

Role of two of the influenza virus core P proteins in recognizing cap 1 structures (m⁷GpppNm) on RNAs and in initiating viral RNA transcription

(UV-induced crosslinking/two-dimensional gel electrophoresis)

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Communicated by Aaron J. Shatkin, August 21, 1981

ABSTRACT Purified influenza viral cores catalyze the entire process of viral RNA transcription, which includes the endonucleolytic cleavage of heterologous RNAs containing cap 1 (m⁷GpppNm) structures to generate capped primers 10–13 nucleotides long, the initiation of transcription via the incorporation of a guanosine residue onto the primers, and elongation of the viral mRNAs [Plotch, S. J., Bouloy, M., Ulmanen, I. & Krug, R. M. (1980) *Cell* 23, 847–858]. To identify which viral core protein (nucleocapsid protein, P1, P2, or P3) recognizes the cap 1 structure on the RNA primer, we irradiated (UV) endonuclease reactions carried out by viral cores in the absence of ribonucleoside triphosphates, with a primer RNA labeled in its cap 1 structure with ³²P. The labeled cap was crosslinked to a protein that had a mobility similar to that of the P3 protein, the smaller of the two basic P proteins, in both one- and two-dimensional gel electrophoresis. This strongly suggests that this crosslinked protein is the viral P3 protein. Competition experiments with unlabeled RNAs containing or lacking a cap 1 structure established that this protein recognizes the cap 1 structure on RNAs. This protein remained associated with the cap throughout the transcription reaction, even after the viral mRNA molecules were elongated. To identify the viral core protein that catalyzes the initiation of transcription via the incorporation of a guanosine residue onto primer fragments, we irradiated transcription reactions carried out by viral cores in the presence of [α -³²P]GTP as the only ribonucleoside triphosphate with an unlabeled primer RNA. A labeled guanosine residue was crosslinked to a protein that had a mobility similar to that of the P1 protein, the larger of the two basic P proteins, in both one- and two-dimensional gel electrophoresis. The transcription reaction conditions required to bring this protein in close association with a labeled guanosine residue so that crosslinking could occur indicated that this association most likely occurred coincident with the guanosine residue's being incorporated onto the primer. These results suggest that the viral P1 protein catalyzes this incorporation and hence initiates transcription.

The unique mechanism by which influenza virus initiates the synthesis of its mRNA has recently been delineated. Transcription *in vitro* and *in vivo* is initiated by a primer derived from RNAs containing a 5'-terminal methylated cap (cap 1) structure m⁷GpppNm (1–5). As shown by studies *in vitro* using the virion-associated transcriptase, these capped RNAs are cleaved 10–13 nucleotides from their 5' ends, preferentially after a purine residue, by a viral endonuclease that requires the presence of a cap 1 structure in the RNA (6). Most of the capped RNA fragments generated by this endonuclease are most likely the actual primers that initiate viral RNA transcription because they were found to be linked to one or more guanosine residues in transcriptase reactions containing GTP as the only ribonucleoside

triphosphate (6). This guanosine incorporation is apparently directed by the penultimate cytosine residue at the 3' end of the eight virion RNA (vRNA) templates (6). In the presence of all four triphosphates, the viral RNA transcripts are then elongated.

This entire reaction is catalyzed by purified viral cores (nucleocapsids) (6), which contain four known virus-specific proteins: the nucleocapsid protein (NP), which constitutes the majority (about 92%) of the protein, and the three P proteins (6, 7). Studies with temperature-sensitive virus mutants indicate that at least two of these P proteins are required for transcription (8, 9).

We undertook the present study to establish the actual specific functions of individual P proteins in transcription. Using UV light-induced crosslinking, we found that the P3 protein, the smaller of the two basic P proteins of the WSN strain of influenza A virus, most probably is the protein that recognizes the 5'-terminal cap 1 structure on RNAs, and that the P1 protein, the larger of the two basic P proteins, is the protein that probably catalyzes the initiation of transcription via the incorporation of a guanosine residue onto the 3' end of a capped RNA fragment.

