, 37). The reaction products were then analyzed by electrophoresis in polyacrylamide gels containing 7 M urea and autoradiography.

It was anticipated, from results previously reported (37, 42), that in the presence of CTP, both α- and β-globin mRNAs would be used by the viral polymerase to yield m^7Gpppm^6AmCACUUCUGG(10)p*C and m^7Gpppm^6AmCACUUGCUUUUG(13)p*C labeled RNA products, respectively. As shown in Fig. 3, two discrete bands with the mobilities expected for these two products were detected in the autoradiogram. In the presence of antibodies G/1, G/2, and PA/16, the band indicated by a black arrowhead, which presumably corresponds to the β-globin mRNA-derived fragment, was much more abundant than the shorter band, indicated by a white arrowhead, which presumably corresponds to the α-globin mRNA-derived fragment. This was expected since it has been reported that β-globin mRNA is more active in priming activity than α-globin mRNA (7, 38, 42). In contrast, when antibodies PB2/28 and NP/3 were included in the reaction, the relative intensity of the two bands was altered, and in the presence of MAb NP/3 the faster-moving band was the most abundant.

To confirm the identities of the two major labeled products and to rule out the possibility that the faster-moving band was

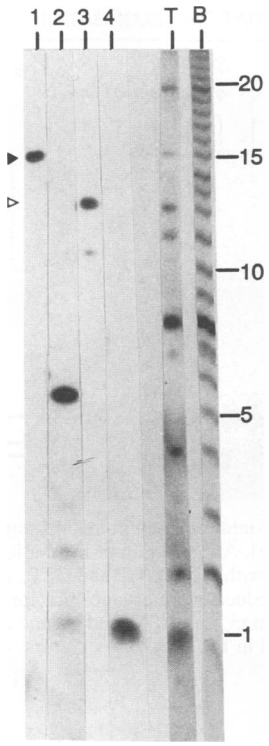

FIG. 4. Analysis of RNA products obtained in the presence of CTP. The two major labeled products obtained in the in vitro transcription reaction shown in Fig. 3 were eluted from the gel, treated with RNase T_1, and separated by electrophoresis in a 20% denaturing polyacrylamide gel as described in Materials and Methods. Lanes 1 and 3, the upper (indicated by a black arrowhead in Fig. 3) and lower (indicated by a white arrowhead in Fig. 3) bands after being eluted from the gel, respectively; lanes 2 and 4, the RNase T_1 digestion products of the corresponding bands; lane T, RNA uniformly labeled with [α-^{32}P]CTP which was partially digested with RNase T_1; lane B, ladder generated by alkali treatment of an RNA labeled at its 5′ end with [γ-^{32}P]ATP. Numbers on the right refer to the chain lengths of the alkali digestion products, counting the labeled band which moved faster as the first base.

