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Influenza Virion Transcriptase: Synthesis In Vitro of Large, Polyadenylic Acid-Containing Complementary RNA

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The influenza virion transcriptase is capable of synthesizing in vitro complementary RNA (cRNA) that is similar in several characteristics to the cRNA synthesized in the infected cell, which is the viral mRNA. Most of the in vitro cRNA is large (approximately 2.5×10^5 to 10^6 daltons), similar in size to in vivo cRNA. The in vitro transcripts initiate in adenosine (A) or guanosine (G) at the 5′ end, as also appears to be the case with in vivo cRNA (R. M. Krug et al., 1976). The in vitro transcripts contain covalently linked polyadenylate [poly(A)] sequences, which are longer and more heterogeneous than the poly(A) sequences found on in vivo cRNA. The synthesis in vitro of cRNA with these characteristics requires both the proper divalent cation, Mg^{2+}, and a specific dinucleoside monophosphate (DNMP), ApG or GpG. These DNMPs stimulate cRNA synthesis about 100-fold in the presence of Mg^{2+} and act as primers to initiate RNA chains, as demonstrated by the fact that the 5′-phosphorylated derivatives of these DNMPs, ^{32}pApG or ^{32}pGpG, are incorporated at the 5′ end of the product RNA. The RNA synthesized in vitro differs from in vivo cRNA in that neither capping nor methylation of the in vitro transcripts has been detected. The virion does contain a methylase activity, as shown by its ability to methylate exogenous methyl-deficient *Escherichia coli* tRNA.

The genome of influenza virus consists of eight segments of single-stranded RNA ranging in size from 2.5×10^5 to 10^6 daltons (6, 32). Recently, it has been demonstrated that the entirety of the viral mRNA is opposite in polarity, i.e., complementary, to virion RNA (vRNA) (10, 13, 14, 23a, 24, 30). Viral complementary RNA (cRNA) is comprised of segments slightly larger than the corresponding vRNA segments, and this difference in size is due to the polyadenylic acid [poly(A)] sequences in cRNA that are absent in vRNA (23a; P. R. Etkind, C. Herz, and R. M. Krug, manuscript in preparation). These results suggest that the cRNA segments correspond to complete transcripts of the vRNA segments, i.e., that initiation of the synthesis of cRNA occurs at or near the 3′ terminus of each of the template vRNA segments. As viral cRNA contains an m^{7}G (7-methylguanosine) cap structure at its 5′ end (24), the penultimate nucleoside in this cap structure is most probably the residue that initiates synthesis of cRNA in the infected cell. Several cap structures are found in cRNA: 70% of the cap structures contain methylated adenosine (A), and 30% contain methylated guanosine (G), as the penultimate nucleoside (24), suggesting that the synthesis of viral cRNA chains initiate with A or G at the 5′ end. Viral cRNA also contains internal m^{6}A residues (24), a feature distinguishing this mRNA from those viral mRNA's known to be synthesized in the cytoplasm (2, 15, 36, 38).

Purified influenza virions contain an RNA transcriptase which synthesizes in vitro RNA complementary to vRNA (7, 8, 29, 37). According to the "negative-strand" virus concept (4), this virion-associated transcriptase would be expected to be capable of synthesizing authentic viral mRNA, i.e., RNA identical to the cRNA found in the infected cell. However, the in vitro transcripts described in previous studies differed in several important characteristics from in vivo cRNA. In reaction mixtures containing the four nucleoside triphosphates and divalent cation(s), the virion transcriptase was reported to synthesize only RNA considerably smaller than vRNA (7, 16). The limited amount of poly(A) synthesized in vitro was found in molecules of small size, which were not covalently linked to the molecules containing the cRNA sequences (16). The in vitro transcripts were reported to initiate only in G, and not in A (19, 27). Consistent with initiation in G, it was shown that the rate of transcription is

stimulated by guanosine and the dinucleoside monophosphates (DNMPs) GpG and GpC and that ^{3}H-labeled guanosine is incorporated at the 5′ end of the in vitro product RNA (27).

We report here that in fact the transcriptase, as it is found in purified virions, is capable of synthesizing RNA transcripts that possess some, but not all, of the characteristics of in vivo cRNA. Specifically, the in vitro RNA transcripts are of large size, contain poly(A), and initiate in A or G. The synthesis of these transcripts requires the presence of both the correct divalent cation, Mg^{2+}, and specific DNMPs, which act as initiators. However, neither capping nor methylation of the product RNA has been detected.

MATERIALS AND METHODS

Cells and virus. The procedures for culture of the MDCK and MDBK cell lines and for the growth of WSN (influenza A) virus in MDBK cells have been described previously (21, 22). Virus was purified by two sequential bandings in sucrose-D_2O gradients (21).

Purification of viral cRNA from infected cells. The procedures for obtaining [^{3}H]adenosine-labeled viral cRNA have been described (14).

RNA synthesis by detergent-disrupted virus. Purified WSN virus was pelleted by centrifugation for 1 h in an SW41 rotor at 39,000 rpm and suspended in a small volume of 1% Triton N-101, 0.05 M NaCl, 0.005 M Tris-hydrochloride, pH 7.8. Approximately 15 μg of viral protein was added to an assay mixture containing, in a final volume of 0.05 ml: 50 mM Tris-hydrochloride (pH 7.8), 100 mM KCl, 10 mM NaCl, 1 mM dithiothreitol, 0.2% Triton N-101, $MgCl_2$ or $MnCl_2$ as indicated, 0.4 mM DNMP (usually ApG or GpG) as indicated, three ribonucleoside triphosphates at 1 mM each, and a labeled ribonucleoside triphosphate. The labeled ribonucleoside triphosphate was [^{3}H]UTP (0.4 mCi/μmol) at 0.2 mM, [α-^{32}P]GTP (0.3 mCi/μmol) at 0.4 mM, or [α-^{32}P]ATP (0.3 mCi/μmol) at 1 mM. Reaction mixtures were incubated at 31°C. To determine the amount of radioactivity incorporated, the reactions were stopped by the addition of 0.1 ml of a 1:1 mixture of saturated sodium phosphate and sodium pyrophosphate, followed by 3 ml of cold 10% trichloroacetic acid. The precipitated RNA was washed as described by Gomatos (18), collected on membrane filters (Millipore Corp.), and counted using toluene-based scintillation fluid.

