RNA polymerase of influenza virus. IV. Catalytic properties of the capped RNA endonuclease associated with the RNA polymerase

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ABSTRACT

Catalytic properties of the capped RNA-specific endonuclease associated with the influenza virus RNA polymerase were analyzed with use of synthetic hetero- and homopolymers containing ³²P-labeled CAP structures at their 5' termini. The endonuclease displays its intrinsic activity provided that substrate RNA contains both the CAP-1 structure (m⁷GpppGm) and either A or U residues at 9 to 11 nucleotides distant from the CAP structure. Independent recognition of multiple RNA signals by the endonuclease was further supported by the findings that dinucleotide ApG, free CAP structures and RNA without the CAP structure inhibited the endonuclease activity to different extents.

In the presence of four species of ribonucleoside 5'-triphosphates, the endonucleolytically cleaved fragments with the CAP-1 structure were incorporated into polynucleotides, supporting the concept that they are used as the primers for the transcription. The initial nucleotide linked to the primers was a G residue, the nucleotide complementary to the second base of the 3' termini of the vRNA segments.

INTRODUCTION

The genomes of RNA viruses multiply in various modes and each viridae exhibits its own unique mechanisms of transcription, replication and translation. Parallel with the variety of the multiplication modes, viral RNA polymerases are complex with respect to their functions and structures. For example, three types of transcription initiation are known: i) de novo initiation by vesicular stomatitis virus RNA polymerase (1,2); ii) RNA-primed initiation by influenza virus RNA polymerase (3,4); and iii) protein-primed initiation by polio virus RNA polymerase (5,6). Of these three initiation types, the RNA-primed initiation depends most on host cell functions, because it requires a constant supply of host cell mRNA (7,8).

Various RNAs with CAP structures such as globin mRNA, reovirus mRNA, CPV (cytoplasmic polyhedrosis virus) mRNA, several plant virus RNAs recapped with the CAP-1 structure, and synthetic polymers with the CAP-1 structure are effective primers for the influenza virus-associated RNA polymerase reaction in vitro and transfer short 5' RNA fragments containing the CAP-1 structure

into viral mRNA (3,9-12). Evidence is being accumulated indicating that the host cell mRNAs are utilized as primers for in vivo initiation of viral transcription (4,13-15). Krug and colleagues have shown that the first step of the transcription initiation is the endonucleolytic cleavage of capped RNA and the second step is the addition of G or C residues to the cleaved capped RNA fragments (16). For a detailed understanding of the mechanism of transcription initiation and the roles of the influenza virus-associated endonuclease-RNA polymerase complex, we have prepared various hetero- and homopolyribonucleotides with ^{32}P -labeled CAP structures and investigated their availability as primers for RNA synthesis. The effects of dinucleotides, free CAP structures and RNAs without CAP structures on the endonuclease reaction were also examined to confirm our conclusion that the capped RNA endonuclease-RNA polymerase complex carries multiple recognition sites for RNA signals. The effect of CAP structures on RNA synthesis is described in the following report (Kawakami et al., submitted for publication).

MATERIALS AND METHODS

Viruses --- Influenza virus A/PR/8/34 (H1N1) was used throughout this study. Viruses were grown in allantoic sacs of 10 day-old embryonated eggs for 48 hr at 35.5°C. Viruses were purified as described previously (17,18).

