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Mechanism for Inhibition of Influenza Virus RNA Polymerase Activity by Matrix Protein

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Influenza virus M1 protein has been shown to inhibit the transcription catalyzed by viral ribonucleoprotein complexes isolated from virions. Here, this inhibition mechanism was studied with the recombinant M1 protein purified from *Escherichia coli* expressing it from cDNA. RNA mobility shift assays indicated that both soluble and aggregate forms of the recombinant M1, which were separated by the glycerol density gradient, were bound to RNA. Once an M1-RNA complex was formed, free M1 was bound to the M1-RNA complex cooperatively rather than to free RNA. In addition, the recombinant M1 was capable of binding to preformed RNA-nucleocapsid protein complexes. The mechanism for inhibition of the viral RNA polymerase activity was analyzed by the in vitro RNA synthesis systems that depend on an exogenously added RNA template. These systems were more sensitive for evaluating the inhibition by M1 than the RNA synthesis system depending on an endogenous RNA template. The RNA synthesis inhibition was examined at four steps: cleavage of capped RNA; incorporation of the first nucleotide, GMP; limited elongation; and synthesis of full-size product. M1 inhibited RNA synthesis mainly at the early steps. The experiments with M1 mutant proteins containing amino acid deletions suggested that the M1 region between amino acid residues 91 and 111 was essential for anti-RNA synthesis activity, RNA binding, and oligomerization of M1 on RNA.

Type A influenza virus has eight single-stranded RNAs of negative polarity as its genome. The RNA genome in the virion exists as a ribonucleoprotein (RNP) complex with RNA-dependent RNA polymerase and nucleocapsid protein (NP). RNA dependent RNA polymerase, which is composed of three subunits, PB2, PB1, and PA, plays crucial roles in transcription and replication of the virus genome (12, 13, 15). NP is required for the elongation of RNA chains (11, 14). RNP is surrounded by the membrane-associated matrix (M1) protein. M1 is thought to function as a structural template for assembly of transmembrane glycoproteins, hemagglutinin and neuraminidase, that are located in the envelope of virion. It is likely that M1 associates with the lipid bilayer derived from the host cell membrane (8).

Infection of the host cell by influenza virus is initiated by adsorption of the hemagglutinin spike to sialic acid-containing glycoprotein receptors on the plasma membrane, followed by receptor-mediated endocytosis and membrane fusion between virus and the intracellular membrane at a low pH (23, 27). RNP complexed with M1 is, then, cast into the cytoplasm, where M1 protein is dissociated from the RNP complex. RNP is then transported to the nucleus (17), and this incoming RNP supports the primary transcription. In consequence, early gene products including PB2, PB1, PA, NP, and NS1 (nonstructural protein 1) are synthesized. Concomitantly, the first step of replication of the viral genome, the synthesis of cRNA from viral RNA (vRNA), occurs. During the infection phase, late gene products such as hemagglutinin, neuraminidase, M1, M2, and NS2, which are essential for formation of a mature virus particle, are synthesized. Translation of M1 protein is stimulated by NS1 protein (5). Some of newly synthesized M1 moves from the cytoplasm to the nucleus and becomes associated with the newly formed RNP complex. Formation of an RNP-M1 complex is essential for transportation of the progeny RNP to the cytoplasm (16). Assembly and budding occur on the host cell membrane, where hemagglutinin, neuraminidase, and M2 are located.

It has been suggested that M1 is involved in regulation of transcription of the viral genome. M1 is shown to inhibit the in vitro RNA synthesis catalyzed by RNP complexes isolated from virions (36). The extent of M1-mediated inhibition is about 50% at most. Ye et al. reported that the region involved in the transcription inhibition lies within the carboxy-terminal two-thirds of M1 (33). Analysis using a variety of monoclonal anti-M1 antibodies revealed that the regions on M1 in the vicinity of amino acids residues 70 and 140 are critical for the transcription inhibition (9). It has been shown that M1 is an RNA-binding protein for single-stranded RNA (25). There are three hydrophobic domains in the primary structure of M1 which are involved in incorporating it into the lipid bilayers (2, 8, 9, 32, 33). Similar properties of RNP binding, inhibition of viral transcription (3), and association with membrane bilayers (4, 19) have been assigned to the M protein of vesicular stomatitis virus.

Recently, we developed an in vitro RNA synthesis system which is dependent on the exogenously added RNA template. With this system in hand, it becomes possible to test the effect of M1 on the progeny RNP being formed with newly synthesized viral RNA, RNA polymerase, and NP after replication. In this study, we systematically examined the effect of M1 on various steps of viral RNA synthesis, i.e., RNA binding, cleavage of the capped RNA, incorporation of the first nucleotide, and elongation of RNA chains. These assays were carried out with the recombinant M1 protein, which was purified from extracts of *Escherichia coli* expressing M1 from its cDNA.

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Furthermore, we showed functional domains of M1 using a series of mutant recombinant M1 proteins.