Isolation of product RNA from reaction mixtures. Reaction volumes were increased to 0.5 to 1.0 ml. After incubation for 1 to 2 h at 31°C, the reaction mixtures were made 0.5% in sodium dodecyl sulfate, 0.001 M in EDTA, and 0.1 M in Tris-hydrochloride, pH 9.0, and the RNA was deproteinized by extracting three times with phenol-chloroform (1:1) at pH 9.

Analysis of the RNA product. (i) Oligo(dT)-cellulose chromatography. The RNA was precipitated with ethanol and subjected to chromatography on oligo(dT)-cellulose as described previously (14).

(ii) Polyacrylamide gel electrophoresis. The RNA was desalted by chromatography through Sephadex G-50 in water and precipitated with ethanol after the addition of LiCl to 0.2 M. The RNA was dissolved in 0.10 ml of 0.01 M sodium phosphate, pH 6.8, 0.002 M EDTA, and 10% sucrose, heated at 100°C for 2 min, fast-cooled, and layered onto a 12-cm cylindrical gel containing 2.1% acrylamide, 0.7% agarose, 6 M urea, and Peacock-Dingman (P-D) buffer (3, 32, 35). After electrophoresis at 140 V for 2 or 5 h in the P-D buffer, the gels were cut into 2-mm slices, which were processed for counting in a scintillation counter as previously described (23). The 18*S* and 28*S* rRNA's from MDCK cells, run on separate gels, served as molecular weight markers.

(iii) Ribonuclease sensitivity of product RNA. The product RNA in 0.01 M Tris-hydrochloride, pH 7.4, 0.002 M EDTA was heated for 5 min at 100°C and fast-cooled. The salt concentration was increased to 2.4× SSC (SSC = 0.15 M NaCl, 0.015 M sodium citrate), and the RNA was digested with pancreatic (3 μg/ml) and T1 (20 U/ml) RNases for 45 min at 37°C. Acid-insoluble radioactivity was then determined.

(iv) RNA annealing. The product RNA was mixed with 15 μg of vRNA and heated for 5 min at 100°C. The salt concentration was increased to 2.4× SSC, and the mixture was incubated for 4 h at 68°C. After cooling to room temperature, the percentage of the product that was RNase (pancreatic and T1) resistant was determined.

Analysis of the size of the poly(A) sequences in cRNA. A mixture of in vitro product RNA (^{32}P labeled), in vivo cRNA (^{3}H labeled), and yeast RNA (100 μg/ml) was heated, fast-cooled, and digested with pancreatic and T1 RNases as described above. After phenol-chloroform extraction, the poly(A) fragments were selected by oligo(dT)-cellulose chromatography, ethanol precipitated, and dissolved in 0.10 ml of a solution containing P-D buffer, 8 M urea, 0.2% sodium dodecyl sulfate, and 20% glycerol. The sample was applied to a 15% acrylamide gel containing 8 M urea and P-D buffer. Electrophoresis was at 250 V for 5 h. As molecular weight markers, 5*S* and 4*S* RNA from BHK-21 cells were run on separate gels.

Synthesis of ^{32}pApG and ^{32}pGpG. ApG (1 mM) or GpG (1 mM) was incubated for 6 h at 37°C with 15 U of polynucleotide kinase in a final volume of 0.3 ml containing: 70 mM Tris-hydrochloride, pH 7.8; 10 mM $MgCl_2$; 2 mM spermidine-HCl; 2.5 mM dithiothreitol; and 1 mM [γ-^{32}P]ATP (3 Ci/mmol). After the incubation, the mixture was diluted to 6 ml with 0.02 M Tris-hydrochloride, pH 7.8, 0.05 M NaCl, 7 M urea and subjected to chromatography on a DEAE-Sephadex A-25 column (0.9 by 25 cm), employing a linear gradient of NaCl (0.05 to 0.35 M) in 7 M urea, pH 7.8, buffer. The ^{32}pApG or ^{32}pGpG eluted from the column between the peaks of ADP and ATP. During electrophoresis on Whatman 3MM paper, using 0.05 M ammonium formate buffer, pH 3.5, each of these compounds migrates as a single homogeneous peak, with less than 1% of the radiolabel migrating in the ATP region. The purified com-

pounds were desalted by binding to, and elution from, DEAE-Sephadex using triethylammonium bicarbonate buffer, pH 7.5, and were stored at −20°C. The ^{32}pApG, at pH 7, has a maximum absorbancy at 256 nm, and its millimolar absorbancy ($\alpha_m \times 10^{-3}$) was assumed to be 25.4, the same as ApG at pH 1 (P and L Biochemicals catalogue). The ^{32}pGpG, at pH 7, has a maximum absorbancy at 254 nm, and its millimolar absorbancy was assumed to be 25, approximately 10% less than two times the millimolar absorbancy of GMP.

Determination of the site(s) of incorporation of ^{32}pApG or ^{32}pGpG in the in vitro product RNA. A reaction mixture containing 0.2 mM ^{32}pApG or ^{32}pGpG (10,000 cpm/pmol) and [^{3}H]UTP (65 cpm/pmol) in a total volume of 0.9 ml was incubated for 2 h at 31°C. After phenol-chloroform extraction, the RNA was separated from most of the labeled precursors by chromatography through Sephadex G-50 in water, ethanol precipitated, and taken up in 0.2 ml of 0.01 M Tris-hydrochloride, pH 7.4, 0.002 M EDTA, 95% dimethyl sulfoxide (Me_2SO). The RNA was heated to 60°C for 2 min and layered onto a 5 to 20% sucrose gradient in this buffer containing 95% Me_2SO in an SW50 polyallomar tube. After centrifugation for 26 h at 48,000 rpm, 5-drop fractions were collected. A separate gradient containing ^{32}P-labeled vRNA was run, and the fractions from the in vitro product gradient corresponding to the region in which vRNA sedimented were pooled. The RNA was precipitated with ethanol, dissolved in 0.2 ml of 0.03 M sodium acetate, pH 4.5, and digested for 18 h at 37°C with 10 U of RNase T2 per ml. After phenol extraction, the hydrolysate was subjected to chromatography on a DEAE-Sephadex A-25 column in 7 M urea, as described above. The material eluted from the column was analyzed by paper electrophoresis, as described above.