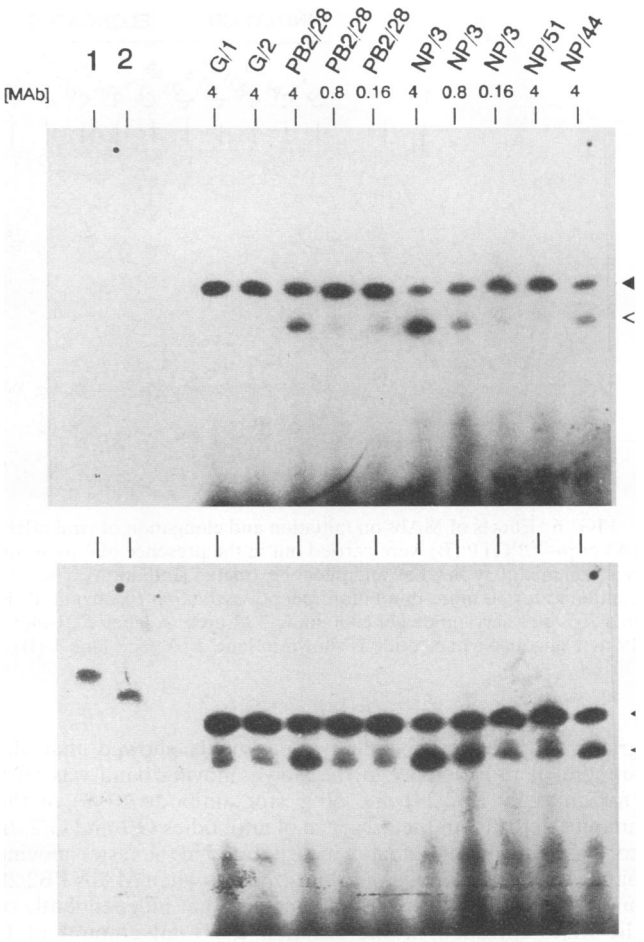

FIG. 5. Effects of MAbs NP/3, NP/44, NP/51, and PB2/28 on initiation of viral RNA transcription. Experimental details are as indicated in the legend to Fig. 3. The MAbs tested and their final concentrations (expressed as micrograms per microliter) in the transcription reaction are indicated (top). Lanes 1 and 2, 21- and 19-mer oligodeoxynucleotides labeled with ^{32}P, respectively. The lower panel is a longer exposure of the same gel.

a degradation product of the larger band, both bands were eluted from a 20% polyacrylamide gel and treated with RNase T_1 and the digestion products were resolved by electrophoresis (Fig. 4). If the two bands were derived from the predicted α- and β-globin primers, the labeled RNase-T_1 digestion products had to be Gp* and CUUUUGp*, respectively. Two size markers were included in the gel; one of them was a ladder generated by alkali treatment of a 5′-end-labeled RNA, and the other was an RNA (transcribed in vitro in the presence of [α-^{32}P]CTP) partially digested with RNase T_1. The fastest-moving band of the latter marker would be Gp* and therefore should comigrate with one of the expected RNase T_1 digestion products. The RNA products eluted from the gel comigrated approximately with alkali-derived fragments of 15 and 12 nucleotides (Fig. 4), a result which agreed with the expected sizes of the two products (37, 42). In addition, the mobility of the RNase T_1 digestion products was in agreement with the predicted sizes of 1 and 6 nucleotides, therefore demonstrating that the two labeled RNA products obtained in the presence of CTP were in fact the expected α- and β-globin mRNA-derived fragments. It should be noted that the eluted bands and the RNase T_1 digestion product of the larger eluted band did not exactly comigrate with bands observed in the marker lanes. This result is probably because the alkali-derived fragments contain a 2′,3′ cyclic phosphate group and because the nucleotide composition of the alkali-derived fragments differs from that of the globin mRNA-derived fragments.

Transcription initiation reactions were repeated with the other two anti-NP inhibitory MAbs and different amounts of the NP/3 and PB2/28 MAbs. As shown in Fig. 5, the effect observed with antibodies NP/3 and PB2/28 was dose dependent. The effect was observed only at the highest level of MAb PB2/28 tested, whereas antibody NP/3 displayed an intermediate effect at a final concentration of 0.8 μg/μl. In addition, antibody NP/44, but not MAb NP/51, displayed an effect similar to that shown by MAbs NP/3 and PB2/28. Although it appears that antibody NP/44 inhibits overall levels of initiation of transcription (Fig. 5), this effect was not consistently observed (see Fig. 6A). However, it is clear in both experiments that in the presence of antibody NP/44 the labeled band that corresponds to the α-globin mRNA-derived fragment is more abundant than that obtained in the presence of control antibodies G/1 and G/2. Densitometric analysis of the autoradio-