Hetero- and Homopolyribonucleotide Synthesis --- Hetero- and homoribopolymers were synthesized enzymatically using ^3H -labeled ribonucleoside diphosphates as substrates, ppGpCpA as a primer and primer-dependent polynucleotide phosphorylase (PNPase) from Micrococcus luteus as an enzyme source (19). Trinucleotide ppGpCpA was prepared from ppG>p and CpA by the reverse reaction of ribonuclease T1 (20). The reaction conditions for ppGpCpA-primed poly(A₂U₂G) were as described previously (20,21), while the reaction conditions for the synthesis of ppGpCpA-primed homopolymers were modified with respect to pH and NaCl concentration. The pH was 8.25 for poly(G) and poly(C) synthesis, and 8.50 for poly(A) and poly(U) synthesis. The NaCl concentration was 0.10 M for poly(U), 0.20 M for poly(A) and poly(C), and 0.32 M for poly(G), respectively. For homopolymer synthesis, [^3H]ADP (0.42 cpm/pmol), [^3H]GDP (0.12 cpm/pmol), [^3H]CDP (0.5 cpm/pmol) or [^3H]UDP (0.35 cpm/pmol) was used as substrate. The reaction was generally carried out for 4 hr at 37°C and terminated by adding 0.3 ml of the stopping solution (7 M urea, 0.5% SDS, 20 mM Tris-HCl (pH 7.9), 0.2 mM EDTA and 0.2 M NaCl) to 0.1 ml reaction mixture. RNA products were phenol-chloroform extracted, ethanol precipitated twice and dissolved in 0.2 ml of 40 mM NH₄ acetate-1 mM EDTA solution, and then purified

by chromatography on a Sephadex G-25 column.

Capping Reaction --- Synthetic hetero- and homopolymers were subjected to the capping reaction using the vaccinia virus capping enzyme. The reaction mixture contained, in a final volume of 50 μ l: 60 mM Tris-HCl (pH 7.9 at 37°C), 2 mM $MgCl_2$, 28 mM NaCl, 20 mM dithiothreitol (DTT), 30 μ M S-adenosyl-L-methionine (SAM), 1.3 μ M [α - ^{32}P]GTP (1.2×10^5 cpm/pmol) and either crude capping enzyme fraction from the vaccinia virus for the synthesis of the CAP-1 ($m^7GpppNm$) structure or purified capping enzyme for the synthesis of the CAP-0 (m^7GpppN) structure. The reaction was carried out for 1.5 hr at 37°C and terminated by adding 0.3 ml of the stopping solution. Capped RNA was phenol-chloroform extracted in the presence of *E. coli* tRNA, ethanol precipitated twice, and purified by Sephadex G-50 column chromatography.

For analysis of CAP structures, capped RNA was completely digested with nuclease P1 and subjected to thin-layer chromatography on a polyethyleneimine cellulose sheet. For development, 0.6 M LiCl was used.

Assay for Endonuclease and RNA Polymerase --- The standard reaction mixture for RNA polymerase-associated endonuclease activity contained in a final volume of 100 μ l: 50 mM Tris-HCl (pH 7.7 at 30°C), 5 mM $MgCl_2$, 2.5 mM DTT, 0.1 M NaCl, 0.1% Nonidet P-40 (NP-40), synthetic polynucleotides with ^{32}P -labeled CAP structures as substrates and purified virions as the enzyme source. The reaction was carried out for 30 min at 30°C and terminated by adding SDS to the final concentration of 0.2%. RNA was extracted with phenol-chloroform and, after addition of tRNA carrier, precipitated with ethanol. The ethanol precipitates were dissolved in 15 μ l of the RNA sample buffer (80% formamide, 8% glycerol, 8 mM EDTA, 0.01% xylene cyanol and 0.01% bromophenol blue) and analyzed by electrophoresis on 18% polyacrylamide gel containing 7 M urea in 50 mM Tris-borate (pH 8.3)-1 mM EDTA buffer. Gels were exposed to X-ray films and traced with a Joyce-Loebl microdensitometer.

RNA synthesis by influenza virus-associated RNA polymerase was carried out as described previously (18). To examine the elongation of ^{32}P -labeled oligonucleotide primers generated by the endonuclease reaction, one or several species of nucleoside triphosphates were added simultaneously with the initiation of the endonuclease reaction.

Chemicals and Enzymes --- 3H -Labeled ribonucleoside diphosphates (ADP, GDP, CDP and UDP) were obtained from Amersham, England, while unlabeled ribonucleoside diphosphates were from Yamasa Shoyu, Japan. [α - ^{32}P]GTP and unlabeled ribonucleoside triphosphates were obtained from Amersham, England, and PL-Biochemicals, USA, respectively.