Assays for in vitro methylation. For the analysis of the methylation of in vitro synthesized RNA transcripts, reaction mixtures contained 1 mM of the four unlabeled nucleoside triphosphates and 7 μM neutralized [^{3}H]S-adenosylmethionine ([^{3}H]SAM) (12.5 mCi/μmol) in a total volume of 0.1 ml. Other additions are indicated in Results and in Table 5. For the analysis of the methylation of an exogenous RNA, 0.5 mg of methyl-deficient tRNA per ml from *Escherichia coli* strain W was added to the assay mixture, and the four nucleoside triphosphates were omitted to prevent RNA synthesis (1, 9). After incubation for 2 h at 31°C, the assay mixture was extracted with phenol and was then subjected to the trichloroacetic acid precipitation and washing procedure described above.

Materials. [^{3}H]UTP, [α-^{32}P]GTP and -ATP, [γ-^{32}P]ATP, and [^{3}H]SAM were purchased from New England Nuclear Corp., Boston, Mass. Oligo(dT)-cellulose was obtained from Collaborative Research, Inc., Waltham, Mass. Enzymes were purchased from P and L Biochemicals, Inc. (polynucleotide kinase) and Sankyo Co., Tokyo (T1 and T2 RNases). ^{3}H-labeled 18*S* and 28*S* rRNA's from MDCK cells were obtained as described previously (15). ^{3}H-labeled 4*S* and 5*S* RNA from BHK-21 cells was supplied by D. Sawicki of The Sloan-Kettering Institute. The DNMPs were purchased from Collaborative Research, Inc., or from Calbiochem, San Diego, Calif. Adenosine 3',5'-diphosphate (pAp) was purchased from Sigma Chemical Co. Purified vesicular stomatitis virus (VSV) and methyl-deficient tRNA from *E. coli* strain W were kindly supplied by Amiya K. Banerjee of the Roche Institute of Molecular Biology, Nutley, N.J. ApGpC was supplied by H. G. Khorana of the Massachusetts Institute of Technology, Cambridge, Mass.

RESULTS

Effect of Mg^{2+} and Mn^{2+} on the rate of transcription in the presence and absence of a stimulating DNMP. In the absence of the stimulating DNMP GpG, Mn^{2+} at an optimal concentration of 5 mM is the preferred divalent cation (Table 1). Mg^{2+} at an optimal concentration of 5 mM is only about 15% as effective as Mn^{2+}. At suboptimal levels of Mn^{2+} (1.5 mM), the addition of Mg^{2+} up to 8 mM increases the rate to almost that found in the presence of Mn^{2+} alone at its optimal concentration. However, at optimal levels of Mn^{2+}, the addition of Mg^{2+} is inhibitory (not shown). In the presence of GpG, the preferred diavlent cation is Mg^{2+} rather than Mn^{2+}. The optimal concentration of Mg^{2+} in the presence of GpG is also 5 mM. The stimulation by GpG is almost 90-fold in the presence of Mg^{2+} alone, whereas only a 5-fold stimulation is observed in the Mn^{2+}-containing reactions. The optimal pH (7.8 to 8.0), temperature (30 to 32°C), and ionic strength (0.1 to 0.2) are independent of the divalent cation, or the presence or absence of GpG. The reaction in the presence or absence of GpG is linear for at least 1.5 h at 31°C.

Effect of different DNMPs on the rate of transcription. All 16 DNMPs were tested for their effect on the rate of RNA synthesis in the presence of Mg^{2+}. The most effective DNMP has consistently been ApG, followed by GpG and GpC (Table 2). Maximal stimulation of the reaction occurs at 0.3 to 0.4 mM DNMP and 5 mM Mg^{2+}. Other DNMPs stimulate poorly or not at

TABLE 1. *Effect of Mn^{2+} and Mg^{2+} on the rate of RNA synthesis catalyzed by the influenza virion transcriptase*

Divalent cation (mM)		pmol of [^{3}H]UMP incorporated[a]	
Mn^{2+}	Mg^{2+}	−GpG	+GpG
5	-	5.5	24.8
-	5	1.0	87.5
1.5	8	4.4	22.0

[a] Assays were performed as described in Materials and Methods. Incubation was for 1 h at 31°C.

TABLE 2. *Stimulation of the influenza virion transcriptase by DNMPs*

DNMP	pmol of UMP incorporated[a]
None	1.0
ApG	110.0
GpG	90.0
GpC	19.8
UpG	5.3
CpG	5.2
GpU	3.5
All others	1.0–1.5
ApG + GpG[b]	110.0

[a] Assay mixtures contained [^{3}H]UTP and 5 mM Mg^{2+} and were incubated for 1 h at 31°C.

[b] Each of these two DNMPs was at 0.4 mM.

all. The effect of ApG and GpG is not additive, indicating that they probably do not have independent sites of action. Only one trinucleoside diphosphate, ApGpC, has been examined: the stimulation observed is intermediate between that caused by ApG and GpC. Other compounds are much less active, or are inactive, in stimulating RNA synthesis. Guanosine and 5'-GMP stimulate only 4- and 2.5-fold, respectively. Compounds that stimulate poorly, or not at all, include: d-(GpG), IpI, GpppG, SAM, adenosine, 5'-AMP, and poly(G). Only those compounds active in the presence of Mg^{2+} are also active in the presence of Mn^{2+}, but the fold stimulation in the presence of Mg^{2+} is 8 to 20 times that observed in the presence of Mn^{2+}.