MATERIALS AND METHODS

Construction of wild-type and mutant M1 protein expression vectors. For generation of an insert for pET14b, cloned M1 cDNA (30) was used for amplification by PCR with synthetic oligonucleotide primers 5′-CCCCCCCATATG AGTCTTCTAACCGAG-3′ and 5′-AAAAAACATATGTTACTCCAGCTCT A-3′. Amplified DNA was digested with *Nde*I and inserted into *Nde*I-digested pET14b. The resultant plasmid was designated as pET14b-M1. For construction of pET14b-M1Δ2-75, insert DNA was amplified by PCR with pET14b-M1 as a template using synthetic oligonucleotide primers 5′-AAAAAACATATGCGT AGACGCTTTGTCCAA-3′ and 5′-AAAAAACATATGTTACTCCAGCTCT A-3′. Amplified DNA was digested with *Nde*I and inserted into *Nde*I-digested pET14b. For construction of pET14b-M1Δ76-116, insert DNA was amplified by PCR with pET14b-M1 as a template using synthetic oligonucleotide primers 5′-AAAAAACTGCAGCTCAGTTATTCTGCTGGT-3′ and 5′-AAAAAACAT ATGTTACTCCAGCTCTA-3′. Amplified DNA was digested with *Nde*I and *Pst*I. A DNA fragment of 1,272 bp was prepared by digestion of pET14b with *Nde*I and *Pst*I. These two fragments were inserted into pET14b-M1 digested with *Nde*I and *Pst*I. For construction of pET14b-M1Δ91-, pET14b-M1 was digested with *Bam*HI, and the isolated 4,924-bp fragment was subjected to self-ligation. For construction of pET14b-M1Δ113-, pET14b-M1 was digested with *Nco*I. Fragments of 413 and 4,962 bp were ligated.

Expression and purification of recombinant M1 proteins. *E. coli* (BL21/DE3) was transformed with each plasmid. A 10-ml culture in L broth was induced by the addition of 0.8 mM isopropylthio-β-D-galactoside to synthesize recombinant M1 proteins. After incubation for 3 h, cells were collected by centrifugation and the pellet was suspended in an ice-cold buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl [pH 7.9]). The cell suspension was subjected to sonication, and recombinant M1 proteins were purified by using Ni chelation resins according to the method suggested by the manufacturer (Novagen). The eluate was dialyzed against STE buffer (100 mM NaCl, 10 mM Tris-HCl [pH 7.9], 1 mM EDTA). The pH of the solution was then adjusted to 1 by addition of HCl and lyophilized. Lyophilized protein was dissolved in an acidic solution at pH 4 to 6 and was centrifuged on a 15 to 35% linear glycerol gradient in 100 mM NaCl–10 mM Tris-HCl (pH 7.9)–1 mM EDTA in a Beckman TLS55 rotor at 54,000 rpm at 4°C for 6 h. Fractions of 120 μl were collected from the top of the gradient. Proteins were analyzed by electrophoresis on 10% polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS). Approximately 20 and 4 μg of the soluble M1 and aggregate M1, respectively, were obtained.

Preparation of the RNA template. RNA V53wt, consisting of 53 nucleotides containing 15 nucleotides of the 3′-terminal sequence and 22 nucleotides of the 5′-terminal sequence, which are identical to those of segment 8 vRNA, and a 16-nucleotide spacer between both terminal sequences were prepared as described elsewhere (22). RNA M53wt, complementary to V53wt, was also prepared as described elsewhere (22). RNA V172wt, consisting of 172 nucleotides, was prepared by in vitro transcription of *Ksp*632I-digested pUC119-V172 with T7 RNA polymerase. V172wt contains 20 nucleotides of the 3′-terminal sequence and 31 nucleotides of the 5′-terminal sequence, which are identical to those of segment 6 vRNA, and a 121-nucleotide spacer between these terminal sequences. The in vitro transcription of *Bgl*II-digested pUC119-V2000 resulted in the synthesis of RNA of 277 nucleotides containing 31 nucleotides of the 5′-terminal sequence derived from segment 6 vRNA, followed by a 246-nucleotide sequence. Detailed methods for construction of pUC119-V172 and pUC119-V2000 are described elsewhere (26).

Preparation of RNP, MN RNP, NP, and RAF. Influenza virus RNP cores were isolated from purified influenza virus A/PR/8/34 essentially as described elsewhere (10). Micrococcal nuclease (MN)-treated RNP cores (MN RNP) were prepared as described elsewhere (20). Briefly, RNP was treated with 1 U of MN per ml in 1 mM $CaCl_2$ at 25°C for 2 h, and the reaction was stopped by addition of 3 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]. The sample was stored at −80°C. NP was purified from RNP by the CsCl gradient method as described elsewhere (31). The sample was dialyzed and stored at −80°C. RAF (RNA polymerase activating factor) fractions were prepared from HeLa nuclear extracts (22). The flowthrough fractions of the phosphocellulose column contained RAF stimulatory activities that stimulate the viral polymerase in RNA synthesis from both endogenous and exogenous RNA templates.

RNA binding assays. The ^{32}P-labeled RNA probe was prepared by the in vitro transcription of a plasmid digested with an appropriate restriction endonuclease with T7 RNA polymerase in the presence of [α-^{32}P]UTP. ^{32}P-labeled RNA was incubated at 30°C for 10 min with M1 in a solution containing 120 mM Tris-HCl (pH 7.9), 50 mM NaCl, and 4 mM dithiothreitol. For the RNA mobility shift assay, the reaction mixture was subjected to electrophoresis on a 4.5% native polyacrylamide gel containing 45 mM Tris-borate, 100 mM NaCl, and 1 mM EDTA and the gel was autoradiographed. For the filter binding assay, the reaction mixture was filtered through a nitrocellulose filter (HAWP; Millipore) and the amount of ^{32}P-labeled RNA retained on the filter was determined with the AMBIS radioanalytic imaging system.