MATERIALS AND METHODS

Purification of Viral Cores. Viral cores were prepared by treating purified influenza virions (WSN strain) with lysolecithin followed by sedimentation in glycerol gradients (6, 10). Gel electrophoretic analysis of the proteins in the resulting core preparations showed the presence of only the three P proteins, the NP protein, and a small amount of the viral matrix (M) protein, as detected both by [³⁵S]methionine label and by Coomassie blue staining.

Assay for the Binding of RNA Fragments to Viral Cores. Alfalfa mosaic virus (ALMV) RNA 4 containing ³²P in its 5'-terminal cap 1 structure (m⁷GpppGm) (6), or a fragment of this RNA generated as described below, was incubated with cores for 30 min at 31°C under transcription conditions but in the absence of the four ribonucleoside triphosphates (6). The mixture was then subjected to centrifugation on a 15–40% (vol/vol) glycerol gradient for 3 hr at 48,000 rpm in the SW 50.1 rotor to separate cores from free RNA or RNA fragments. The amount of ³²P in each gradient fraction was determined, and, after phenol extraction, the free RNA and the RNA bound to cores were analyzed by electrophoresis on 20% acrylamide/7 M urea gels (6). The following fragments of cap-labeled RNA 4 were used: (i) the cap fragment cleaved at the adenosine 13 nucleotides from the cap (A-13 fragment) by the viral endonuclease

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Abbreviations: vRNA, virion RNA; NP, nucleocapsid protein; ALMV, alfalfa mosaic virus.

and isolated by gel electrophoresis (6); (ii) the A-7 cap fragment [$m^7GpppGm(U)_5A(p)$] generated by digestion of the RNA with RNase U2 and isolated by gel electrophoresis; (iii) $m^7GpppGmpUp$ generated by complete digestion of the RNA with RNase T2; and (iv) the cap itself, $m^7GpppGm$, generated by complete digestion of the RNA with nuclease P1.

Assays for the Viral Endonuclease and for the Initiation of Viral RNA Transcription. The viral endonuclease was assayed by incubating viral cores (25–30 μ g of protein) for 30 min at 31°C with AIMV RNA 4 containing ^{32}P in its cap 1 structure in the absence of ribonucleoside triphosphates (6). To assay for the initiation of transcription, an unlabeled capped RNA was incubated with viral cores for 30 min at 31°C in the presence of [α - ^{32}P]GTP (or [α - ^{32}P]CTP in some experiments) (6). With both assays, 5 μ l of the reaction mixture (100 μ l) was extracted with phenol, and the labeled RNA fragments were resolved by electrophoresis on 20% acrylamide/7 M urea gels.

Photochemical Crosslinking. The remainder of the reaction mixtures from the endonuclease and initiation assays were irradiated at 254 nm for 1 hr at 0°C at a distance of 4 cm with a Mineralight USV-11 UV lamp, yielding a dose of about 3×10^5 ergs/mm². The samples were digested for 1 hr at 37°C with pancreatic and T1 RNases at a final concentration of 200 μ g/ml and 150 units/ml, respectively. The proteins were then analyzed by gel electrophoresis.

Electrophoresis of Viral Proteins on Polyacrylamide Gels. Viral proteins were resolved on one-dimensional 8% acrylamide/4 M urea gels (11). For analysis on two-dimensional gels, the viral protein mixture was adjusted to O'Farrell lysis buffer A (which lacks sodium dodecyl sulfate) (12). The first dimension was a nonequilibrium pH gradient electrophoresis (12–14) run at 500 V for 3.5 hr, and the second dimension was the 8% acrylamide/4 M urea gel described above. Gels were stained with 0.01% Coomassie brilliant blue in 50% trichloroacetic acid and were destained with several washes of 7% acetic acid.

