Polyadenylation Sites for Influenza Virus mRNA

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Polyadenylated transcripts of influenza virus RNA are incomplete copies of the individual genome segments, lacking sequences complementary to the 5'-terminal nucleotides of the virion RNA. By using a procedure which depends on the polyadenylic acid tail of the mRNA being encoded in part by the genome, we have determined that the common tract of uridine residues, approximately 17 to 22 nucleotides from the 5' end of each segment, is the site of polyadenylation of influenza virus mRNA.

Polyadenylated transcripts of the individual RNA segments of influenza virus, synthesized either in vivo or in vitro, are incomplete copies of the virion RNA (4–6). When synthesized in vivo, these transcripts also contain 5' cap structures with associated host-derived sequences and constitute the virus mRNA (2, 12, 13). In contrast, non-polyadenylated transcripts are complete copies of the virus genome and are synthesized in vivo only (4, 6). They are presumed to function as templates for the synthesis of progeny virion RNA molecules.

Single-strand–specific nuclease digestion of hybrids formed between virion RNA and either polyadenylated or non-polyadenylated complementary RNA has indicated that the polyadenylated transcripts corresponding to each segment lack the sequence complementary to the 5'-terminal 20 to 30 nucleotides of virion RNA (4, 26). A specific feature in this region of the virion RNA, which is common to all eight segments, is a tract of five to seven uridine residues at approximately 17 to 22 nucleotides from the 5' end (20, 26). Such a feature is involved at the site of polyadenylation of each of the five mRNA species of vesicular stomatitis virus (17, 21, 23). The analysis described in this report demonstrates that in at least four of the eight RNA segments of the influenza virus genome, the tract of uridine residues common to each segment of virion RNA is the site of polyadenylation of virus mRNA.

MATERIALS AND METHODS

Preparation of virus-specific RNA. Fowl plague virus (A/FPV/Rostock/34) was grown in 11-day-old fertile hen eggs; the virus was purified by sucrose velocity sedimentation, and the virion RNA was extracted as previously described (8, 20). Cytoplasmic RNA was extracted from fowl plague virus-infected primary chicken embryo fibroblasts, and the polyadenylic acid [poly(A)]-containing mRNA was selected by using oligodeoxythymidylic acid [oligo(dT)]-cellulose chromatography (1, 10). Individual segments of the genome RNA were labeled at their 5' termini with polynucleotide kinase and [γ-^{32}P]ATP and separated by polyacrylamide gel electrophoresis, and the bands were located by autoradiography (20). The individual segments were eluted from the gel in a solution of 0.5 M ammonium acetate, 5 mM MgCl₂, and 1% sodium dodecyl sulfate, pH 7.4, and precipitated twice with ethanol (20).

Selection of specific T₁ oligonucleotides. Selection of specific T₁ oligonucleotides protected by the 3'-terminal region of virus mRNA was performed essentially as described elsewhere (23). Individual genome segments prepared from 20 μg of virion RNA were digested to completion with T₁ RNase, and the larger oligonucleotides were selected by gel filtration through Sephadex G-25 in 10 mM Tris-hydrochloride (pH 7.6)–1 mM EDTA–0.4 M LiCl–0.5% sodium dodecyl sulfate. The material in the excluded volume was precipitated with ethanol, washed with 80% ethanol, and stored in a small volume of water. Aliquots of these oligonucleotides were 5' terminally labeled with polynucleotide kinase and [γ-^{32}P]ATP, and the mixture was deproteinized by phenol extraction. The labeled oligonucleotides were precipitated twice in ethanol with 10 μg of tRNA as a carrier and resuspended with an approximate 10-fold molar excess of total infected-cell mRNA in a final volume of 9 μl of water. The mixture was heated at 100°C for 30 s, adjusted to 10 mM sodium phosphate (pH 6.8)–0.4 M NaCl, and incubated at 37°C. After 2 h, T₁ and pancreatic RNases were added (final concentrations of 10 U/ml and 50 μg/ml, respectively) in the same buffer, and incubation at 37°C was continued for 20 min. At this time, pronase (final concentration of 1 mg/ml) was added, and the mixture was incubated at 37°C for an additional 20 min.