RNA synthesis in vitro. The full-size RNA synthesis reaction was performed at 30°C for 60 min in a final volume of 25 μl, which contained 50 mM Tris-HCl (pH 7.9); 5 mM $MgCl_2$; 80 mM NaCl; 1.5 mM dithiothreitol, 250 μM each ATP, GTP, and CTP; 10 μM UTP; 5 μCi of [α-^{32}P]UTP; 10 U of RNasin; 25 μg of actionomycin D per ml; 0.25 mM ApG; 10 ng of a 172-nucleotide RNA template, and 100 ng NP eq of RNP or MN RNP in the absence or presence of recombinant M1. RNA was extracted with phenol-chloroform, precipitated with ethanol, and then analyzed by electrophoresis on a 5% polyacrylamide gel in the presence of 50% (wt/vol) urea. The gel was subjected to autoradiography. The limited elongation reaction was essentially the same as the full-size RNA synthesis reaction except for the absence of UTP and addition of 10 μM GTP and 5 μCi of [α-^{32}P]GTP. RNA products were analyzed by electrophoresis on a 20% polyacrylamide gel in the presence of 50% (wt/vol) urea. The GMP transfer reaction was identical to the limited elongation reaction except for omission of ATP and CTP and addition of 20 ng of globin mRNA instead of ApG as a primer. The capped-RNA cleavage assay was performed essentially identically to the GMP transfer assay, except for omission of nucleotide triphosphates and addition of 3 pmol of [^{32}P]m^{7}GpppGpCp-poly(A_2U_2G) instead of globin mRNA as a primer. [^{32}P]m^{7}GpppGpCp-poly(A_2U_2G) was synthesized from synthetic ppGpCp-poly(A_2U_2G) (average chain length, 40 to 80 nucleotides) with [α-^{32}P] GTP and vaccinia virus capping enzymes as described elsewhere (18).

RESULTS

Purification of recombinant M1. In order to study the function of M1 in detail, we decided to prepare recombinant M1 proteins. First, we tried to purify M1 from extracts of *E. coli* expressing the protein from its cDNA by either the acid-chloroform–methanol method (6) or the acid extraction method (35), which have been used as the standard methods for purification of M1 from virions. These methods, however, were not successful because of significant contamination of proteins derived from *E. coli*. We next prepared histidine-tagged M1. This M1 was yielded in an inclusion body and then solubilized by urea (Fig. 1A, lane 2). The solubilized M1 was purified by using Ni chelaion resins. The eluate from Ni chelation resins contained M1 with a trace amount of contamination proteins (Fig. 1A, lane 4). Western blotting (immunoblotting) analysis using the anti-virion antibody confirmed that the purified protein was M1 (data not shown). For assays described below, the eluate was dialyzed to remove high concentrations of imidazole and urea. The low (2 M or lower) concentration of urea caused aggregation of M1. It has been reported that M1 is soluble and stable at a low pH (35). An aggregation form of M1 after dialysis was solubilized by decreasing pH and lyophilized. Lyophilized M1 was dissolved in an acidic solution, pH 4 to 6, in which a major part of M1 was kept as a soluble form.

RNA binding activity of M1. It has been reported that the RNA binding activity of M1 is reduced under conditions expected to favor M1 aggregation (25). To determine the molecular form of purified M1, it was subjected to fractionation by a glycerol density gradient (Fig. 1B). M1 sedimented in the top fractions (fractions 1 to 5) and the bottom fraction (fraction 10), corresponding to a soluble form and an aggregate, respectively. To confirm whether recombinant M1 has the RNA binding activity, the RNA mobility shift assay was carried out with the single-stranded RNA probe of 172 bases. Both soluble and aggregate forms of M1 showed the RNA binding activity (Fig. 1C). At the low concentration of the soluble M1, a fast-migrating band corresponding to an intermediate M1-RNA complex was formed, while at the increasing concentrations of the soluble M1 RNA probe was bound by M1 and stayed at the gel top as a highly associated form of the M1-RNA complex (lanes 2 to 7). An aggregate form of M1 formed only a slowly migrating complex even at the low concentration of M1. The stepwise formation of a highly associated M1-RNA complex can be explained by the concept that a soluble form of M1 binds to the RNA probe and then a free form of M1 preferentially binds to this M1-RNA complex rather than the free RNA probe by M1-M1 cooperative interaction. A series of