Material. Capped RNA primers, globin mRNA, AIMV RNA 4, and brome mosaic virus RNA 4, were obtained as described (6). Globin mRNA lacking a terminal m^7G was prepared by β -elimination (2, 6). Ribonucleoside [α - ^{32}P]triphosphates were purchased from Amersham, New England Nuclear, and International Chemical and Nuclear. [^{35}S]Methionine-labeled virus was prepared as described (15).

RESULTS

Specific Binding of Capped RNA Fragments to Viral Cores.

When AIMV RNA 4 containing a ^{32}P -labeled methylated cap (cap 1) structure (m^7G^*pppGm) was incubated with viral cores for 30 min at 31°C in the absence of ribonucleoside triphosphates, a large fraction (about 70%) of the ^{32}P label cosedimented with viral cores in glycerol gradients (Fig. 1 *Left*). The radiolabeled material bound to the cores was all in the form of the A-13 cleavage product of AIMV RNA 4 generated by the viral endonuclease, whereas the material that did not bind was largely uncleaved RNA. Neither cleavage nor binding occurred when the incubation was carried out at 0°C. The presence of a methylated cap structure was required for binding because AIMV RNA 4 containing a G^*pppG 5' end did not bind (not shown). The A-13 capped fragment isolated from a viral endonuclease reaction also bound to cores with an efficiency that was high but slightly lower than that observed when the incubation was carried out with the intact RNA.

A shorter fragment, $m^7GpppGm(U)_5A(p)$, also bound with relatively high efficiency, about half that observed with the intact RNA (Fig. 1 *Middle*). In contrast, the cap 1 structure alone, m^7G^*pppGm , bound with extremely low efficiency (Fig. 1

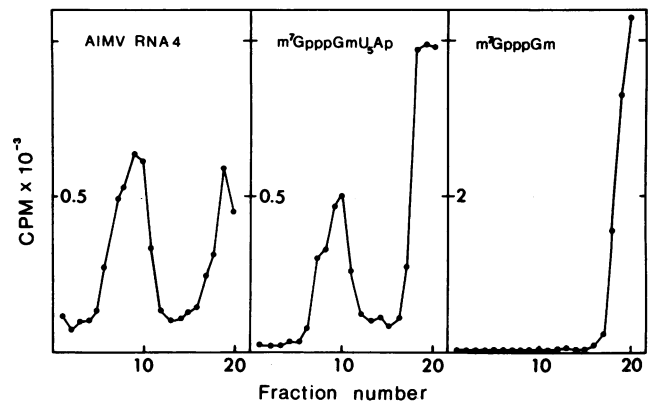


FIG. 1. Binding of capped RNA fragments to viral cores. Purified viral cores were incubated for 30 min at 31°C with intact AIMV RNA 4 containing a ^{32}P -labeled cap 1 structure (*Left*), $m^7G^*pppGm(U)_5Ap$ (*Middle*), or m^7G^*pppGm (*Right*). The incubation mixtures were subjected to glycerol gradient centrifugation; the direction of sedimentation was from right to left. Cores sedimented in fractions 7–11.

Right). Mixing experiments (m^7G^*pppGm plus intact cap-labeled AIMV RNA 4) indicated that this low binding was not due to the presence of an inhibitor in the m^7G^*pppGm preparation. Similar low binding was observed with $m^7G^*pppGmpUp$. Consequently, although a 5' cap 1 structure is required for binding to cores, it by itself is not sufficient for efficient binding. Some additional structure is also needed, apparently including a RNA chain length of about seven nucleotides.