The reaction mixture was diluted into 200 μl of 10 mM Tris (pH 7.6)–1 mM EDTA–0.4 M LiCl–0.5% sodium dodecyl sulfate, and the hybrids were selected by oligo(dT)-cellulose chromatography in the same buffer. After washing the cellulose with buffer contain-

ing 0.4 M LiCl and then with buffer containing 0.1 M LiCl, the bound material was eluted with 10 mM Tris (pH 7.6)–1 mM EDTA–0.5% sodium dodecyl sulfate. Both the flow-through and the eluted fractions were precipitated in ethanol with carrier tRNA and analyzed by electrophoresis in a 12% polyacrylamide gel containing 7 M urea–90 mM Tris-borate (pH 8.3)–2.5 mM EDTA.

Sequence analysis. Standard methods of sequence analysis of terminally labeled oligonucleotides were employed (3, 16, 24). Partial digestion products were analyzed by one- or two-dimensional gel electrophoresis (16, 22).

Materials. Oligo(dT)-cellulose was obtained from Collaborative Research Inc. T_1 RNase, pancreatic RNase, and pronase were obtained from Calbiochem. Polynucleotide kinase was supplied by Boehringer Mannheim Corp., and [γ-^{32}P]ATP was obtained from the Radiochemical Centre.

RESULTS

The following procedure was adopted to determine the site of polyadenylation of influenza virus mRNA within the individual segments of the virus genome. We began by making the assumption that the tract of uridine residues close to the 5' end of each genome segment is involved in polyadenylation. If this assumption is correct, then, after complete digestion of the individual genome segments with T_1 RNase, the T_1 oligonucleotide which contains the uridine tract should form a duplex structure with the 3'-terminal region of virus mRNA. This duplex should contain the first five to seven adenine residues of the 3' poly(A) tail of the mRNA. Furthermore, after digestion with T_1 and pancreatic RNases, the duplex should remain at-

tached to the poly(A) tail and be specifically selected by oligo(dT)-cellulose chromatography. Such a procedure has been successful in determining that polyadenylation of the L mRNA of vesicular stomatitis virus occurs at a specific tract of uridine residues within the genome RNA (23).

For such an analysis to be feasible, the uridine tracts must be part of an oligonucleotide large enough to form a stable hybrid with the mRNA. The 5'-terminal nucleotide sequences of the eight segments of fowl plague virus (20) (Fig. 1) indicate that in segments 8, 7, 5, 4, and 1, the uridine tracts were situated at or close to the 5' end of suitably large T_1 oligonucleotides. In segment 6, the uridine tract was not in a specific T_1 oligonucleotide. In the remaining segments (2 and 3), the uridine tract was not located in a suitable T_1 oligonucleotide.

An additional necessary condition to enable this analysis to be carried out is the availability of suitable quantities of mRNA corresponding to the individual segments. It is well documented that there is unequal representation of the various virus mRNA's in infected cells (5, 11). Polyadenylated RNA from the cytoplasm of infected cells contains adequate quantities of virus mRNA for this analysis, corresponding to segments 4 to 8, but not to segments 1 to 3, the latter two of which are, in any event, not especially suitable for the above type of analysis. After taking the above factors into consideration, we elected to analyze segments 8, 7, 5, and 4. For these segments, the highly selective procedure outlined above should select a specific 19-residue T_1 oligonucleotide from segment 8, a

Segment   25   50
                5'

8   AGUAGAAACAAGGGUGUUUUUUAUCAUUAAAUAAGCUGAAACGAGAAAGUUCUUAUCUCU

7   AGUAGAAACAAGGUAGUUUUUUACUCCAGCUCUAUGUUGACAAAAUGACCAUCGUCAACA

6   AGUAGAAACAAGGAGUUUUUUUGAACAAACUACUUGUCAAUGGUGAAUGGCAACUCAGCA

5   AGUAGAAACAAGGGUAUUUUUCUUUAAUUGUCAUACUCCUCUGCAUUGUCUCCGAAGAAA

4   AGUAGAAACAAGGGUGUUUUUUUCCAAACUUAUAUACAAAUAGAGCACCGCAUGUUUCCG

3   AGUAGAAACAAGGUACUUUUUUGGACAGUAUGGAUAGCAAAUAGUAGCAUUGCCACAACU

2   AGUAGAAACAAGGCAUUUUUUCAUGAAGGACAAGCUAAAUUCACUAUUUCUGCCGUCUGA

1   AGUAGAAACAAGGUCGUUUUUAAACAAUUCGACAUUAAUUGAUGGCCAUCCGAAUUCUUU

FIG. 1. *Terminal nucleotide sequences (5') of the individual segments of the fowl plague virus genome (20). Solid lines indicate the uridine tract-containing T_1 oligonucleotides. The dashed line indicates the additional T_1 oligonucleotide selected from segment 5 (see Fig. 2 and the text).*

specific 13-residue T_1 oligonucleotide from segment 7, a specific 16-residue T_1 oligonucleotide from segment 5, and a specific 27-residue T_1 oligonucleotide from segment 4 (Fig. 1).