Crude capping enzyme from the vaccinia virus (first DEAE-cellulose fraction) was prepared as described by Martin *et al.* (23) from purified viruses, which were kindly supplied by T. Urushibara, Kitasato University. Purified capping enzyme was a product of BRL, USA. Ribonuclease T1, nuclease P1 and polynucleotide phosphorylase (PNPase) were purchased from Sankyo (Japan), Yamasa Shoyu (Japan) and PL-Biochemicals (USA), respectively. Alkaline phosphatase was a product of Boehringer, Mannheim, Germany.

RESULTS

RNA Signals for the Endonuclease

To examine the roles of CAP structures, RNA sequences and distances between these two signals on the nucleolytic cleavage by influenza virus-associated endonuclease, we synthesized various homo- and heteropolyribonucleotides modified with various forms of ^{32}P -labeled CAP structures at their 5' termini and followed the fate of ^{32}P radioactivity after incubation with influenza virus.

Role of CAP Structures: A synthetic heteropolymer, ppGpCpA-poly($\text{A}_2\text{U}_2\text{G}$) with the chain length of 30~70 nucleotides was capped by the vaccinia virus capping enzyme system and using [$\alpha\text{-}^{32}\text{P}$]GTP and unlabeled S-adenosyl methionine as substrates. For the addition of the CAP-0 structure (CAP structure without ribose-2'-O-methyl), we used a purified enzyme preparation containing both guanylyltransferase and guanine-7-methyltransferase but lacking ribose-2'-O-methyltransferase, whereas a crude capping enzyme fraction containing all the three enzyme activities was used to add the CAP-1 structure (fully methylated CAP structure). The nature of CAP structures thus formed was analyzed by thin-layer chromatography of nuclease P1 digests on PEI cellulose sheet. The CAP-0 product contained both the m^7GpppG and GpppG structures in the molar ratio of 1:3.7, while more than 95% of the CAP-1 RNA preparation contained the m^7GpppGm structure (data not shown).

When the CAP-1 RNA (RNA with CAP-1 structure) was incubated with purified influenza virus in the presence of a non-ionic detergent, radioactive materials shifted to bands corresponding to 9~11 nucleotide long RNA fragments, confirming the nucleolytic cleavage of RNA modified with fully methylated CAP structure (16). In contrast, the migration pattern of the CAP-0 RNA (RNA without ribose-2'-O-methyl) did not change appreciably (data not shown). We therefore concluded that not only unmethylated CAP-core RNA (16) but also CAP-0 RNA are insensitive to cleavage by influenza virus endonuclease and the CAP-1 structure is absolutely required.

Role of RNA Sequences: To determine the sequence specificity, we synthesized four species of homopolymers by elongating primer trinucleotide, ppGpCpA, with polynucleotide phosphorylase, added the [32 P]CAP-1 structure at their 5' termini and investigated their cleavage by influenza virus-associated endonuclease. As shown in Figure 1, the migration pattern on gel electrophoresis changed only for poly(A), poly(U) and poly(A₂U₂G) (lanes 1-2, 7-8 and 9-10), while the pattern did not change appreciably for poly(G) and poly(C) (lanes 3-4 and 5-6). In the presence of four species of ribonucleoside tri-