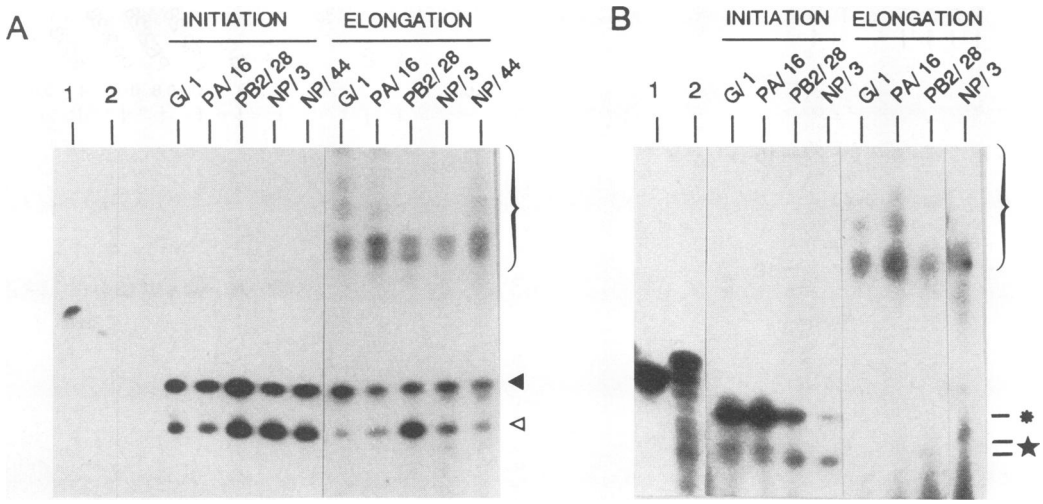

FIG. 6. Effects of MAbs on initiation and elongation of viral mRNAs. Transcription reactions containing globin mRNA and either [α-^{32}P]CTP (A) or [α-^{32}P]GTP (B) were carried out in the presence of 4 μg of the MAbs indicated (top) per μl. After 10 min of incubation, an aliquot of the reaction mixture was taken and phenol extracted (Initiation). The other aliquot was supplemented with ATP, CTP, and GTP, and the reaction was continued for 30 more min before phenol extraction (Elongation). Finally, the RNA reaction products were ethanol precipitated and separated in a 20% polyacrylamide gel containing 7 M urea. A labeled 21-mer oligodeoxynucleotide is shown in lane 1 (A) and in lane 2 (B), and a labeled 19-mer oligodeoxynucleotide is shown in lane 2 (A) and lane 1 (B). Other symbols are explained in the text.

grams of three independent experiments showed that the amount of radioactivity in the slowly moving band was 80% (for antibody PB2/28) and 50% (for antibody NP/3) of the amount detected in the presence of antibodies G/1 and G/2. In contrast, the amount of label incorporated in the faster-moving band was increased by more than 10 times when MAbs PB2/28 and NP3 were used. It should be noted that independently of the MAb included in the reaction the total amount of C residues incorporated into globin mRNA-derived primers was not significantly affected.

To determine whether the RNA transcripts initiated in the presence of CTP could be elongated, an aliquot of the reaction mixture was supplemented with ATP, CTP, and GTP and incubated for 30 more min (Fig. 6A). Under these assay conditions, transcription should proceed until a UTP residue has to be incorporated, i.e., the initiated products would be elongated between 10 and 17 nucleotides. Since the concentration of the labeled nucleotide was diluted 80 times during the elongation reaction, the labeled products observed in the autoradiography correspond to RNA products initiated in the presence of antibody. Independently of the antibody used, a fraction of the initiated products were not elongated; and although elongated products (enclosed within brackets in the figure) were observed in the presence of all MAbs tested, the largest transcripts were observed only in the presence of antibodies G/1, PA/16, and NP/44.