For this analysis, the individual genome segments of fowl plague virus, an avian strain of influenza virus, were separated by gel electrophoresis, eluted from the gel, and digested to completion with T_1 RNase. To increase the efficiency of labeling of the larger T_1 oligonucleotides, the smaller nonspecific oligonucleotides were removed by gel filtration. Aliquots of the larger T_1 oligonucleotides of segments 8, 7, 5, and 4 were individually 5'-end labeled and annealed with an approximate 10-fold molar excess of total infected-cell mRNA. After digestion with T_1 and pancreatic RNases (T_1 RNase only with segment 5; see below), the resistant hybrids were fractionated by oligo(dT)-cellulose chromatography, and the bound and flow-through fractions were analyzed by gel electrophoresis (Fig. 2). With each of the segments analyzed, the predicted oligonucleotide was apparently selected by the oligo(dT)-cellulose (Fig. 2, arrows). With segments 8 and 7, single products of approximately 19 and 13 residues in length (respectively) were selected, whereas in the analysis of segment 5, a second specific oligonucleotide of approximately 13 residues was selected in addition to a 16-residue fragment. With segment 4,

a series of smaller products was selected (the yield of which varied between different experiments) in addition to a product of approximately 27 residues. With segments 8, 7, and 5, the bands in the eluate fractions were of similar intensities to those in the flow-through fractions, indicating a quantitative selection of the predicted fragments.

To confirm that the selected oligonucleotides contained the specific uridine tracts and also to investigate the additional products selected from segments 5 and 4, these species were further characterized by analysis of their nucleotide sequences. Analysis of the single oligo(dT)-cellulose-selected product of segment 8 confirmed that it was the predicted 19-residue T_1 oligonucleotide (Fig. 3). Analysis of the T_1 oligonucleotides selected from segments 7 and 5 (Fig. 2, arrows) also confirmed that they were the expected 13-residue and 16-residue (respectively) T_1 oligonucleotides of which the uridine tracts are part (data not shown). Analysis of the smaller 13-residue oligonucleotide specifically selected from segment 5 showed that it was the T_1 product adjacent to the uridine tract-containing oligonucleotide, representing residues 31 to 43 from the 5' end of the genome (Fig. 1 and 4).

Analysis of the large fragment selected from segment 4 (Fig. 2) indicated that it was the predicted uridine tract-containing oligonucleo-
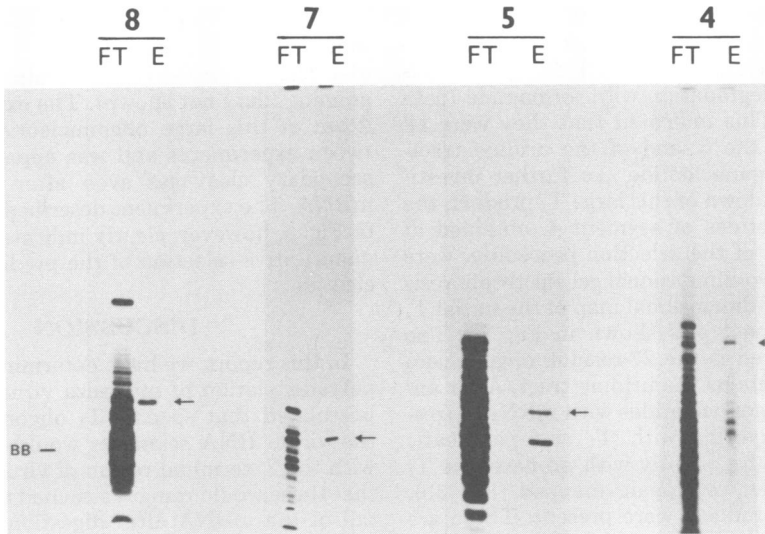