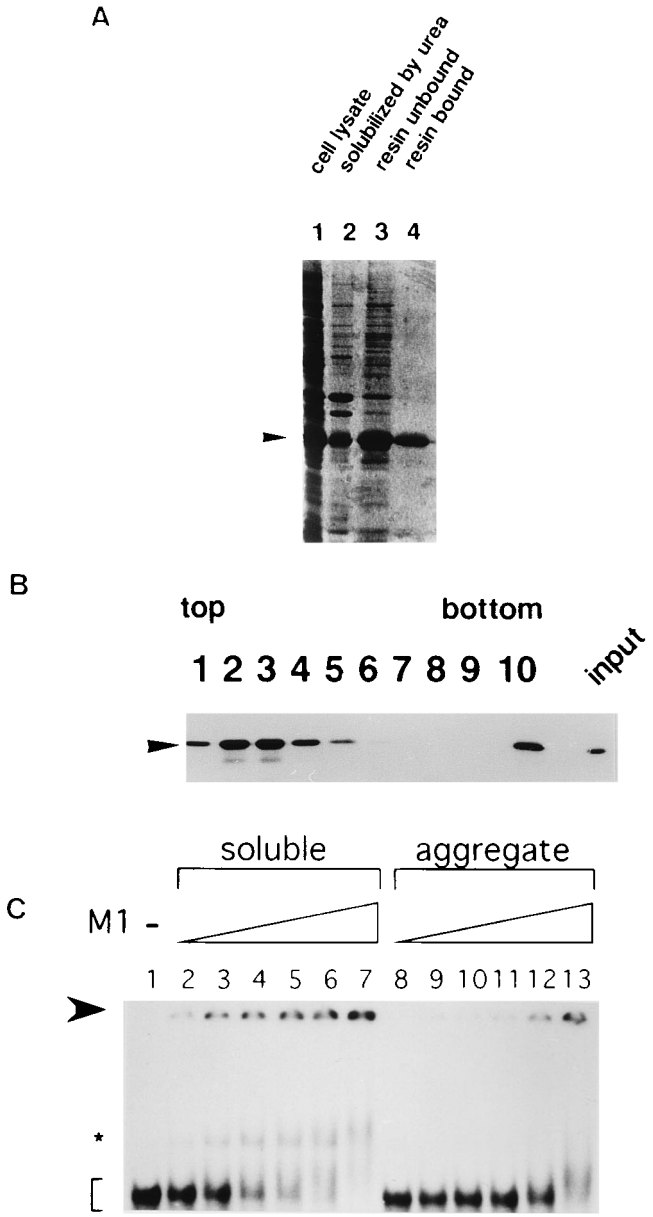

FIG. 1. Preparation of recombinant M1 protein. (A) Purification of histidine-tagged recombinant M1. The purification procedure is described in Materials and Methods. Proteins (lane 1, total cell lysate; lane 2, urea-solubilized fraction; lane 3, flowthrough fraction; lane 4, bound fraction) were separated on a 10% polyacrylamide gel in the presence of SDS and visualized by staining with CBB. Arrowhead, M1 protein. (B) Sedimentation of M1 protein through the glycerol density gradient. Twenty micrograms of purified M1 protein was subjected to the glycerol density gradient as described in Materials and Methods. The direction of sedimentation was from left to right. An aliquot (9 μl) of each gradient fraction or input (1 μl) was analyzed on a 10% polyacrylamide gel in the presence of SDS, and proteins were visualized by silver staining. Arrowhead, M1 protein. (C) RNA mobility shift assay. In a final volume of 15 μl, 0.48 fmol of a 172-nucleotide RNA probe was incubated without (lane 1) or with increasing amounts (1, 2, 3, 4, 5, and 11 ng) of a soluble form (lanes 2 to 7) and an aggregate form (lanes 8 to 13) of M1 protein as described in Materials and Methods. Arrowhead, asterisk, and bracket, highly associated M1-RNA complexes, intermediate M1-RNA complexes, and the free probe, respectively.

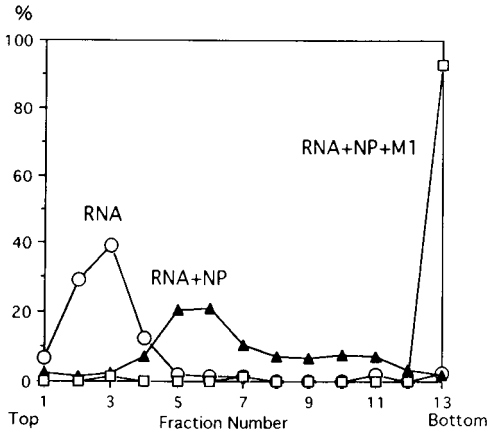

FIG. 2. M1 binding to a preformed RNA-NP complex. Two-hundred-seventy-seven-nucleotide ^{32}P-labeled RNA (25 ng) was incubated with (triangles and squares) or without (circles) 150 ng of purified NP in 10 mM Tris-HCl (pH 7.9)–90 mM NaCl–5 mM dithiothreitol–30 U of RNasin at 30°C for 10 min. Recombinant M1 (1.25 μg) was then added to the preformed RNA-NP complex (squares), and the complexes were incubated for another 10 min. The sample was layered onto a 32 to 60% (wt/vol) linear glycerol gradient in a buffer containing 100 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA and centrifuged at 54,000 rpm in a Beckman TLS55 rotor at 20°C for 4 h. An aliquot (10 μl) of each gradient fraction was subjected to determination of radioactivity with the AMBIS radioanalytic imaging system.

M1-RNA complexes in which different amounts of M1 are bound to the RNA probe was not observed. This could be due to the fact that the rate-limiting step of M1-RNA binding is the formation of the initial complex between the soluble M1 protein and the RNA probe and the following M1-M1 interaction step is kinetically much faster than the initial complex formation. Along this line, it has been reported that influenza virus NP and adenovirus DNA-binding protein, both of which are cooperatively bound to RNA and single-stranded DNA, respectively, form the highly associated complex without any intermediate complexes (24, 31).

Next, we tested whether M1 is capable of binding to preformed RNA-NP complexes (Fig. 2). NP is cooperatively bound to RNA every 20 nucleotides. NP-RNA complexes consisting of eight to nine NP molecules on the RNA probe of 172 nucleotides sedimented in the middle fractions in the glycerol density gradient. When M1 was added to the preformed NP-RNA complexes, all of the RNA probe sedimented in the bottom of the gradient, suggesting that M1 interacts with NP-RNA complexes. Soluble M1, NP, and a virion in a parallel gradient sedimented in fractions 2, 3, and 13, respectively. These results clearly indicate that M1 binds to not only naked RNA but also NP-RNA complexes or RNA in NP-RNA complexes.

Anti-RNA synthesis activity of M1. Next, the anti-RNA synthesis activity of the recombinant M1 was examined in in vitro RNA synthesis systems. RNP complexes isolated from virions support RNA synthesis from the endogenous RNA template in the presence of either an oligonucleotide containing a cap structure or dinucleotide ApG as a primer. In this system, the recombinant M1 inhibited RNA synthesis as did M1 purified from virions (data not shown; also, see below). Transcription inhibition by M1 was moderate, and maximal inhibition was around 30 to 50%. As noted previously (9), this is possibly due to a heterogeneous population of RNP complexes containing M1-sensitive and M1-insensitive RNP species. In infected cells, transcription inhibition caused by binding of M1 to newly synthesized RNA or RNP complexes should take place at the late stages of infection. Therefore, an in vitro RNA synthesis system that depends on the exogenously added template is needed to study the mechanism of M1-mediated transcription inhibition in detail. Recently, we developed an in vitro RNA