Effect of Mg^{2+} and Mn^{2+} and ApG or GpG on the size of the RNA transcripts. The size of the RNA product was analyzed by polyacrylamide gel electrophoresis. Detergent-treated virus containing RNA radiolabeled with [^{3}H]adenosine was used to synthesize RNA with [^{32}P]GTP as the labeled precursor. In the first series of experiments, the RNA was subjected to gel electrophoresis for a relatively short time so as to allow the full range of the RNA species synthesized to be visualized, even though resolution of the vRNA (and cRNA) segments suffers as a result. Most of the RNA synthesized in the presence of Mn^{2+} and in the absence of stimulating DNMPs is much smaller than the template vRNA (Fig. 1, upper panel). The addition of ApG (Fig. 1, middle panel) leads to little increase in size of the product. In contrast, in the presence of both Mg^{2+} and ApG, conditions that optimally stimulate the rate of RNA synthesis, the RNA product is predominantly large, migrating in the molecular weight region of the vRNA segments (Fig. 1, lower panel). Similar results were obtained when GpG was used instead of ApG. Therefore, for the synthesis of large-size RNA, both Mg^{2+} and ApG or GpG are required.

For better resolution, the gels were run for longer times. Figure 2 (upper panel) shows a representative gel pattern of a vRNA preparation (in this case, labeled with ^{32}P). The vRNA is resolved into six distinct RNA peaks, ranging

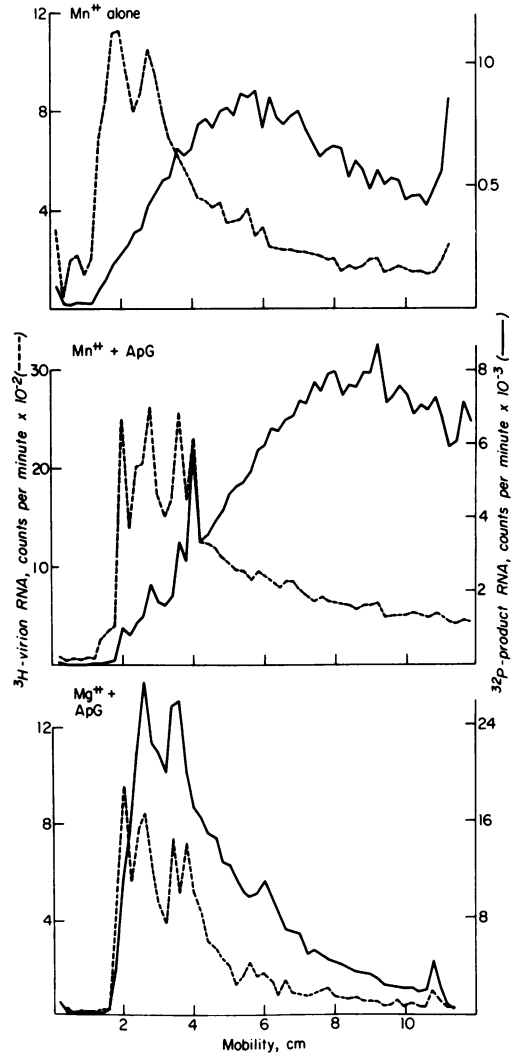

FIG. 1. *Size of the RNA product synthesized by the virion transcriptase in the presence of Mn^{2+}, Mn^{2+} + ApG, or Mg^{2+} + ApG. Reaction mixtures contained detergent-treated virus (radiolabeled with [^{3}H]adenosine), [α-^{32}P]GTP, $MnCl_2$ (5 mM) or $MgCl_2$ (5 mM), and, where indicated, ApG (0.4 mM) in a final volume of 1.0 ml. The mixtures were incubated for 2 h at 31°C. The RNA products were isolated from the reaction mixture as described in Materials and Methods and, after heating to 100°C and fast-cooling, were subjected to electrophoresis for 2 h.*

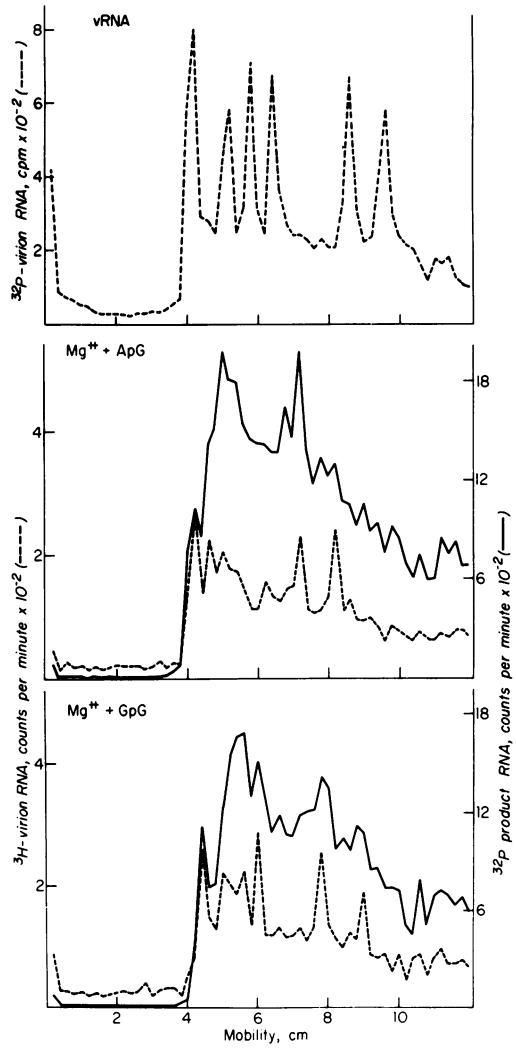

FIG. 2. *Size of vRNA and of the RNA product synthesized by the virion transcriptase in the presence of Mg^{2+} and either ApG or GpG. The top panel shows the gel pattern of [^{32}P]vRNA. ^{3}H-labeled 18S and 28S MDCK rRNA's (0.7 × 10^6 and 1.9 × 10^6 molecular weights, respectively) were run on separate gels as molecular weight markers. The middle and bottom panels show the gel pattern of the ^{32}P-labeled product synthesized by the transcriptase of virus labeled with [^{3}H]adenosine. Reaction mixtures contained [α-^{32}P]GTP, $MgCl_2$ (5 mM), and either ApG (0.4 mM) or GpG (0.4 mM) in a final volume of 1.0 ml. Gel electrophoresis was for 5 h.*