FIG. 2. *Selection of T_1 oligonucleotides protected by the 3'-terminal region of influenza virus mRNA. Individual segments 8, 7, 5, and 4 of influenza virus genome RNA were digested to completion with T_1 RNase, radiolabeled at their 5' termini, and annealed to excess virus mRNA. After treatment with T_1 and pancreatic RNases (T_1 RNase only with segment 5), the resistant duplexes were selected by oligo(dT)-cellulose chromatography, and the unbound flow-through fractions (FT) and the bound and eluted fractions (E) were analyzed in a 12% polyacrylamide gel. The products in the eluate fractions identified by arrows correspond in size to the uridine tract-containing oligonucleotides. BB, Bromophenol blue dye marker (~12 nucleotides).*
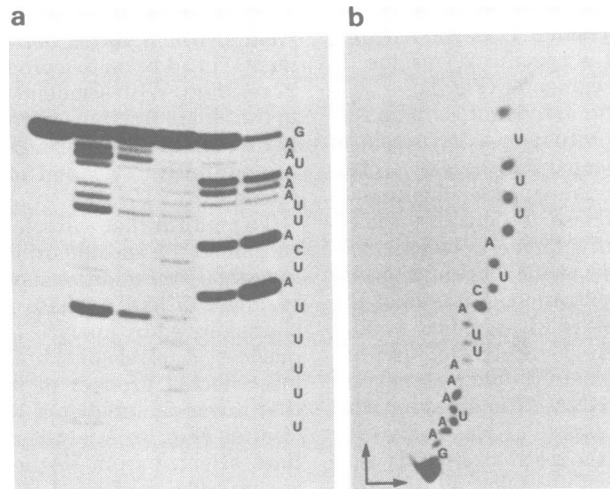
FIG. 3. *Characterization of the T_1 oligonucleotide of segment 8 protected by the 3'-terminal region of virus mRNA. The single T_1 oligonucleotide of genome segment 8 specifically selected by oligo(dT)-cellulose chromatography (Fig. 2, arrows) was subjected to partial enzymatic or chemical degradation, and the products were analyzed by one- or two-dimensional gel electrophoresis. Because of the effect any adjacent nucleotide has on the ability of PhyI RNase to discriminate between cytosine and uridine residues (18), the cytosine and uridine assignments were based on mobility shifts in the wandering spot analysis and not after PhyI digestion. The lack of resolution of mono- and dinucleotides in the two-dimensional gel prevented conclusive identification of the 5'-terminal nucleotides. (a) From left to right, tracks are; control, PhyI, PhyI, ladder, U_2, U_2. (b) Two-dimensional gel electrophoresis after formamide degradation.*

tide but that it contained only 25 residues, having lost the 3'-terminal adenine and guanine. The nucleotide sequences of the smaller species in the eluate fraction of segment 4 were compared by polyacrylamide gel electrophoresis after partial degradation with formamide (data not shown). This indicated that they were all derived from the 5' end of the uridine tract-containing oligonucleotide. To further investigate the breakdown of this large T_1 product, the T_1 oligonucleotides of segment 4, obtained at various stages of the selection procedure, were analyzed by two-dimensional gel electrophoresis (Fig. 5). A two-dimensional map of the initial T_1 digest of segment 4 is shown in Fig. 5a. The product arrowed is the 27-residue oligonucleotide which contains the uridine tract. After annealing the oligonucleotides with mRNA, digestion of the hybrids with T_1 and pancreatic RNases and subsequently with pronase, the T_1 oligonucleotides were again mapped (Fig. 5b). Several new products were present. These are presumably derived from some of the largest of the T_1 products (including the 27-residue uridine tract-containing oligonucleotide) which are correspondingly reduced in intensity. Of the RNase-treated hybrids shown in Fig. 5b, only two specific products were selected with oligo(dT)-cellulose; these mapped in a similar region to the

specific 27-residue oligonucleotide (Fig. 5c). Sequence analysis of these two products indicated that the larger represented the complete 27-residue oligonucleotide, whereas the other was identical but lacked the 3'-terminal adenine and guanine (data not shown). The extent of breakdown of this large oligonucleotide varied between experiments and was apparently due to secondary cleavages even after annealing to mRNA. The experiment described in the legend to Fig. 5, however, clearly indicates the specific quantitative selection of the predicted oligonucleotide.