Figure 1. Role of RNA sequences in the endonucleolytic cleavage. [A] 32 P-Labeled capped homo- and heteropolyribonucleotides were prepared as described in MATERIALS AND METHODS, and incubated with 5 μ g of purified influenza virus for 0 (lanes 1, 3, 5, 7 and 9) or 30 min (lanes 2, 4, 6, 8 and 10) at 30°C. RNA was extracted by phenol treatment and analyzed by electrophoresis on 18% polyacrylamide gel containing 7.3 M urea and the gel was exposed for autoradiography. Lanes 1-2, poly(A) (6.8 fmol); lanes 3-4, poly(G) (7.1 fmol); lanes 5-6, poly(C) (9.8 fmol); lanes 7-8, poly(U) (8.8 fmol); lanes 9-10, poly(A₂U₂G) (6.6 fmol). The open and closed arrowheads indicate the migration position of xylene cyanol and bromophenol blue, respectively, while the arrow indicates the gel origin. [B] 32 P-Labeled poly(A) (lanes 1-3; 26 fmol each), poly(U) (lanes 4-6; 26 fmol each) and poly(A₂U₂G) (lanes 7-9; 26 fmol each) were incubated with 4 μ g of purified virions in the absence (lanes 2, 5 and 8) or presence (lanes 3, 6 and 9) of 0.2 mM each of ATP, GTP, CTP and UTP for 30 min at 30°C. Samples without incubation are in lanes 1, 4 and 7.

phosphates, the cleaved fragments of poly(A), poly(U) and poly(A₂U₂G) were elongated into high molecular-weight polynucleotides which remained on the gel top after electrophoresis (Fig. 1B). These observations indicate that the endonuclease recognizes and cleaves either A or U sequences, and the cleavage products are ultimately elongated by the RNA polymerase into polynucleotides. Our conclusion on the sequence signal is apparently inconsistent with the proposal of Plotch *et al.* (16) that the endonuclease cleaves capped RNA at the 3' side of A or G residues. Possible explanations for this discrepancy are given later.

Substrate activities for the endonuclease and primer activities for the RNA polymerase were then compared between the two capped homopolymers, poly(A) and poly(U). Figure 2(I) shows the kinetics of the cleavage reaction for capped poly(A). The cleaved fragments appear first at 5 min and increase thereafter while the rest of capped poly(A) remains uncleaved (Fig. 2(IA)).

Figure 2. [I] Time course of the endonuclease reaction. ³²P-labeled capped poly(A) (26 fmol) and purified virions (4 µg) were incubated in the absence [A] or presence [B] of ATP, GTP, CTP and UTP for various times at 30°C. The reaction times are: 0 (lane 1), 1 (lane 2), 2 (lane 3), 3 (lane 4), 5 (lane 5), 10 (lane 6), 20 (lane 7) and 30 min (lane 8), respectively. Markers are as shown in Fig. 1. [II] Elongation of the cleaved RNA fragments. ³²P-labeled capped poly(A) (26 fmol) [A] or ³²P-labeled capped poly(U) (26 fmol) [B] was incubated with purified virions (10 µg) in the absence (lane 1) or presence of 0.2 mM of GTP (lane 2), ATP (lane 3), CTP (lane 4) or UTP (lane 5) for 30 min at 30°C.

Since no degradation intermediates exist during the cleavage reaction, we suggest that the activity is not due to an exonuclease but an endonuclease with a high specificity with respect to the cleavage site. In the presence of four species of ribonucleoside triphosphates, the appearance of high molecular-weight RNA is closely coupled with the disappearance of capped poly(A) (Fig. 2(1B)). With use of the same amount of enzyme, capped poly(U) was cleaved faster than capped poly(A), *e.g.*, 50% cleavage time was within 10 min for poly(U) but between 15 and 20 min for poly(A). In another experiment, 5-fold more enzyme was required for poly(A) to achieve the same cleavage rate as that for poly(U).

Figure 2(II) shows the single addition of nucleotide to either capped poly(A) fragment [A] or capped poly(U) fragment [B]. The bands of poly(A) fragments became slower migrating bands, by one nucleotide, only when the incubation was carried out in the presence of GTP alone. On the other hand, the migration of cleaved poly(U) fragments in the presence of GTP decreased by two nucleotides. No changes were observed for the migration patterns of both capped poly(A) and poly(U) fragments when the reaction was carried out in the presence of either a single species of another three nucleotides (ATP, CTP or UTP) or any combination of these nucleotides lacking GTP. Plotch *et al.* (16) reported that one to five G residues were incorporated into the primer fragments. But, we found that such an erroneous reaction took place only for the capped poly(U) fragment and in the presence of high concentrations (above 30 μ M) of GTP alone (Kawakami, K. *et al.*, in preparation). In the capped poly(A)-primed reaction, only a single G residue is added to the primer fragment even at higher GTP concentrations and the second base to be added is only C residue, which is complementary to the third base of the 3' termini of vRNAs.