in molecular weight from approximately 2.5 × 10^5 to 10^6. In gels that are stained rather than sliced, the RNA peak of largest size is resolved into three bands, demonstrating the presence of eight vRNA segments. This gel pattern of WSN virus vRNA is identical to that recently described by Pons (32). The middle and lower panels of Fig. 2 show the gel pattern of the RNA obtained from reaction mixtures using [^{3}H]adenosine-labeled virus and containing Mg^{2+} and ApG or GpG. It is apparent that during incubation the ^{3}H-labeled vRNA has undergone some degradation: an increased amount of radiolabeled material is present between less well-resolved peaks. A large proportion of the ^{32}P-labeled product RNA migrates in the same region of the gel as the vRNA segments. The RNA product of largest size comigrates with the largest vRNA segment, whereas the other large-size product RNAs are heterogeneous and consistently migrate in two broad regions of the gel (e.g., in Fig. 2, middle panel, between 4.5 and 5.5 cm and between 6.5 and 7.5 cm). These results indicate that in the presence of Mg^{2+} and ApG or GpG the virion transcriptase is capable of synthesizing in vitro RNA which is at least as large as the template vRNA segments.

Poly(A) of in vitro synthesized RNA. Influenza viral cRNA synthesized in the infected cell contains poly(A) (10, 13, 17). To determine whether the RNA synthesized in vitro also contains poly(A), the RNA products were tested for their ability to bind to oligo(dT)-cellulose (Table 3). Only a very small percentage of the product made in the presence of Mn^{2+}, with or without ApG, binds to oligo(dT)-cellulose. In contrast, almost half of the RNA product synthesized in the presence of Mg^{2+} and either ApG or GpG binds. These results demonstrate that Mg^{2+} and not Mn^{2+} is required for the addition of poly(A) to in vitro cRNA.

Further experiments showed that the oligo(dT)-cellulose-bound RNA from the products synthesized in the presence of Mg^{2+} and ApG or GpG actually contains an RNase-resistant poly(A) fragment (Table 4). Thus, after

TABLE 3. *Binding of in vitro synthesized RNA to oligo(dT)-cellulose*[a]

Divalent cation	DNMP	% of product bound to oligo-(dT)-cellulose
Mn^{2+}	None	5
Mn^{2+}	ApG	7
Mg^{2+}	ApG	45
Mg^{2+}	GpG	45

[a] Reaction mixtures of 0.5 to 1.0 ml containing [α-^{32}P]GTP and the indicated divalent cation (at 5 mM) and DNMP (0.4 mM) were incubated for 2 h at 31°C. The RNA was isolated and subjected to oligo(dT)-cellulose chromatography as described in Materials and Methods. The percent binding shown is the average of three determinations. For each determination, 2 × 10 × 10^5 cpm of product RNA were applied to the column.

TABLE 4. *Poly(A) content and complementarity of the RNA product synthesized in the presence of ApG*[a]

Oligo(dT)-cellulose chromatography	Labeled precursor	% RNase resistance of product RNA after:	
		Heating and fast-cooling	Annealing to excess vRNA
Bound	[^{32}P]ATP	22	101
	[^{32}P]GTP	2.5	96
Not bound	[^{32}P]ATP	3	99
	[^{32}P]GTP	2	98

[a] Reaction mixtures, in a final volume of 0.75 ml, contained 5 mM $MgCl_2$, 0.4 mM ApG, and either [α-^{32}P]ATP or [α-^{32}P]GTP as the labeled ribonucleoside triphosphate. The mixture was incubated for 2 h at 31°C. The isolation of the RNA product, oligo(dT)-cellulose chromatography, treatment with pancreatic and T1 RNases, and annealing were carried out as described in Materials and Methods. Prior to RNase treatment, the heated and fast-cooled or annealed samples contained 5,000 to 12,000 cpm.

heating and fast-cooling, this RNA has a 22% resistance to pancreatic and T1 RNases when labeled with [^{33}P]ATP as precursor and only a 2 to 3% resistance when labeled with [^{32}P]GTP as precursor. The RNA not bound to the column has only a 2 to 3% RNase resistance after heating and fast-cooling, whether labeled with a [^{32}P]ATP or a [^{32}P]GTP precursor. Moreover, the poly(A) fragment was shown to be covalently linked to the heteropolymeric complementary sequences of the product RNA. All of the RNA bound to oligo(dT)-cellulose is complementary to vRNA (Table 4): whether labeled using [^{32}P]ATP or [^{32}P]GTP, the bound RNA is rendered 96 to 100% resistant to RNase after annealing to excess vRNA. All of the RNA that does not bind to oligo(dT)-cellulose is also complementary to vRNA. Furthermore, the labeled RNA bound to the column is of large size similar to the unfractionated ApG- or GpG-stimulated product RNA shown in Fig. 1 and 2, with no indication of the presence of a low-molecular-weight poly(A) fragment (data not shown).

The size of the poly(A) sequences synthesized in vitro was compared to that of the poly(A) found in the viral mRNA isolated from the infected cell. The poly(A) fragments were obtained by digesting these cRNA's with pancreatic and T1 RNases and were analyzed by polyacrylamide gel electrophoresis (Fig. 3). The in vivo poly(A) migrates as a relatively homogeneous peak, corresponding to a length of 140 to 160 nucleotides. In contrast, in vitro poly(A) is considerably more heterogeneous and is comprised of species ranging from 60 to 350 nucleotides in length. The bulk of the in vitro poly(A) is 175 to 300 nucleotides in length and is, thus, longer than the in vivo poly(A).