DISCUSSION

In this report, we have determined the site of polyadenylation of influenza virus mRNA. We postulated that specific T_1 oligonucleotides of the virion RNA segments would form hybrids with the 3'-terminal region of virus mRNA and that these would remain attached to the poly(A) tail of the mRNA after digestion with T_1 and pancreatic RNases. Selection of the predicted T_1 oligonucleotides with oligo(dT)-cellulose (Fig. 2) proved our assumption that the tract of uridine residues common to the 5'-terminal region was the site of polyadenylation of the virus mRNA. Although we have presented data concerning only four of the eight virion segments, the uri-
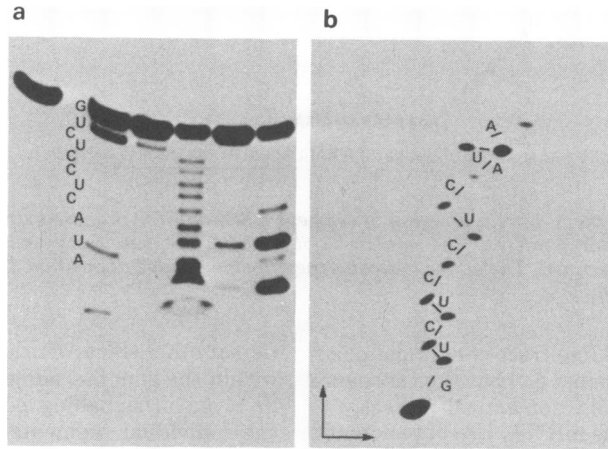
FIG. 4. *Characterization of the smaller of the two T_1 oligonucleotides of segment 5 protected by the 3'-terminal region of virus mRNA. The smaller of the two T_1 oligonucleotides of genome segment 5 specifically selected by oligo(dT)-cellulose chromatography (see Fig. 2) was subjected to partial enzymatic or chemical degradation, and the products were analyzed by one- or two-dimensional gel electrophoresis. Because of the effect any adjacent nucleotide has on the ability of PhyI RNase to discriminate between cytosine and uridine residues (18), the cytosine and uridine assignments were based on mobility shifts in the wandering spot analysis and not after PhyI digestion. The lack of resolution of mono- and dinucleotides in the two-dimensional gel prevented conclusive identification of the 5'-terminal nucleotides. (a) From left to right, tracts are; control, PhyI, PhyI, ladder, U_2, U_2. (b) Two-dimensional gel electrophoresis after formamide degradation.*
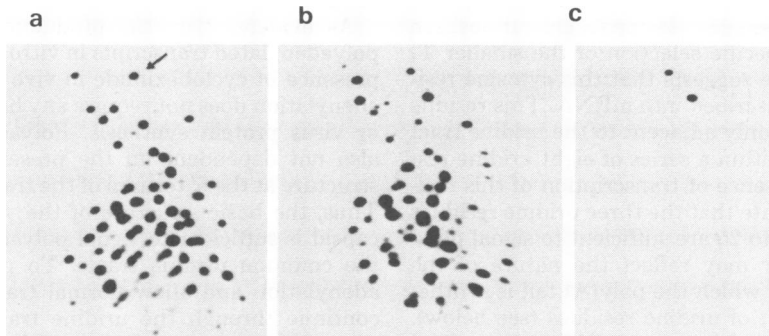

FIG. 5. *Two-dimensional gel electrophoresis of 5'-end-labeled T_1 oligonucleotides of segment 4 obtained at various stages of the analysis. (a) Two-dimensional map of a T_1 digest of segment 4. (b) Two-dimensional map of a T_1 digest of segment 4 after annealing to excess virus mRNA and digestion with T_1 and pancreatic RNases and with pronase. (c) The mixture analyzed in b was selected by oligo(dT)-cellulose chromatography, and the material which bound to the column was analyzed by two-dimensional gel electrophoresis.*

dine tracts in the remaining four segments are also presumably the sites of polyadenylation of mRNA. It has recently been determined that a second virus mRNA is transcribed from segment 8 (7, 14). This mRNA is approximately one-half the size of genome segment 8 and appears in small amounts late in infection. This small mRNA shares a common 3'-terminal region with the normal mRNA of segment 8 (9, 15) and so can also be presumed to be polyadenylated at the common uridine tract. The analysis described in this report only determined whether

or not the virus mRNAs terminate at a specific tract of uridine residues; the possibility that other polyadenylation sites exist, although unlikely, cannot be ruled out.