Identification of the Cap-Recognizing Protein in Viral Cores. Because of the above results, we devised an assay for the viral cap-recognizing protein that used intact RNAs as probes and specific competitors. As demonstrated (6), when AIMV RNA 4 containing a ^{32}P -labeled cap 1 structure (m^7G^*pppGm) is incubated with viral cores, the predominant cleavage product generated by the cap-dependent endonuclease is the A-13-capped fragment (Fig. 2A). This cleavage was totally blocked by preincubating the cores with unlabeled globin mRNA (0.2 μ M), an RNA containing a cap 1 structure (lane 2). In contrast, cleavage of AIMV RNA 4 was not blocked when the cores were preincubated with 0.2 μ M unlabeled globin mRNA lacking a 5' terminal m^7G (β -eliminated globin mRNA) (lane 3) or with 0.2 μ M unlabeled brome mosaic virus RNA 4 which contains a cap lacking a 2'-O-methyl group (cap 0, m^7GpppG) (not shown). Consequently, this competition shows the strict specificity of the viral endonuclease for a cap 1 structure and thus constitutes an assay for the cap-recognizing protein associated with the endonuclease activity.

To identify the cap-recognizing protein, the reaction mixtures of lanes 1–3 of Fig. 2A were exposed to UV light to crosslink the cap to the protein recognizing it. These samples were treated with pancreatic and T1 RNases and then analyzed on protein gels for the presence of ^{32}P label in viral proteins (Fig. 2B). In the absence of a competitor RNA, two proteins were crosslinked to the cap: the nucleocapsid protein (NP) and a protein migrating in the P3 region of the gel. The crosslinking of the latter protein was completely blocked by competition with unlabeled capped globin mRNA but not by competition with unlabeled globin mRNA lacking a terminal m^7G . Crosslinking of this protein required a methylated cap structure in the labeled AIMV RNA 4 primer; no crosslinking occurred with AIMV RNA 4 containing a ^{32}P -labeled 5' end G^*pppG (not shown). Consequently, this P protein exhibits the expected characteristics of the cap-recognizing protein. In contrast, the crosslinking of NP to the cap was not specific because it was partially

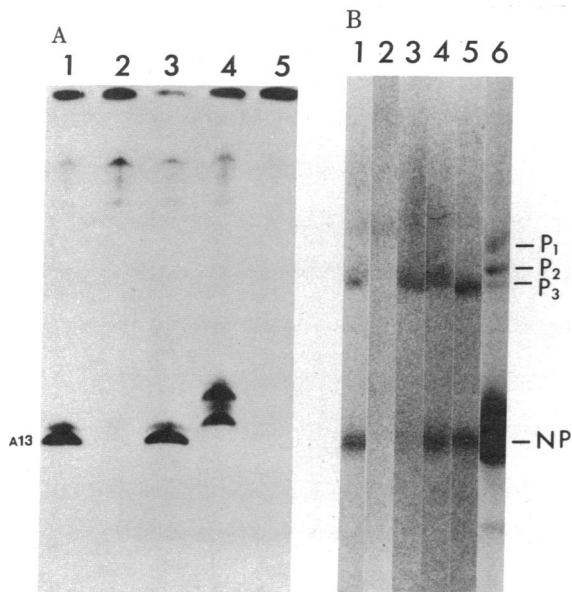


FIG. 2. Assay for the viral core protein that recognizes cap 1 structures on RNAs. Purified viral cores were incubated for 30 min at 31°C with AIMV RNA 4 (100,000 cpm per reaction) in the absence of ribonucleoside triphosphates (lanes 1–3), in the presence of only 50 μ M GTP (lane 4), or in the presence of all four ribonucleoside triphosphates (each at 1 mM) (lane 5). Before the addition of the labeled AIMV RNA 4 in lanes 2 and 3, the viral cores were preincubated for 10 min at 31°C with either 0.2 μ M unlabeled globin mRNA (lane 2) or 0.2 M unlabeled, β -eliminated globin mRNA (lane 3). A 5- μ l aliquot of each of the reaction mixtures after phenol extraction was analyzed on a 20% acrylamide/7 M urea gel (A). The remainder of each reaction mixture was exposed to UV light, treated with RNases, and analyzed on protein gels (8% acrylamide/4 M urea) (B). Lane 6 in B shows the gel pattern of the [³⁵S]methionine-labeled proteins in purified viral cores.