Distance between the Two Signals: As an attempt to locate the two recognition sites on the endonuclease-RNA polymerase complex, we next determined the distance between the two RNA signals on the substrate RNA molecules. The cleavage fragments of capped poly(A), poly(U) and poly(A₂U₂G) were electrophoresed along their partial digests by nuclease P1. Based on nucleotide ladder analysis, we concluded that the chain length of major cleavage products was 10~11 for poly(A) and poly(U), and 9~10 for the heteropolymer (data not shown).

Inhibitors for the Endonuclease

Inhibition by Dinucleotides: To confirm the existence of multiple recognition sites on the enzyme complex, we next investigated the effect of dinucleo-

tides, free CAPs and RNAs without CAP structures on the endonuclease reaction. The dinucleotide, ApG, is the best primer for *in vitro* transcription by most of the influenza virus strains so far examined (17,24). So the effect of ApG on the endonuclease reaction was investigated and a typical result is shown in Fig. 3. Capped poly(A) and capped poly(U) were subjected to endonucleolytic cleavage in the presence of various concentrations of ApG. About 65% of the poly(A) cleavage was inhibited at 0.25 mM ApG, the concentration required for the maximum priming of *in vitro* transcription, while a similar extent of cleavage inhibition of poly(U) was seen at 0.5 mM ApG. The reciprocal replot figure shown in Fig. 3C indicates that the K_i value for ApG is 0.139 mM for poly(A) cleavage and 0.143 mM for poly(U) cleavage. This suggested that, on the enzyme complex with both endonuclease and RNA polymerase activities, the RNA sequence recognition site for the endonuclease reaction is located in the vicinity of or overlaps the dinucleotide primer binding site for RNA synthesis.

Among various dinucleotides with different base sequences, the dinucleo=

Figure 3. Inhibition of the endonuclease reaction by dinucleotide ApG. ^{32}P -Labeled capped poly(A) (21 fmol) [A] or ^{32}P -labeled capped poly(U) (26 fmol) [B] was incubated with 4 μg of purified influenza virus in the absence or presence of ApG. Lane 1, 0; lane 2, 0.05; lane 3, 0.1; lane 4, 0.25; lane 5, 0.5 mM ApG. The endonuclease activity measured from tracings of the autoradiograms is shown in [C] (○—○, capped poly(A) endonuclease; ●—●, capped poly(U) endonuclease). The reciprocal replot is shown in the insert.

tide, GpG, is another effective primer for the *in vitro* transcription by influenza virus-associated RNA polymerase (17,24,25). A set of dinucleotides with various base compositions was investigated for the inhibitory activity toward the endonuclease reaction and the result is shown in Fig. 4. The cleavage of both capped poly(A) and capped poly(U) was effectively inhibited only by ApG. Another three A-containing dinucleotides, ApA, ApC and ApU, exhibited slight inhibition, but other dinucleotides had little effect at the concentration (0.5 mM) tested. This suggests that the ApG-binding reaction is related to the sequential reactions involving the recognition, association and cleavage of natural mRNA and subsequent initiation of transcription using the cleavage products as primers.