When segment 5 was analyzed, two specific T_1 oligonucleotides were selected: one corresponded to the predicted uridine tract-containing fragment, whereas the other smaller product represented the 3'-adjacent T_1 oligonucleotide corresponding to positions 31 to 43 (Fig. 1 and 6). With segment 5, T_1 RNase alone was used in the digestion of the duplexes. This was because
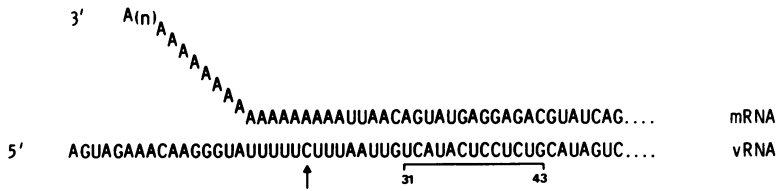
FIG. 6. *Sequence of the 5'-terminal region of segment 5 (20) and the proposed structure of the 3'-terminal region of the corresponding mRNA. The smaller T_1 oligonucleotide, specifically selected as described in the legend to Fig. 2, is underlined. The arrow indicates the cytosine residue at position 22 which is apparently not transcribed into mRNA.*

the 5' end of the uridine tract-containing oligonucleotide of segment 5 has the sequence UAU..., which would not anneal perfectly to the poly(A) tail of the mRNA. Use of pancreatic RNase in the selection procedure would probably have removed the 5'-terminal uridine residue with the terminal radiolabel. A consequence of using T_1 RNase alone is that the poly(A) tail would be removed only from those structures in which a guanine residue intervenes in the mRNA between the poly(A) tail and the duplex formed between any oligonucleotide and its complementary region of the mRNA. Between the uridine tract and the T_1 oligonucleotide derived from position 31 to 43 in genome segment 5, there was a single cytosine residue at position 22 (Fig. 6). Specific selection of the smaller T_1 oligonucleotide suggests that this cytosine residue is not transcribed into mRNA. This residue is located not only adjacent to the uridine tract but actually within a series of eight uridine residues. The absence of transcription of this residue may indicate that the three uridine residues at position 23 to 25 are sufficient to signal polyadenylation or may reflect the nature of the mechanism by which the poly(A) tail is synthesized at a tract of uridine residues (see below). When both T_1 and pancreatic RNases were used in the procedure, no products were selected with the oligo(dT)-cellulose as would be predicted (data not shown).

The procedure described in this report was previously utilized to demonstrate that a specific tract of uridine residues is the site of polyadenylation of the L mRNA of vesicular stomatitis virus (23). Direct sequencing methods have shown that the other four mRNA species of vesicular stomatitis virus are also polyadenylated at specific tracts of uridine residues in the genome RNA (17, 21). It has been suggested that a possible mechanism of polyadenylation of such mRNA's is a "stuttering" or "chattering" mechanism which allows repetitive copying of the uridine tract by the transcriptase complex. A similar model can be proposed for influenza virus. However, to avoid premature polyadenyla-

tion at other tracts of uridine residues elsewhere within the genome, additional features must be involved in signalling polyadenylation. Within the individual segments of the virus genome, there is no common nucleotide sequence before the specific uridine tracts (20) which might be involved in signalling polyadenylation, as has been demonstrated in the genome of vesicular stomatitis virus (17, 21, 23). One possible structural feature that might be involved is the partial sequence complementarity between the 5' and 3' ends of individual segments, which could result in the formation of a "panhandle" duplex structure of the genome adjacent to the common uridine tracts (20).

As evidenced by the production of normal polyadenylated transcripts in vitro (19) or in the presence of cycloheximide in vivo (5, 10), polyadenylation does not require any host cell factor or virus protein synthesis. Polyadenylation is also not dependent on the presence of a cap structure at the 5' termini of the transcripts (19). Thus, the basic structure of the virion nucleocapsid is sufficient to signal polyadenylation at the common uridine tracts. To prevent polyadenylation and allow normal transcription to continue through the uridine tracts to the 5' ends of the genome segments, a host cell factor or a newly synthesized virus protein(s) or both must be required. Possible candidates for modification of the virus polymerase or of the genome template are the nonstructural proteins (NS_1 and NS_2) which are not present in mature virions (25).

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