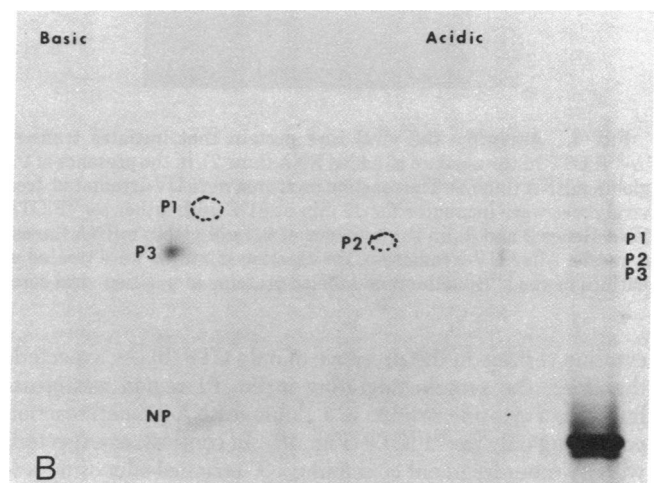
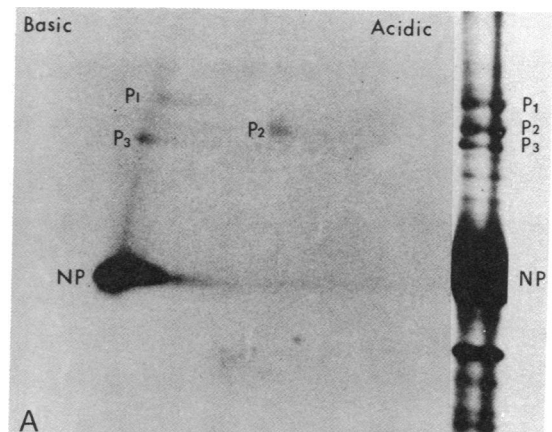


FIG. 3. Two-dimensional gel electrophoresis of [³⁵S]methionine-labeled proteins in viral cores (A) and of the crosslinked ³²P-labeled cap 1-recognizing viral core protein (B). For B, a reaction mixture similar to that for lane 1 of Fig. 2 was incubated, UV-irradiated, and treated with RNases. The viral cores (A) were also treated with RNases prior to gel electrophoresis. The marker lanes show the mobility of the [³⁵S]methionine-labeled proteins of virus (A) or of viral cores (B) in the second dimension. The expected positions of the P1 and P2 proteins are marked in B.

inhibited by either capped or β -eliminated globin mRNA or, in fact, by any RNA.

The protein in the P3 region of the gel was also capable of being crosslinked to the labeled cap 1 structure of AIMV RNA 4 when the transcription reaction was carried out in the presence of only GTP or in the presence of all four ribonucleoside triphosphates (Fig. 2B, lanes 4 and 5). In the presence of only GTP, a few guanosine residues were added to the A-13 fragment; in the presence of all four triphosphates, the A-13 fragment was elongated to form completed viral mRNA chains which did not enter the 20% gel (Fig. 2A, lanes 4 and 5). Consequently, this P protein remains associated with the 5' cap throughout the transcription reaction.

For more definitive identification of the cap-recognizing protein, we used two-dimensional O'Farrell gels (12–14). In such gels, the three P proteins associated with viral cores distribute into two basic proteins (P1 and P3) and one acidic protein (P2) (Fig. 3A), as shown previously (14). When subjected to the same analysis, the crosslinked cap-recognizing protein clearly migrated as a basic protein with a mobility like that of P3 (Fig. 3B). Electrophoretic analysis of a mixture of the crosslinked cap-recognizing protein and the unreacted P3 core protein revealed a small difference in their mobilities (not shown), consistent with the former containing a few covalently linked nucleotides. A small change in the electrophoretic mobility of a protein due to the UV-induced crosslinking of a few nucleotides has been noted (16). These results strongly suggest that the viral cap-recognizing protein is P3, the smaller of the two basic P proteins.