Other series of experiments were performed using [α-^{32}P] GTP as the only triphosphate in the reaction mixture. It was previously demonstrated (37) that in the presence of [α-^{32}P] GTP one labeled G residue is added to the G13 fragment of β-globin mRNA to yield RNA molecules with the structure m^7Gpppm^{6}AmCACUUGCUUUUG(13)p*G, whereas no labeled fragment of α-globin mRNA is found. When antibodies G/1 and PA/16 were included in the reaction a major labeled band (Fig. 6, band indicated by an asterisk) with the electrophoretic mobility expected for the β-globin mRNA-derived fragment was observed. The intensity of this band was reduced when the nucleocapsids were preincubated with antibodies PB2/28 (50% reduction) and NP/3 (90% reduction), showing therefore that both MAbs interfere with the initiation step of viral mRNA synthesis. With all antibodies tested, other minor bands (marked with a star in the figure) were also observed. These products most likely correspond to the U11 and U12 fragments of β-globin mRNA as previously reported (37, 42). It should be noted, however, that the relative intensity of the two bands marked in the figure was changed when MAbs NP/3 and PB2/28 were present during the initiation reaction. As shown in the same figure, virtually all initiated products were elongated when the reaction mixture was supplemented with CTP, GTP, and ATP. Again, the largest elongated products were not observed in the presence of MAbs PB2/28 and NP/3.

A major difference between the elongation reactions shown in Fig. 6 is the fact that practically all products initiated in the presence of GTP (panel B) were elongated whereas a significant fraction of those initiated in the presence of CTP were not elongated (panel A). This suggests that the latter products are inefficient primers for RNA elongation (abortive initiation), as has been described for other RNA molecules (16, 46).

DISCUSSION

The main objective of this study was to gain insights into the roles of the influenza virus NP, PB2, and PA polypeptides during viral mRNA transcription by analyzing the effects of MAbs to these proteins on the RNA polymerase activity associated with influenza virus nucleocapsids. For this purpose, we have utilized three anti-NP antibodies (31) and 21 MAbs specific for the influenza virus PA (13 MAbs) and PB2 (8 MAbs) polypeptides whose preparation and characterization are also described in this paper.

None of the 13 anti-PA antibodies inhibited viral transcriptase, and therefore no hint on the function of the PA protein was obtained. However, one of the anti-PB2 MAbs (PB2/28) and the three anti-NP MAbs (NP/3, NP/44, and NP/51) reduced by more than 50% the amount of RNA synthesized by the viral polymerase in vitro. This inhibitory

effect was due to specific binding of the antibodies, since transcription inhibition was not observed when nucleocapsids isolated from influenza virus B/Singapore/222/79, which are not recognized by the MAbs, were tested under the same assay conditions.

The effects of MAbs PB2/28 and NP/3 on transcription initiation were examined by carrying out transcription reactions in the presence of globin mRNA (a mixture of α- and β-globin chains) and only one NTP. When the reaction mixture contained GTP, both MAbs inhibited transcription initiation, whereas in the presence of CTP the amount of label incorporated into globin mRNA-derived fragments was unaffected. In the latter case, however, the amount of radioactivity incorporated into the β-globin mRNA-derived primer was reduced compared with that with control antibodies, and there was a concomitant increase in the amount of label incorporated into the α-globin mRNA-derived primer. In addition, antibody NP/44, but not antibody NP/51, displayed a similar effect. It should be mentioned that antibodies NP/3 and NP/44 bind to an antigenic region in the NP molecule that is different from that recognized by MAb NP/51 (31).

The PB2 polypeptide is the subunit of the viral polymerase that binds to the cap structure of mRNAs, and therefore it was not unexpected to find that the MAb PB2/28 affected initiation of viral mRNA synthesis. However, there is no direct evidence that NP plays a role during initiation of mRNA transcription, and it was thus striking that anti-NP antibodies displayed effects similar to those shown by antibody PB2/28. In fact, transcription complexes devoid of NP (17, 23) or reconstituted in the absence of NP (16) can catalyze the endonucleolytic cleavage of capped RNAs and are able to incorporate a few NTP residues to the cleavage products, but they are inactive in catalyzing RNA elongation. These results, however, do not exclude the possibility that the transcriptional activity of the complex can be different depending on whether the vRNA is complexed with NP.