Demonstration that ApG and GpG act as initiators of in vitro RNA synthesis. The fact that both ApG and GpG stimulate in vitro RNA synthesis suggests that these DNMPs act as initiators (11, 20) and are therefore incorporated at the 5′ end of the product RNA. To demonstrate this, it was necessary to label these DNMPs with radioactivity. Our approach was to add ^{32}P to the 5′ end of these DNMPs, utilizing polynucleotide kinase. The resulting compounds, ^{32}pApG and ^{32}pGpG, stimulate RNA synthesis approximately one-fourth as well as the parent DNMPs and are incorporated into the product RNA. To determine the site of incorporation, the product RNA labeled with ^{32}pApG (or ^{32}pGpG) and [^{3}H]UTP was isolated free of all the unincorporated substrate molecules, as described in Materials and Methods. The last step in this procedure is a centrifugation through a sucrose gradient containing 95% Me_2SO (Fig. 4). A large proportion of the product labeled with both ^{32}P and ^{3}H sedimented in the same region as vRNA (between the two arrows). This product is somewhat smaller than the ApG-stimulated product, and, in the molecular weight range of vRNA, is estimated to have a median length of approximately 800 to 1,000 nucleotides. From the specific activity

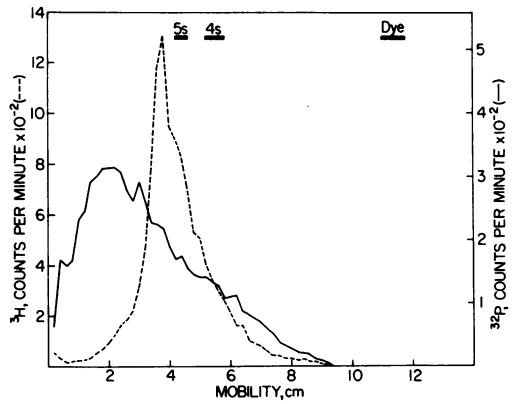

FIG. 3. *Comparison of the size of the poly(A) sequences linked to in vivo and in vitro cRNA. [^{3}H]adenosine-labeled in vivo cRNA was purified as described previously (14), and in vitro cRNA was synthesized in a reaction mixture containing unlabeled virus, $MgCl_2$, ApG, and [α-^{32}P]ATP. The poly(A) sequences were isolated from these cRNA's and were subjected to electrophoresis on 15% acrylamide gels containing 8 M urea as described in Materials and Methods. ^{3}H-labeled BHK-21 cell 4S and 5S RNAs (~80 and 120 nucleotides in length, respectively) were run on separate gels as markers.*

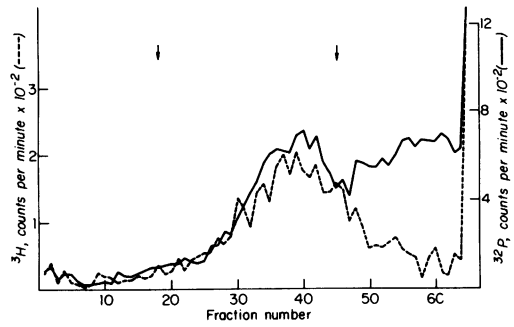

FIG. 4. *Analysis of the ^{32}pApG-stimulated product on a sucrose gradient containing 95% Me_2SO. Reaction mixtures containing 5 mM $MgCl_2$, 0.2 mM ^{32}pApG (10,000 cpm/pmol), and [^{3}H]UTP (65 cpm/pmol) were incubated for 2 h at 31°C. The RNA product was isolated and subjected to centrifugation as described in Materials and Methods. The arrows denote the region in which ^{32}P-labeled vRNA, run on a separate gradient, is found.*

of the precursors, it was calculated that the product RNA in this molecular weight range contains about 30 [^{3}H]UMP residues per ^{32}P atom incorporated from ^{32}pApG. If it is assumed that cRNA contains approximately 20% U residues (14, 22) and that all of the ^{32}pApG is incorporated as a dinucleotide at the 5′ end of the product RNA, then the median chain length would be 150 nucleotides, or only about 17% of the chain length estimated from the Me_2SO gradient. These results can be explained if more than one atom of ^{32}P is incorporated per RNA chain, i.e., if internal as well as 5′ terminal incorporation of ^{32}P occurs.

The labeled RNA in the size range of vRNA was digested with T2 RNase, and the hydrolysate was analyzed by chromatography on DEAE-Sephadex in 7 M urea (Fig. 5, upper panel). The ^{3}H-containing peak at a charge of −2 is the UMP incorporated internally. Two peaks of ^{32}P were found, one at a charge of −2 containing 75% of the ^{32}P radiolabel and the other at a charge of −4 containing 25% of the radiolabel. Analysis of the −2 peak by paper electrophoresis (Fig. 5, middle panel) indicated that it is comprised of all four ^{32}P-labeled mononucleotides. This clearly demonstrates that this ^{32}P had been incorporated internally into the product RNA, with T2 RNase hydrolysis resulting in nearest-neighbor transfer of the label. This internal incorporation of ^{32}P results from the formation of [α-^{32}P]ATP by the sequential action of a virion-associated nuclease splitting ^{32}pApG into ^{32}pA and pG and a phosphokinase converting ^{32}pA into pp^{32}pA (data not shown). The more significant result for the present aim is the ^{32}P incorporated at the 5′ end of the product RNA as represented by the −4 peak. When analyzed by paper electrophoresis at pH 3.5, this material comigrates with authentic pAp (Fig. 5, lower panel). Thus, approximately 25% of the ^{32}P derived from ^{32}pApG is incorporated at the 5′ end of the product RNA. With this level of 5′ end incorporation, the calculated median chain length of the product RNA is about 600 nucleotides, which is close to the chain length estimated from the sucrose-Me_2SO gradient. The fact that the 5′ end is pAp and is not in the form of a cap structure, either GpppAp or m^{7}GpppA^{m}p, indicates that, under these conditions, the product RNA is not capped by virion-associated enzymes. Using ^{32}pGpG as the precursor, ^{32}pGp was found at the 5′ end.