Identification of the Viral Protein Catalyzing the Initiation of Transcription. With capped RNAs as primers, the initiation of transcription occurs via the incorporation of a guanosine onto

the 3' end of the primer fragments generated by the viral endonuclease (6). The viral protein catalyzing this initiation should be closely associated with, and hence capable of being crosslinked to, this incorporated residue. Consequently, we incubated cores with [³²P]GTP in the presence or absence of globin mRNA at 31°C and then exposed these reaction mixtures to UV light. In the presence of the capped globin mRNA primer, two proteins were crosslinked to a guanosine residue: the NP protein, and a protein migrating in the P1 region of the gel (Fig. 4A). The crosslinking of this P protein, but not that of NP, was specific. Thus, the P protein was not crosslinked in the absence of a primer RNA or in the presence of a nonprimer RNA (lane 2), β -eliminated globin mRNA (lane 4), or when the incubation with capped globin mRNA was carried out at 0°C rather than 31°C (not shown). Consequently, this P protein exhibits the characteristics expected of the protein catalyzing the primer RNA-dependent initiation of transcription via the initial incorporation of a guanosine residue.

Further evidence for this conclusion was obtained in experiments using the other triphosphates in single-triphosphate reactions. Because the primer fragments of globin mRNA contain a 3'-terminal guanosine residue, they can also be linked to a

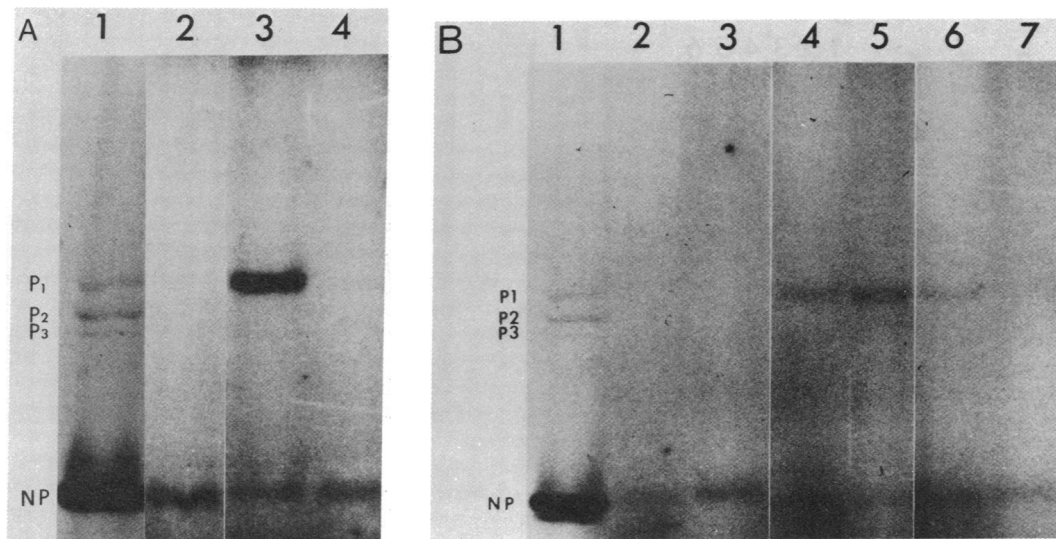


FIG. 4. Assay for the viral core protein that initiates transcription. (A) Purified viral cores were incubated for 30 min at 31°C with [α - 32 P]GTP in the absence of added RNA (lane 2), in the presence of 0.2 μ M capped globin mRNA (lane 3), or in the presence of 0.2 μ M β -eliminated globin mRNA (lane 4). The reaction mixtures were UV-irradiated, treated with RNases, and analyzed on a one-dimensional protein gel. (B) Purified viral cores were incubated for 30 min at 31°C with either [α - 32 P]GTP (lanes 2, 4, and 6) or [α - 32 P]CTP (lanes 3, 5, and 7) in the absence of added RNA (lanes 2 and 3), in the presence of 0.2 μ M globin mRNA (lanes 4 and 5), or in the presence of 0.2 μ M 2'-O-methylated AIMV RNA 4 (lanes 6 and 7). After UV-irradiation, the reaction mixtures were treated with RNases and analyzed on a protein gel. Lanes 1 in A and B show the gel pattern of the [35 S]methionine-labeled proteins in purified viral cores.