Inhibition by CAP structures: The free CAP-1 structure ($m^7\text{GpppNm}$) competes in the capped RNA-primed transcription *in vitro* by influenza virus-associated RNA polymerase (18; Kawakami *et al.*, submitted for publication). In the presence of the free CAP-1 structure alone, however, the level of RNA synthesis is significantly higher than that in its absence presumably

Figure 4. Inhibition of the endonuclease reaction by various dinucleotides. ^{32}P -Labeled capped poly(A) (21 fmol) [A] or ^{32}P -labeled capped poly(U) (26 fmol) [B] was incubated with 4 μg of purified virions in the absence or presence of various dinucleotides at 0.5 mM. Lane 1, control; lane 2, ApA; lane 3, ApG; lane 4, ApC; lane 5, ApU; lane 6, GpA; lane 7, GpG; lane 8, GpC; lane 9, GpU. Markers are as shown in Fig. 1.

TABLE 1
Effect of Free CAP Structures on the Endonuclease Reaction

Free CAP structure	Concentration (A_{260nm})				
	0	0.3	1.0	3.0	10
CAP-1 (m^7 GpppGm)	<u>100</u>	99	66	38	12
CAP-0 (m^7 GpppG)	<u>100</u>	100	87	53	13
CAP-core (GpppG)	<u>100</u>	100	98	87	31

32 P-Labeled capped poly(A) (38 fmol) and purified virions (4 μ g) were incubated in the absence or presence of the indicated free CAP structure in 25 μ l of the standard endonuclease assay mixture for 30 min at 30°C. The cleaved RNA fragments were quantitated from tracings of the autoradiograms of respective gels. The endonuclease activities are represented as the percent activities of the control reaction in the absence of CAP structures.

because the free CAP-1 structure functions as an allosteric effector for the stimulation of influenza virus RNA polymerase (Kawakami *et al.*, submitted for publication). It was therefore reasonable to expect the competitive inhibition of capped RNA cleavage by the free CAP-1 structure. The effects of various CAP structures on capped poly(A) cleavage were investigated and the results are summarized in Table 1. The CAP-1 structure inhibited the endonuclease reaction by 62% at the concentration of 3 A_{260nm} and by 88% at 10 A_{260nm} , while the CAP-0 (CAP structure without ribose-2'-O-methyl group) inhibited it by 47 and 87% at 3 and 10 A_{260nm} , respectively. The CAP core (CAP structure without methyl groups) inhibited it 13 and 69% at 3 and 10 A_{260nm} , respectively. Strong inhibition by the CAP-1 structure is consistent

TABLE 2
Effect of Uncapped RNA on the Endonuclease Reaction

RNA	Concentration (A_{260nm})						
	0	0.001	0.02	0.05	0.2	0.5	10
Poly(U)	<u>100</u>	67	31	0	0	0	ND
Poly(G)	<u>100</u>	100	88	26	7	2	ND
Poly(AU)	<u>100</u>	ND	ND	ND	ND	60	6
Poly(A)	<u>100</u>	ND	ND	ND	ND	100	90
tRNA	<u>100</u>	ND	ND	ND	ND	ND	100

32 P-Labeled poly(A) (34 fmol for poly(U) and poly(G) reactions, or 51 fmol for poly(A), poly(AU) and tRNA reactions) and purified virions (6 μ g for poly(U) and poly(G) reactions, or 8 μ g for poly(A), poly(AU) and tRNA reactions) were incubated in the absence or presence of the indicated uncapped RNA in the standard endonuclease reaction mixture for 30 min at 30°C. The endonuclease activities were determined by measuring the amount of cleaved poly(A) fragments, and are represented as the percent activities of the control reaction without RNA addition. ND, not determined.

with our proposal that the enzyme complex specifically recognizes and interacts with the CAP-1 structure with both 7-methyl and ribose-2'-O-methyl groups.

Inhibition by RNA without CAP structures: RNA without a CAP structure was a strong inhibitor of the capped RNA-primed transcription by influenza virus-associated RNA polymerase, presumably due to the competition in capped RNA-binding on the enzyme protein (Kawakami *et al.*, submitted for publication). The effect of uncapped RNA was then investigated for the endonucleolytic cleavage of capped RNA and the results are summarized in Table 2. Capped poly(A) cleavage was inhibited to different extents by various homo- or heteropolyribonucleotides. The order of the inhibitory activity was poly(U) > poly(G) > poly(AU) > poly(A). tRNA had little effect on the endonuclease reaction within the concentration range examined. The cleavage of capped poly(U) was also interfered with by the uncapped polynucleotides.