Is a virion-associated enzyme(s) capable of methylating the in vitro synthesized cRNA? Influenza viral cRNA synthesized in the infected cell is both capped and methylated at its 5′ end and also contains methylated internal A residues (24). As shown above, cRNA synthesized in vitro by detergent-disrupted virus is not capped at the 5′ end when pApG or pGpG is used to prime the reaction. To determine whether the in vitro cRNA was methylated by a virion-associated methylase(s), assays were performed using [^{3}H]SAM as methyl donor (Table 5). In the presence of Mg^{2+} alone, conditions in which VSV efficiently methylates its RNA transcripts, detergent-disrupted WSN virus does not methylate its RNA transcripts. Similarly, no detectable methylation of in vitro synthesized RNA by WSN virus was observed in the presence of Mn^{2+} alone, or in the presence of Mg^{2+} and ApG, GpG, pApG, or pGpG. The virion does, however, possess a methylase, as shown by its ability to methylate exogenous methyl-deficient tRNA from *E. coli* under conditions in which no cRNA synthesis occurs. This methylase activity is present in an amount comparable to that found associated with VSV.

DISCUSSION

In the present study we have shown that the influenza virion transcriptase is capable of synthesizing in vitro cRNA that is similar in a number of characteristics to authentic viral cRNA synthesized in the infected cell. Specifically, in the presence of Mg^{2+} and the DNMP ApG or GpG, the RNA synthesized in vitro is large, contains covalently linked poly(A), and initiates in A or G at the 5′ end. This synthesis is completely dependent on the presence of both Mg^{2+} and ApG or GpG. These DNMPs stimulate RNA synthesis about 100-fold in the pres-

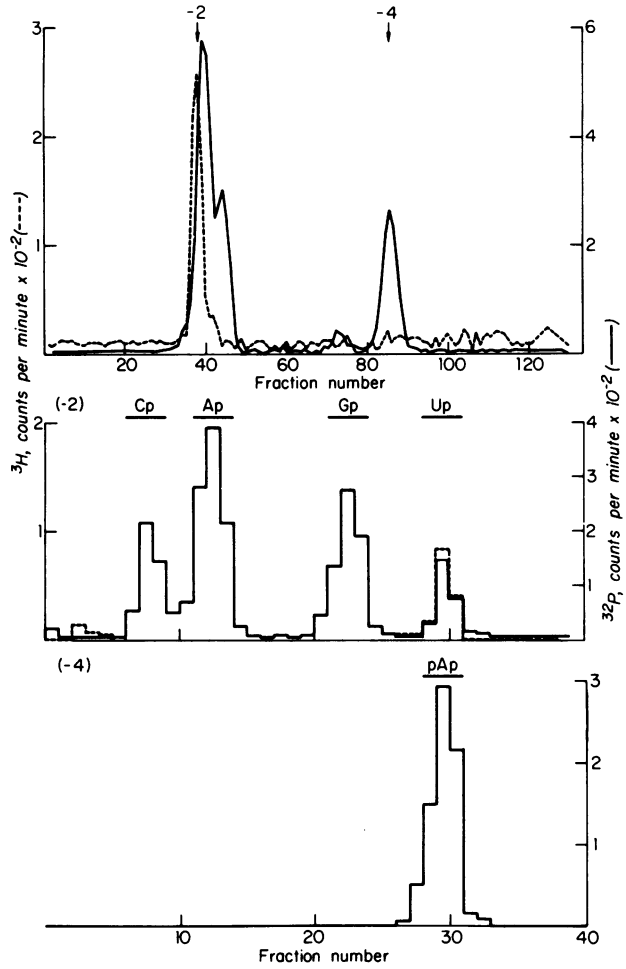

FIG. 5. *Analysis of the RNase T2 hydrolysate of the ^{32}pApG-stimulated product. The labeled RNA sedimenting in the region between the two arrows in Fig. 4 was collected by ethanol precipitation and was digested with RNase T2. The top panel shows the chromatography of the hydrolysate on DEAE-Sephadex A25 in 7 M urea; a mixture of the four 3′ mononucleotides and pAp (unlabeled) was added to the hydrolysate to mark the positions at which molecules with charges of −2 and −4, respectively, elute. The middle and bottom panels show the paper electrophoresis of the material from the DEAE-Sephadex column eluting at a charge of −2 and −4, respectively. Procedures were as described in Materials and Methods.*

ence of Mg^{2+} and act by initiating or priming RNA synthesis, as evidenced by our demonstration that the 5′ phosphorylated derivatives of these DNMPs are incorporated at the 5′ end of the product RNA. Specific DNMPs have also been shown to prime RNA synthesis by *E. coli* DNA-dependent RNA polymerase (11, 20).

In the absence of DNMPs, little RNA synthesis occurs in the presence of Mg^{2+}. This can be presumed to reflect an inability of the transcriptase to initiate efficiently in the presence of Mg^{2+}. However, once the block in initiation is overcome by the priming DNMP, the synthesis of large-size transcripts proceeds at a rapid rate, even to the point of addition of long poly(A) tracts. In contrast, in the presence of Mn^{2+} alone (or in combination with Mg^{2+}), a significant amount of RNA synthesis occurs. Under these conditions, initiation apparently occurs at numerous sites along the template vRNA at C residues, as the transcripts are of small size (7, 16; present study) and reportedly initiate only in G (19, 27). The small amount of poly(A) synthesized is not covalently linked to most of the cRNA sequences (16). This suggests that there is also premature termination in the presence of Mn^{2+} and that only those cRNA chains initiating close to the 5′ end of the vRNA template contain poly(A). The addition of ApG or GpG to an Mn^{2+}-containing reaction mixture

TABLE 5. *RNA methylase activity of influenza and VSV virions*[a]

Virus[b]	Divalent cation	Additions	pmol of [^{3}H]methyl incorporated
VSV	Mg^{2+}	None	0.155
WSN	Mg^{2+}	None	<0.001
WSN	Mn^{2+}	None	<0.001
WSN	Mg^{2+}	ApG	<0.001
WSN	Mg^{2+}	GpG	<0.001
WSN	Mg^{2+}	^{32}pApG	<0.001
WSN	Mg^{2+}	^{32}pGpG	<0.001
VSV	Mg^{2+}	tRNA(-4NTPs)	0.033
WSN	Mg^{2+}	tRNA(-4NTPs)	0.041

[a] Assay conditions were as described in Materials and Methods. Either $MgCl_2$ (5 mM) or $MnCl_2$ (5 mM) was present. Where indicated, ApG (0.4 mM), GpG (0.4 mM), ^{32}pApG (0.2 mM), or ^{32}pGpG (0.2 mM) was added.