cytosine residue in the presence of only CTP (6). As expected, therefore, the protein migrating in the P1 region was cross-linked to a cytosine residue in a globin mRNA-primed reaction containing only [α - 32 P]CTP (Fig. 4B). In contrast, as expected, with a primer fragment containing a 3'-terminal adenosine residue (the A-13 fragment of AIMV RNA 4), this protein was cross-linked to guanosine but not to cytosine, in single-triphosphate reactions. With either globin mRNA or AIMV RNA 4 as primer, this protein was not crosslinked to an adenosine or uridine residue in single-triphosphate reactions (not shown).

In two-dimensional gels this P protein migrated as a basic protein with a mobility like that of P1 (Fig. 5). As with the P3 protein, a small difference was detected between the mobility of the crosslinked P protein and that of the unreacted P1 core

protein (not shown), consistent with the presence of a few covalently linked nucleotides after crosslinking and RNase digestion. These results strongly suggest that the viral protein catalyzing the initiation of transcription is P1, the larger of the two basic P proteins.

DISCUSSION

The transcriptase complex associated with influenza viral cores requires a methylated cap (cap 1) structure m^7 GpppNm on the RNA primer (2, 3, 6, 17). We have most likely identified the viral core protein that recognizes this cap structure. After UV irradiation of endonuclease reaction mixtures in which viral cores had been incubated at 31°C with AIMV RNA 4 containing a 32 P-labeled cap 1 structure, the labeled cap 1 structure was crosslinked to a protein that very likely was the viral P3 protein on the basis of its mobility during electrophoresis in both one- and two-dimensional gels. The specificity of the crosslinking of P3 to the labeled cap of AIMV RNA 4 was demonstrated by competition experiments in which only those unlabeled RNAs that contained a cap 1 structure blocked this crosslinking.

There were several reasons for using an assay for the cap-recognizing protein in which intact RNAs were probes and specific competitors. First, intact capped RNAs or relatively long 5'-terminal fragments of these RNAs are the actual species that, at low concentrations (0.2 μ M), maximally stimulate the viral transcriptase reaction (1, 3, 18, 19). In addition, the 5'-terminal fragments cleaved from these RNAs by the viral endonuclease bind efficiently to cores, whereas the cap 1 structure alone, m^7 GpppGm, binds very poorly. This explains why the concentration of m^7 GpppGm required for effective inhibition of capped RNA-primed transcription and of endonuclease activity is 1000-fold higher (about 0.2 mM) than that required for optimal priming by a capped RNA (ref. 17; unpublished data). At these high concentrations, much of the specificity is lost; the cap 0 fragment m^7 GpppG and even the unmethylated fragment GpppG also inhibit (17).

Our experiments show that the P3 protein is closely associated with the 5'-terminal cap 1 structure throughout the tran-

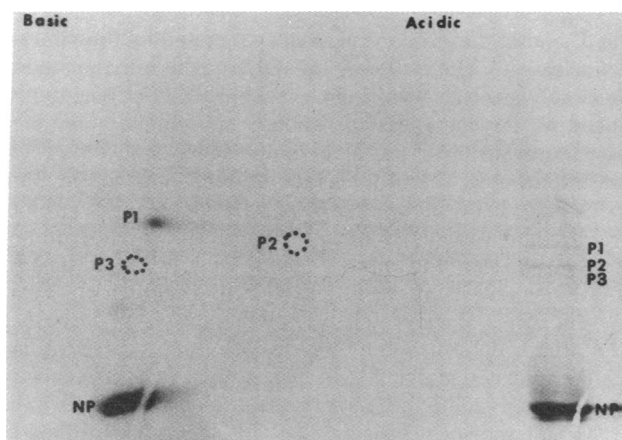


FIG. 5. Two-dimensional gel electrophoresis of the viral core P protein which probably catalyzes the initiation of transcription. A reaction mixture similar to that for lane 3 of Fig. 4A was incubated, UV-irradiated, and treated with RNases prior to gel electrophoresis. The marker lane shows the mobility of the [35 S]methionine-labeled proteins of viral cores in the second dimension. The expected positions of the P2 and P3 proteins are marked.