DISCUSSION

We have shown that the influenza virus-associated endonuclease recognizes three signals on the substrate RNA molecules: i) the 5' CAP-1 structure; (ii) the RNA sequence consisting of A or U residues; and iii) the distance of 9 to 11 nucleotides between the two signals. Previously, Plotch *et al.* (16) reported that both AlMV (alphavirus mosaic virus) RNA-4 and BMV (brome mosaic virus) RNA-4 with the CAP-1 structure were cleaved by the influenza virus endonuclease but the same two species of viral RNA lacking both 7-methyl and ribose-2'-O-methyl groups were not cleaved by the enzyme. We confirmed these observations with use of different RNAs and in addition found that RNAs with only the 7-methyl group (CAP-0) resisted the cleavage by the endonuclease. With respect to the CAP signal, therefore, only the CAP-1 structure functions as an active signal to be recognized by the endonuclease.

Based on cleavage site analyses and the known sequence of substrate RNAs, Krug and colleagues (16,25) proposed that the influenza virus-associated endonuclease is a purine-specific endonuclease (therefore, it cleaves the 3' phosphodiester bond of purine residues). Our present study indicates that, among the four species of capped homopolymers, not only poly(A) but also poly(U) is cleaved by the endonuclease, the latter being a rather better substrate, and capped poly(G) is not cleaved at all. To explain their and our observations together, we propose that the influenza virus endonuclease recognizes A or U sequences and cleaves the 3' phosphodiester bond of the nucleotides located at 5' next to the A or U residues. In good agreement

with this novel hypothesis, we could find either A or U residues at the 5' termini of the cleaved fragments from various capped RNAs so far examined in other laboratories (9,16). If the viral endonuclease cleaves the 3' phosphodiester bond of purine residues, viral mRNA itself should be a substrate for the viral endonuclease because the first G residue at the primer-transcript junction is within the recognition distance from the CAP-1 structure. We found, however, that in vitro viral transcripts primed by capped poly(A) are cleaved no more by the endonuclease (Kawakami, unpublished observation). Our hypothesis explains the situation that the viral endonuclease cleaves host cell mRNA and utilizes the cleaved capped fragments as primers for viral transcription but does not cleave viral mRNA with the sequence of capped primer-G-C-- devoid of both A and U residues within the possible cleavage sites. At present, however, it should be taken reservedly that the insensitivity of capped poly(G) is attributed to the secondary or tertiary structures of the capped poly(G), which prevent the enzyme association with the target site.

In in vitro transcription by influenza virus RNA polymerase, synthetic dinucleotides added as primers are incorporated into the 5' termini of transcripts (24,25) and therefore must interact with the product-binding site in the vicinity of the enzyme catalytic site for RNA synthesis. Since dinucleotide ApG, the best primer for RNA synthesis, is a potent inhibitor for the endonuclease reaction by competing in the binding of capped RNA, it is reasonable to consider that the catalytic sites for RNA synthesis and for the endonuclease are located close to each other. In fact, the cleaved capped fragments remain associated with the enzyme complex and are utilized as primers for the viral transcription by the elongation mechanism (26).

As to the third signal or the distance between the above two signals, we found some fluctuations in the nucleotide number, which might be related to the nucleotide sequence. The endonuclease inhibition experiments with uncapped RNA also suggested sequence-specific interaction of the endonuclease. Modification of the internal sequence between the CAP-1 site and the cleavage site will clarify the possible involvement of the nucleotide sequence in the third signal.

Recently, we have isolated a complex of the three P proteins, P1, P2 and P3, which retains the activities for carrying out both RNA synthesis and endonucleolytic cleavage of capped RNA (Kawakami and Ishihama, in preparation). Structural mapping of the multiple functional sites on the P protein complex is in progress.

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