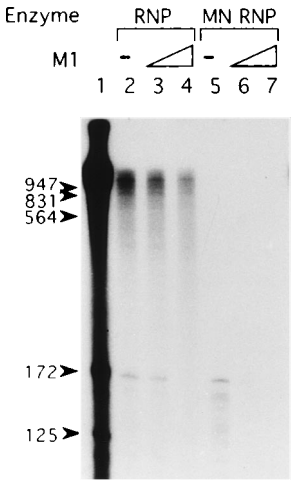

FIG. 3. Effect of recombinant M1 on in vitro synthesis of full-size RNA. The reaction was carried out as described in Materials and Methods by using RNP (lanes 2 to 4) or MN RNP (lanes 5 to 7) as an enzyme source in the presence of 100 ng (lanes 3 and 6) or 1 μg (lanes 4 and 7) or absence (lanes 2 and 5) of soluble M1. Molecular weight markers including ^{32}P-labeled *Eco*RI- and *Hin*dIII-digested lambda DNA and ^{32}P-labeled 172-nucleotide RNA which was synthesized by the in vitro transcription of *Ksp*632I-digested pUC119-V172 by T7 RNA polymerase are shown in lane 1.

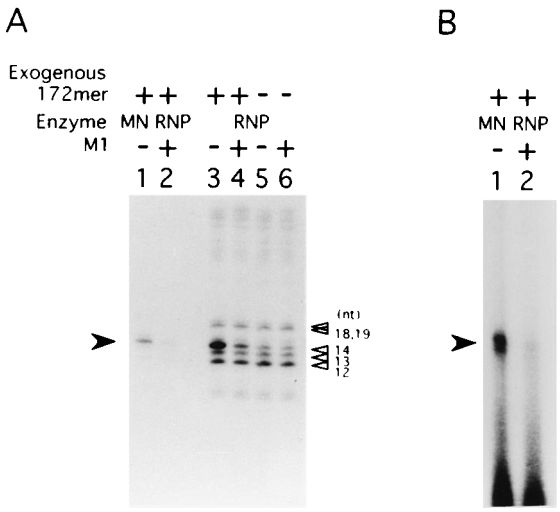

FIG. 4. Effect of M1 on limited elongation and GMP transfer. (A) Limited elongation assay. The reaction was carried out as described in Materials and Methods with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) 1 μg of M1 by using MN RNP (lanes 1 and 2) or RNP (lanes 3 to 6) as an enzyme source in the presence (lanes 1 to 4) or absence (lanes 5 and 6) of an exogenously added 172-nucleotide RNA template. An oligonucleotide synthesized from the exogenously added 172-nucleotide RNA template is indicated (arrowhead). Open arrowheads, products synthesized from endogenous RNP. nt, nucleotides. (B) GMP transfer. The reaction was carried out as described in Materials and Methods with (lane 2) or without (lane 1) 1 μg of M1 by using MN RNP as an enzyme source. The [^{32}P]GMP-labeled oligonucleotide is indicated (arrowhead).

synthesizing system with exogenously added 53-nucleotide model RNA templates which contain conserved sequences of 12 and 13 nucleotides at the 3′ and 5′ ends of segment 8 vRNA, respectively (22). A 172-nucleotide model RNA template containing 20 and 31 nucleotides at the 3′ and 5′ ends of segment 6 vRNA, respectively, was also functional in this system (Fig. 3, lane 2). The labeled material present at the top portion of the gel is the RNA product synthesized from the endogenous template associated with RNP complexes which was used as an enzyme source. The 172-nucleotide transcript was identified as the RNA complementary to the template RNA by the RNase T_2 protection assay using an antisense probe (data not shown). The addition of M1 caused an approximately 50% inhibition in RNA synthesis from the endogenous template, while RNA synthesis from an exogenously added RNA template was more efficiently inhibited than that from endogenous RNP (lanes 2 to 4). Brownlee and colleagues reported an RNA synthesis system depending on an exogenously added template (21) which is different from the system described above. The system is reconstituted with an exogenously added template and RNA polymerase and NP liberated from RNP complexes by MN treatment (MN RNP). M1-mediated transcription inhibition was also observed in this system with 172-nucleotide RNA as a template (lanes 5 to 7). NP binds to RNA every 20 nucleotides at saturation (28). Therefore, in this system, seven or eight molecules of NP were expected to bind to 172-nucleotide RNA. It was reported that the inhibitory effect of M1 protein leveled off at 50% as the M1/NP molar ratio was 2 in the endogenous RNA synthesis system (9). The NP/M1 ratio in lane 6 of Fig. 3 was 1.8. These results indicate that recombinant M1 protein is capable of not only binding to RNA but also inhibiting RNA synthesis.

Inhibition of RNA synthesis by M1. In order to determine the step at which M1 inhibits transcription, three assays were preformed. First, a limited elongation assay was carried out in the absence of UTP (Fig. 4A, lanes 3 to 6). In this assay, the lengths of RNA products are from 12 to 19 nucleotides depending on the species synthesized from endogenous templates. Since the 3′ and 5′ ends of the exogenously added 172-nucleotide template are derived from those of segment 6 vRNA, the RNA product in the limited elongation assay is expected to be 14 nucleotides long. In fact, MN RNP catalyzed the synthesis of a 14-nucleotide RNA product from the exogenously added 172-nucleotide RNA template. The addition of M1 results in a marked reduction of the synthesis of this oligonucleotide (Fig. 4A). This result suggests that M1 inhibits RNA synthesis at or prior to the step of limited elongation.