scription reaction, from when the primer RNA is cleaved to after the viral mRNA chains are completed in the presence of the four ribonucleoside triphosphates. This suggests that P3 may continue to remain associated with the cap of the viral mRNA molecules after they are released from the transcriptase complex. If so, this protein might then serve other functions as well. For example, P3 could facilitate the translation of viral mRNAs, thereby bypassing the use of the cellular cap-binding proteins associated with ribosomes (20, 21). Furthermore, the continued association of P3 with the cap of the completed viral mRNAs argues that P3 alone does not possess endonuclease activity; otherwise, the 5' ends of the newly synthesized viral mRNAs would be removed. In fact, our experiments were specifically designed to identify only the cap-recognizing component of the endonuclease, and this enzyme could be composed of more than one of the P proteins, perhaps P1 and P3 as discussed below.

The first virus-encoded nucleotide added to primer fragments is a guanosine directed by the 3' penultimate cytosine of the vRNA (6). When the primer fragment contains a 3'-terminal guanosine, it is also possible to link a cytosine (directed by the guanosine at the third position from the 3' end of the vRNA) onto these primer fragments in reaction mixtures containing only CTP (6). Here we show that UV irradiation of transcription mixtures that had been incubated at 31°C with either [α -³²P]GTP or [α -³²P]CTP and the appropriate unlabeled capped RNA primer results in crosslinking between the labeled initiating nucleotide and a protein that very likely was the viral core P1 protein on the basis of its mobility during electrophoresis in both one- and two-dimensional gels. The transcriptase reaction conditions required for bringing the P1 protein into close association with a labeled guanosine or cytosine residue so that crosslinking can occur are the same conditions required for covalent bond formation between the endonuclease-generated primer fragment and these residues. This suggests that the P1 protein is closely associated with the guanosine or cytosine residue coincident with its incorporation onto the primer fragment, consistent with this protein catalyzing the initiation of transcription by incorporating these residues.

Consequently, we have shown that the primer fragments to which a guanosine or cytosine has been linked are closely associated with two viral core proteins—P3 with the 5' cap and P1 with the 3' guanosine or cytosine residue. It will be of great interest to determine whether P1 is actually associated with the 3' end of the endonuclease-generated primer fragment before guanosine or cytosine is linked to this fragment. If this were the case, it would explain why longer (seven nucleotides or more) capped RNA fragments bind to viral cores much more effectively than do short cap fragments like m⁷GpppGm and

m⁷GpppGmpUp, because effective binding to cores may require that a capped RNA contain a P1 as well as P3 binding site. Based on this reasoning, we can speculate that a complex containing both P3 and P1 proteins actually constitutes the endonuclease enzyme, with P3 recognizing the 5' cap and P1 cleaving the phosphodiester bond. Then, with P3 remaining at the cap, P1 would link the initial guanosine to the 3' end of the capped RNA fragment. Further experiments are needed to test this hypothesis and to determine whether P1, or some other viral protein—e.g., P2—catalyzes the subsequent elongation of the viral mRNA chains.

To facilitate comparison with the P proteins of other influenza virus strains, we suggest that the P proteins be renamed on the basis of their mobilities in two-dimensional gels. Thus, the P3 protein of our strain (WSN) would be called PB2 (i.e., the smaller of the two basic P proteins); P1 would be called PB1; and P2 would be called PA.

This investigation was supported by U.S. Public Health Service Grants CA 08747 and AI 11772 and by European Molecular Biology Organization Fellowship ALTF 190-1979 to I.U.

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