[b] A 30-μg amount of viral protein was added.

increases the rate of RNA synthesis but leads to little improvement in the nature of the product synthesized, suggesting that initiation still is random and/or premature termination still occurs. These results indicate that Mg^{2+} and not Mn^{2+} is the proper divalent cation for the influenza virion transcriptase.

The sequence of bases on the vRNA at which initiation occurs in vitro can be deduced from the base sequence of the DNMPs that prime cRNA synthesis (11, 20). The order of stimulation by DNMPs is ApG > GpG > GpC. As cRNA is segmented, this result could indicate that the different segments initiate with different sequences. However, the stimulation by ApG and GpG is not additive, suggesting that they do not have independent sites of action and that there is a common initial sequence on all the cRNA segments. Based on the order of stimulation by the three DNMPs and Watson-Crick base pairing, the vRNA sequence at which initiation occurs would be expected to be (5')GCCU(3'). Another sequence is also possible. It has been shown that when GG dinucleotides are bound to a polynucleotide, G-to-G interactions between the dinucleotide and the polynucleotide are at least as strong as the Watson-Crick G-to-C interaction (26). Consequently, the vRNA sequence could also be (5')GCU(3'). This latter sequence would be most compatible with the observed priming ability of ApGpC. In addition, the vRNA sequence at which priming occurs in vitro could be at the 3' terminus of vRNA, as vRNA segments reportedly all contain U at the 3' end (25).

Whether ApG or GpG is effecting initiation of cRNA synthesis at, or near, the same site on vRNA as occurs in vivo cannot be ascertained from the present data. The largest in vitro cRNA product comigrates with the largest vRNA segment (approximately 10^6 daltons), as is also the case with in vivo cRNA (23a; Etkind et al., manuscript in preparation). However, the other large-size in vitro cRNA products are heterogeneous and distribute into two broad molecular weight ranges, whereas in vivo cRNA's, which also fall into these molecular weight ranges, distribute into more sharply defined peaks (23a; Etkind et al., manuscript in preparation). These in vivo cRNA segments, as a consequence of their attached poly(A), are larger than the corresponding vRNA segments. The heterogeneity of the in vitro cRNA's may be due to their larger, heterogeneous poly(A) sequences and/or to the action of nucleases, which have been detected in the virus preparation. To determine whether the initiation of transcription is at the same site on vRNA in vivo and in vitro, sequence analysis at the 5' end of both cRNA's is clearly required.

The poly(A) of in vivo cRNA is fairly homogeneous in length, 140 to 160 nucleotides, whereas the poly(A) of in vitro cRNA is more heterogeneous and is predominantly of larger size. Previous studies indicated that the poly(A) associated with in vivo cRNA was smaller and more heterogeneous (13) than that determined here, but subsequent analysis showed that degradation probably occurred during the isolation procedure used in the earlier study (14). The heterogeneity and larger size of the in vitro poly(A) could reflect a lack of precision in the in vitro system. Alternatively, larger poly(A) sequences may also be synthesized in vivo and may then be reduced by processing to the 140- to 160-nucleotide length observed. A similar difference between the poly(A) synthesized in vitro and in vivo has been described for Newcastle disease virus (39).

In vivo cRNA is both capped and methylated at its 5' terminus and contains internal m^6A residues (24). The in vitro RNA product does not possess these characteristics. In the reactions primed by ^{32}pApG or ^{32}pGpG, the 5' end of the product RNA is ^{32}pAp and ^{32}pGp, respectively, and no cap structures are found. However, as evidence in other systems indicates that some capping enzymes require as substrate an RNA containing a diphosphorylated 5' end (12, 36), we are presently synthesizing ppApG and ppGpG to test for a potential capping enzyme. In addition, no methylation of the in vitro product could be detected under a vari-

ety of conditions in the presence of [^{3}H]SAM. The virion may still have a methylase activity dependent on the prior capping of the in vitro product RNA. In fact, a methylase activity associated with influenza virions was detected, using exogenous methyl-deficient *E. coli* tRNA as substrate. Whether this activity, which is also associated with Newcastle disease virus and VSV virions (1, 9), is due to a virus-specific enzyme capable of methylating the cap of in vitro cRNA or is due to a host-derived enzyme remains to be determined. Evidence obtained with VSV suggests that the tRNA methylase activity is not associated with the same enzyme molecules responsible for virus-specific cRNA methylation (1).

The influenza virion-associated transcriptase is apparently defective in that in the absence of specific primer DNMPs it is unable to synthesize in vitro cRNA of large size containing poly(A). In the infected cell, this initiation function would most probably be provided by the host, either as a primer RNA or as a host protein that effects correct initiation of RNA chains without a primer. The correct initiation of viral cRNA can be postulated to be the host nuclear function required for influenza virus replication. Influenza viral cRNA synthesis in the infected cell is inhibited by actinomycin D and α-amanitin (5, 31, 33, 34), whereas cRNA synthesis in vitro catalyzed by the virion transcriptase is not inhibited by these drugs (8, 29, 37). The simplest hypothesis to explain the sensitivity of viral cRNA synthesis in vivo to both these drugs would be that correct transcription of viral cRNA requires the synthesis of a primer RNA transcribed from host cell DNA by the nucleoplasmic, α-amanitin-sensitive RNA polymerase II. We are presently testing this hypothesis.

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