Next, the effect of M1 on the GMP transfer assay was examined. The first step of transcription of the influenza virus genome is cleavage of capped RNA, followed by incorporation of GMP into the 3′ end of the cleaved capped RNA. The reaction was performed in the presence of [α-^{32}P]GTP and globin mRNA, and the product was detected as an oligonucleotide containing [^{32}P]GMP at its 3′ end. MN RNP supported this reaction (Fig. 4B, lane 1). Figure 4B clearly shows that M1 inhibited production of the GMP-transferred oligonucleotide, suggesting that M1 inhibits transcription at or prior to the addition of the first nucleotide, GMP, into the 3′ end of the cleaved primer. As shown below, M1 inhibited cleavage of the capped RNA. Thus, we have demonstrated inhibition by M1 of the synthesis of RNA of positive polarity from template RNA of negative polarity. Our in vitro system using exogenously added 53-nucleotide RNA is capable of synthesizing not only RNA of positive polarity from RNA of negative polarity but RNA of negative polarity from RNA of positive polarity as long as a dinucleotide primer and a host factor that stimulates the vRNA polymerase activity are added (22). Indeed, host factor RAF stimulated RNA synthesis from RNA of positive polarity (Fig. 5, lane 3 versus lane 5). RNA synthesis from RNA of positive polarity was inhibited by M1 as efficiently as RNA synthesis from RNA of negative polarity (Fig. 5). This observation suggests that M1 inhibits the RNA synthesis directed by promoter sequences located on RNA templates of either negative or positive polarity.

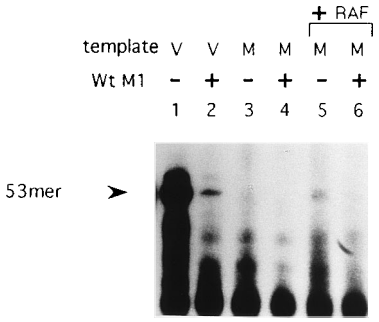

FIG. 5. Effect of M1 on RNA synthesis from templates of negative or positive polarity. In vitro RNA synthesis was carried out as described in Materials and Methods with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) 1 μg of wild-type (wt) M1 by using 100 ng of a 53-nucleotide RNA template of negative polarity (lanes 1 and 2) or positive polarity (lanes 3 to 6) in the presence (lanes 5 and 6) or absence (lanes 1 to 4) of the RAF fraction (1 μg of total protein of the phosphocellulose fraction [22]).

Transcription inhibition and RNA binding by mutant M1 protein. To localize the functional domains of M1 involved in transcription inhibition and RNA binding, a series of deletion mutant M1 proteins was constructed (Fig. 6A). Mutant A was the deletion mutant lacking amino acids 2 to 75. Mutant B was the internal deletion mutant lacking amino acids 76 to 116. Mutants C and D contained the first 90 and 111 N-terminal amino acids, respectively. Mutant M1 proteins were purified by the same method as that for purification of the wild-type recombinant M1 protein, and soluble fractions of wild-type and mutant M1 proteins were used for assays described below. The transcription inhibition activity of each mutant M1 was examined (Fig. 6B). It has been suggested that the transcription inhibition domain of M1 is located in regions between amino acid positions 90 and 108 and/or between positions 135 and 165 (32, 33). The wild type and mutants A and D efficiently inhibited the RNA synthesis from the exogenously added RNA template. In contrast, mutant C lost the transcription inhibition activity. Mutant B, which lacks the first transcription inhibition domain of amino acid positions 90 to 108 but contains the second transcription inhibition domain of amino acid positions 135 to 165 (32), slightly inhibited the RNA synthesis. These results clearly indicate that the region of M1 between amino acid positions 91 and 111 is involved in the transcription inhibition. Capped-RNA cleavage mediated by RNA polymerase, which is the first step of transcription initiation, followed by the incorporation of the first nucleotide, GMP, was inhibited by the addition of the wild type and mutants A and D (Fig. 6C). When mutant A was added, the level of the recovery of the substrate was low. The reason for this phenomenon is presently unknown. However, the fact that mutant D inhibits the cleavage reaction but mutants B and C do not suggests that the region between amino acid positions 91 and 111 is essential for M1-mediated inhibition of the cleavage reaction. In addition, the RNA binding activity of these mutant M1 proteins was examined by RNA filter binding and RNA mobility shift assays (Fig. 7). Figure 7A shows that wild-type M1 and mutants A and D were efficiently bound to RNA whereas mutants B and C were incapable of binding to RNA. Therefore, the region between amino acid positions 91 and 111 was essential for RNA binding, as it was for transcription inhibition. The mode of binding of M1 to RNA was analyzed by the RNA mobility shift assay (Fig. 7B). The highly associated complexes were formed with wild-type M1 and mutants A and D.