It should be noted that for a nucleotide residue to be incorporated into a globin mRNA-derived primer, the viral polymerase has to catalyze a series of steps that include cap binding, endonucleolytic cleavage of the capped RNA, and positioning the 3′ end of the primer close to the vRNA template and in the vicinity of the polymerization site to allow incorporation of the first nucleotide residue. It seems likely that subtle movements of both RNA (primer and template) and protein components (NP and the three P proteins) are required to allow all these steps. In the presence of CTP, MAbs NP/3, NP/51, and PB2/28 did not reduce the total amount of label incorporated into globin mRNA-derived primers, suggesting that the antibodies do not inhibit any of the enzymatic activities of the transcription complex; rather it seems that the antibodies modify the complex in such a way that certain primers are preferentially used for initiation of mRNA synthesis. It might be speculated that the transcription complex has been modified to use preferentially short primers. Accordingly, in the presence of CTP, the viral enzyme, which can choose between two mRNA species, uses the shorter primer (derived from α-globin mRNA), whereas in the presence of GTP the enzyme can use only the large β-globin mRNA-derived fragment; therefore, in this latter case, the effect observed in the initiation assay is a reduction in the total amount of label incorporated.

How might the MAbs modify the transcription complex to utilize the α-globin mRNA fragment preferentially over the β-globin mRNA fragment? One hypothesis is that binding of the antibodies may prevent, by steric hindrance, movements of the components of the transcription complex, or there might be P-protein-specific contacts or interactions between the NP and the P proteins that are not allowed once the antibody is bound to the antigen. Alternatively, binding of anti-NP antibodies may alter the RNP structure and as a consequence modify the activity of the transcription complex. Further studies are required to discriminate between these possibilities.

The transcription initiation complex shows specificity for certain NTP-mRNA combinations in vitro. Thus, each mRNA tested in the initiation transcription reaction is cleaved at specific residues, and only certain NTP-mRNA combinations can be utilized by the viral polymerase. For example, a G residue is added to the G13 residue of β-globin mRNA, to the A13 residue of alfalfa mosaic virus RNA 4, and to the A10 residue of brome mosaic virus RNA4 but is not incorporated into α-globin mRNA, and a C residue can be linked to the G13 residue of β-globin mRNA, to the G10 residue of α-globin mRNA, or to the G12 residue of brome mosaic virus RNA 4, whereas no alfalfa mosaic virus RNA 4 fragment is labeled with CTP (37). Although what determines the specificity of the transcription complex for some NTP-mRNA combinations is not clear, the results reported here indicate that the preferential usage of some mRNAs can be modified by exogenously added proteins. In this regard, it is tempting to speculate that compounds which mimic MAb binding could have an effect similar to that displayed by them and could therefore function as specific antiviral drugs inhibiting viral RNA transcription.

Our results extend those described by van Wyke et al. (52) and Schreir et al. (45), who showed that antibodies to influenza virus nucleoprotein and rabbit serum to the P protein complex, respectively, had inhibitory effects on RNA synthesis in vitro. Although the effect of anti-NP antibodies on transcription initiation was not examined, van Wyke et al. (52) suggested, on the basis of kinetic experiments, that the antibodies inhibit elongation of the nascent RNA chain. Further studies are required to demonstrate whether the inhibitory antibodies described in this report inhibit RNA chain elongation in addition to transcription initiation, although the absence of largest elongated products in the limited elongation assays suggests so. Moreover, antibody NP/51, which showed no effect on transcription initiation, drastically reduced the amount of RNA synthesized by purified viral cores, suggesting an effect in RNA chain elongation.

In conclusion, our results suggest that the RNP structure and/or critical interactions of the NP polypeptide and P proteins are important for initiation of mRNA-primed transcription.

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