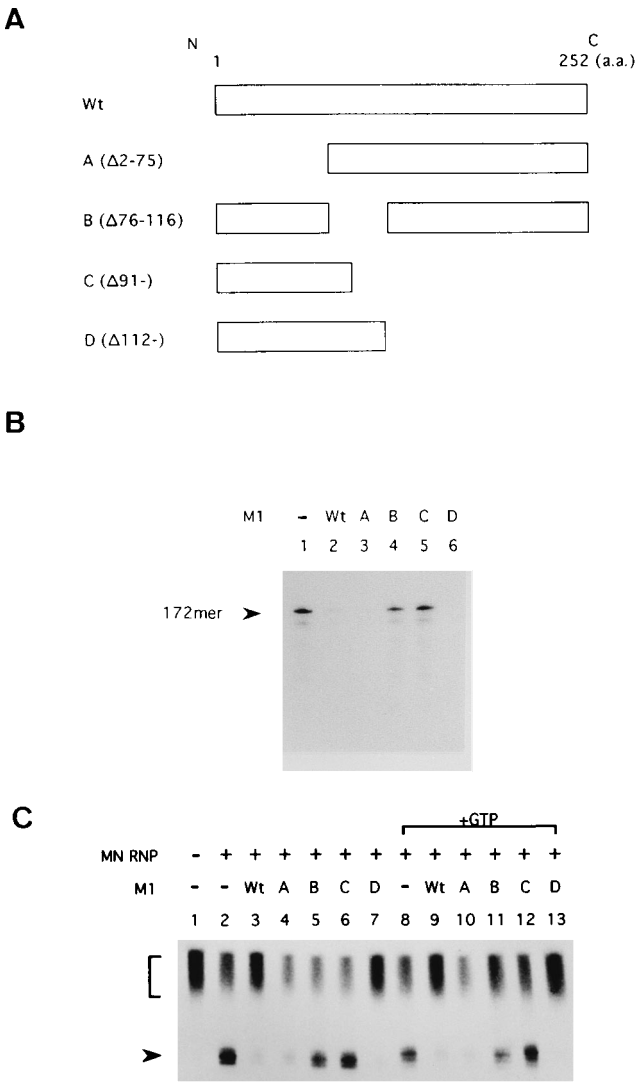

FIG. 6. Analysis of the anti-RNA synthesis domain of M1. (A) Structure of deletion (Δ) mutant M1 proteins. a.a., amino acids; Wt, wild type. (B) determination of the transcription inhibition domain of M1 protein. The reaction was carried out as described in Materials and Methods with 100 ng of the wild-type (lane 2) or mutant (lanes 3 to 6, mutants A, B, C, and D, respectively) M1 protein or without M1 protein (lane 1). (C) Capped-RNA cleavage and incorporation of the first nucleotide, GMP. The reaction was carried out as described in Materials and Methods with 500 ng of the wild-type M1 protein (lanes 3 and 9) or mutant A (lanes 4 and 10), B (lanes 5 and 11), C (lanes 6 and 12), or D (lanes 7 and 13) or without (lanes 1, 2, and 8) M1 protein by using MN RNP (lanes 2 to 13) as an enzyme source in the presence (lanes 8 to 13) or absence (lanes 1 to 7) of unlabeled GTP. Bracket, substrate capped RNA; arrowhead, RNA polymerase-dependent cleaved capped RNA products (lanes 2 to 7) or products that incorporate the first nucleotide, GMP (lanes 8 to 13).

DISCUSSION

Histidine-tagged M1 purified from extracts of *E. coli* expressing it from cDNA easily formed aggregates. It was reported that acid-dependent extracted M1 exists in a monomer form and possesses higher activity in transcription inhibition than acid-chloroform–methanol extracted M1, possibly because the degree of denaturation is lower in the former method. The former method was applied to solubilize recombinant M1 proteins (see Materials and Methods) (Fig. 1). The recombinant technology allowed us to obtain a large quantity of M1 as well as a series of mutant M1 proteins. However, it is

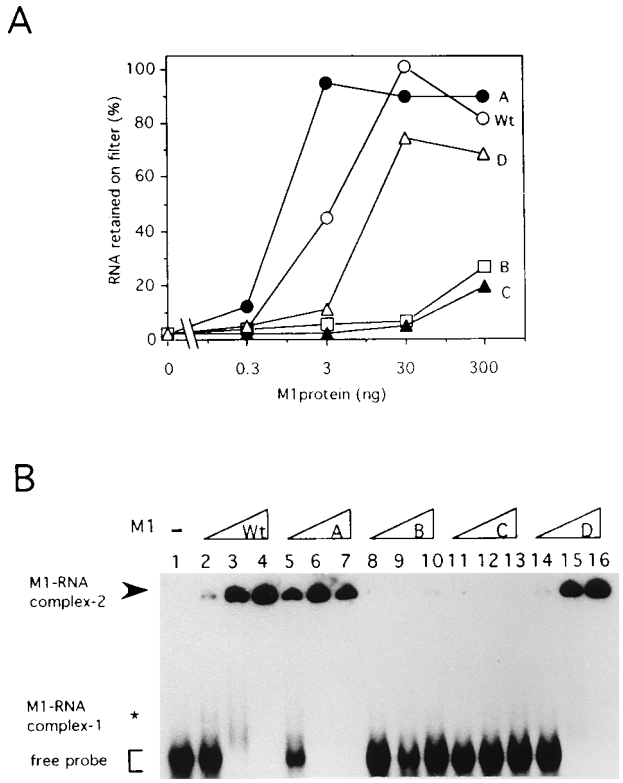

FIG. 7. Analysis of the RNA binding domain of M1. In a final volume of 7.5 μl, 1 ng of ^{32}P-labeled 172-nucleotide RNA was incubated with recombinant M1 proteins as described in Materials and Methods. (A) Filter binding assay. The reaction was carried out in the presence of increasing amounts of wild type (Wt) and mutant (A, B, C, and D) M1 proteins. The mixture was filtered through a nitrocellulose filter (HAWP), and the amount of ^{32}P-labeled RNA retained on the filter was determined with the AMBIS radioanalytic imaging system. (B) RNA mobility shift assay. The reaction was carried out with increasing amounts (3, 10, and 30 ng) of the wild type (lanes 2 to 4) and mutants A (lanes 5 to 7), B (lanes 8 to 10), C (lanes 11 to 13) and D (lanes 14 to 16) or without M1 protein (lane 1). Highly associated M1-RNA complexes (complex 2), intermediate M1-RNA complexes (complex 1), and the free probe are indicated.

possible that the recombinant M1 somehow differs from M1 present in virions or infected cells, for example, in modification. Indeed, phosphorylation of M1 was shown to be responsible for its aberrant nuclear retention (28). To determine the effect of such modification on M1 function, we should prepare M1 as a native form from an infected cell.

To separate a soluble form of M1, purified M1 was subjected to the glycerol density gradient (Fig. 1B). The RNA mobility shift assay revealed that both soluble and aggregate forms of M1 show the RNA binding activity (Fig. 1C). RNA or RNA-NP complexed with M1 formed huge complexes as judged by the RNA mobility shift assay and the glycerol density gradient, whereas the reconstituted RNA-NP complex sedimented at the expected size. NP has been shown to be cooperatively bound to RNA every 20 nucleotides (31). The amino-terminal region of the NP is required to bind RNA (1). Once one NP molecule is bound to RNA, this may accelerate the binding of the second NP molecule to the same RNA. In this report, we suggested that the mode of the formation of highly associated M1-RNA complexes is cooperative. However, the formed M1-RNA complex might be fundamentally different from the NP-RNA complex. Wakefield and Brownlee reported that M1 binding reaches saturation at a minimum of one M1 per 200 nucleotides (25). The RNA probe used here was 172 or 277 nucleotides long. Figure 1C shows that M1 could be bound to the RNA of 172 nucleotides. At a low concentration of soluble M1 an M1-RNA complex was formed, while at high concentrations all complexes are present as highly associated forms without any intermediate complexes. Therefore, the appearance of the highly associated complex may be due to M1-M1 interaction; one M1 molecule is bound to RNA and then the second M1 molecule is bound to M1 on the M1-RNA complex.

The experiments using deletion mutants of M1 indicated that the region of M1 between amino acid positions 91 and 111 is essential for RNA binding. Of interest is the fact that mutant M1 proteins that are capable of binding to RNA preferentially formed the highly associated M1-RNA complexes, suggesting that in our assay the RNA binding and the oligomerization domains were not separable. A variety of functional domains of M1 have been reported. Anti-RNA synthesis and RNA binding domains were localized at the region between amino acid residues 128 and 164 and/or between residues 90 and 108 by Ye et al. (32, 33). The region between amino acid positions 135 and 165 was not essential for transcription inhibition in our experimental conditions. The contradiction between data reported by Ye et al. (32) and our data may be due to different experimental conditions, in particular the concentration of salt used for assays. Previous experiments were performed with low salt concentrations (for example, 10 mM Tris-HCl [pH 7.8] and 10 mM NaCl), whereas our experiments were performed with physiological salt concentrations (50 mM Tris-HCl [pH 7.9] and 80 to 100 mM NaCl). This difference may affect the biochemical activities of M1 protein. Indeed, our preliminary experiments showed that at the low salt concentration mutant B is bound to the RNA more effectively than at the physiological salt concentration (data not shown). Thus, it is likely that the region between amino acid positions 135 and 165 may form an RNA binding domain which is less effective in RNA binding and transcription inhibition than the region between amino acid positions 91 and 111. The hydrophobic regions between amino acid residues 62 and 68 and between residues 114 and 133 have been shown to be lipid binding domains (7). Wakefield and Brownlee noticed a zinc finger motif between amino acid residues 146 and 160 (25). It was suggested that the arginine-lysine-rich region spanning amino acids 95 to 105 may interact with the phosphate backbone of RNP (29). Recently, it was shown that amino acid residues 101 to 105, which are similar to signal sequences for translocation across the nuclear membrane, are required for nuclear localization (34).

Our in vitro transcription system that depends on the exogenously added RNA template was suitable for the study of the mechanism of the anti-RNA synthesis activity of M1 protein. In previous studies, solubilized virion or RNP complexes purified from virions were used as enzyme sources and templates. The RNA synthesis system depending on the endogenous template in RNP complexes is thought to be suitable for analysis of the effect of M1 on the incoming RNP complexes which are utilized for the primary transcription or the progeny RNP complex which is readily covered with M1, if any. However, it is possible that M1 is bound to the progeny RNA under the competitive condition in the presence of NP early in the late stages of infection. The experiment described here, in which the RNP-M1 complex is reconstituted with naked exogenously added RNA, M1, and MN RNP, is one good model of the phenomena that occur at the late stages of infection. Systematic analyses of the step at which M1 inhibits transcription using the RNA synthesis system depending on the exogenously added template revealed that the target step for M1 is the cleavage of capped RNA or events prior to this cleavage. By using mutant M1 proteins, it was determined that the domain

essential for transcription inhibition is exactly the same as that essential for the RNA binding activity (Fig. 6 and 7). It is therefore presumed that M1 is bound to RNA or RNA on the RNP complex (Fig. 1C and 2), thereby inhibiting the initiation of RNA synthesis. RNA synthesis from the exogenously added template was more sensitive to M1 than that from the endogenously added template. It has been known that M1 inhibits transcription from the endogenous template by 50% at most. This phenomenon was interpreted as the presence of M1-sensitive and M1-insensitive species of RNP (9). It has been shown that M1 inhibits the steps of transcription initiation and reinitiation more effectively than that of RNA chain elongation. Experiments to discriminate these populations into defined species, for instance, including preinitiation complex, initiation complex, elongation-competent complex, and elongation complex, are ongoing.

It is unlikely that M1 regulates the primary transcription catalyzed by the incoming RNP, although there is a possibility that the dissociation of M1 from RNP differs among eight segments, and determines the template activity of each segment. M1 could be involved in the switch from replication and secondary transcription to virion assembly. A portion of M1 transported to the nucleus may inhibit the initiation and reinitiation of replication and transcription. RNP associated with M1 is exported into the cytoplasm by an unknown mechanism. Cooperative binding of M1 on RNA results in RNP covered with M1, which is readily incorporated into the envelope. Along this line, it is important to determine the domain of M1 involved in oligomerization.

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