Characterization of Influenza Virus RNA Transcripts Synthesized in vitro

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SUMMARY

Polyadenylated transcripts synthesized in vitro by detergent-disrupted influenza virus resemble virus mRNAs in that they possess the complement of the 3' terminus of the genome RNAs but lack sequences corresponding to the same 5' terminal region, including the homologous sequence of nucleotides 1 to 22. Transcription is initiated at the 3' terminus by both ApG and GpG as well as in the absence of added primer.

INTRODUCTION

During influenza virus infection two classes of genome transcript are synthesized, both of which contain molecules complementary to all eight genome RNAs (Hay et al. 1977). One class of molecules, the mRNAs, are polyadenylated incomplete transcripts which lack sequences complementary to at least 16 nucleotides at the 5' ends of the genome RNAs (Skehel & Hay, 1978) whereas the second class which are not polyadenylated are complete genome transcripts (Skehel et al. 1978).

Influenza virus particles contain an RNA transcriptase which is responsible for the synthesis of mRNA immediately following infection and which in vitro catalyses the synthesis of polyadenylated complementary RNAs (Plotch & Krug, 1977; Skehel & Hay, 1978). The results of electrophoretic analyses have suggested that the products of in vitro synthesis are incomplete genome transcripts equivalent in size to in vivo synthesized polyadenylated transcripts (Hay et al. 1978; Plotch & Krug, 1978). The present communication presents the results of a more detailed investigation of the properties of these molecules.

METHODS

Preparation and analysis of virion RNA. Fowl plague virus (FPV) was produced in 12-day-old fertile eggs and purified as described previously (Hay, 1974). The extraction of virion RNA and complementary RNA from fowl plague virus-infected cells, the isolation of polyadenylated molecules by oligo(dT)-cellulose chromatography and the formation of virion RNA-complementary RNA hybrids in DMSO solutions were carried out as described by Hay et al. (1977). The preparation of 5' terminal 32P-labelled virion RNA and nucleotide sequence analyses were carried out as described by Skehel & Hay (1978).

In vitro transcription reaction. Reactions contained tris-HCl, pH 8·0, 50 mM; NaCl, 150 mM; MgCl2, 8 mM; 2-mercaptoethanol, 0·2%; ATP, GTP and CTP, 1 mM; 3H-UTP, 0·2 mM, 20 μCi/ml; Nonidet P40, 0·2%; Macaloid, 2 mg/ml, and purified influenza virus approx. 1 mg/ml, and were incubated at 32 °C. When ApG (0·5 mM) or GpG (0·5 mM) was included in the reaction, the Nonidet P40 concentration was increased to 0·5%.
The time course of the reaction was followed by determining the TCA-insoluble radioactivity in 20 μl samples removed after various periods of incubation. Preparative reactions contained 1 mmol of all four nucleoside triphosphates and were terminated after 2 h (ApG or GpG present) or 4 h (no dinucleoside monophosphate) by the addition of SDS and EDTA to 1% and 10 mM, respectively. The mixture was extracted twice with an equal volume of phenol–chloroform (1:1) and the RNA precipitated in 70% ethanol at −20 °C.

Preparation of 5' terminal 32P-labelled in vitro transcripts. Polyadenylated transcripts synthesized in the presence of ApG or GpG were isolated by oligo(dT)-cellulose chromatography and labelled by incubating for 60 min at 37 °C in 50 μl reactions containing: RNA (10 to 20 μg); tris-HCl, pH 8.0, 50 mM; MgCl2, 10 mM; 2-mercaptoethanol, 10 mM; γ-32P-ATP, 50 μCi; and polynucleotide kinase (2 units). The reaction mixture was diluted with 0.5% SDS, 50 mM-sodium acetate, pH 5, extracted with an equal vol. of phenol–chloroform (1:1) and the RNA precipitated in 70% ethanol. The RNA was washed twice with 70% ethanol, 0.03 M-NaCl, dissolved in 0.3 M-NaCl, 10 mM-sodium acetate, pH 4.5, 1 mM-ZnSO4 and incubated at 37 °C for 30 min with nuclease S1 (40 units/μg RNA). The reprecipitated RNA was dissolved in 7 M-urea, 10 mM-tris-acetate, pH 7.8, 5 mM-EDTA and the labelled double-stranded RNAs separated by electrophoresis for 16 h at 5 V/cm in polyacrylamide gels containing 4% acrylamide, 0.2% N,N'-methylene bis-acrylamide, 0.4% TEMED, 0.1% SDS, 10 mM-EDTA, 40 mM-tris-acetate, pH 7.8, and ammonium persulphate (1 mg/ml), and eluted as described by Skehel & Hay (1978).

Polyadenylated transcripts synthesized in the absence of dinucleoside monophosphates were incubated at 37 °C for 30 min in 20 mM-tris-HCl, pH 8.0, containing 20 units/ml bacterial alkaline phosphatase. The reaction mixture was extracted twice with phenol–chloroform (1:1) and the RNA precipitated in 70% ethanol and end-labelled as above. The RNA was hybridized with a greater than 10-fold excess of unlabelled virion RNA and the polyadenylated hybrids were isolated by oligo(dT)-cellulose chromatography. This RNA was hybridized with a similar excess of virion RNA and the polyadenylated hybrid molecules containing 5' terminal labelled transcripts recovered.

Preparation of 3' terminal 3H-labelled virus RNA by oxidation with sodium periodate followed by reduction with 3H-sodium borohydride was as described by De Wachter & Fiers (1967).

Materials. 3H-UTP (40 to 60 Ci/mmol), γ-32P-ATP (3000 to 5000 Ci/mmol) and 3H-sodium borohydride (5 to 20 Ci/mmol) were obtained from the Radiochemical Centre, Amersham; bacterial alkaline phosphatase from Boehringer; polynucleotide kinase from PL Biochemicals and nuclease S1 from Sigma. The Phy1 nuclease preparation from Physarum polycephalum which hydrolyses the bonds between 3' AMP, 3' UMP and 3' GMP and the 5'-OH of adjacent nucleotides was given by Helen Donis-Keller, Department of Biochemistry and Molecular Biology, Harvard University. Adenylyl-(3'-5') guanosine and guanylyl-(3'-5') guanosine were obtained from Sigma.

RESULTS

Conditions for transcription

The optimal conditions for transcription in vitro by detergent-disrupted fowl plague virus, as detailed in Methods, are similar to those reported by others (McGeoch & Kitron, 1975; Plotch & Krug, 1977). They are unaffected by the addition of dinucleoside monophosphate ‘primers’ such as ApG and GpG (McGeoch & Kitron, 1975) except that raising the deter-
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Fig. 1. Time course of in vitro synthesis in the presence or absence of ApG. Samples (20 μl) were removed after various periods of incubation from the reactions described in Methods and Fig. 2(a) and the trichloroacetic acid-insoluble radioactivity was determined: ●●, no ApG; ○○, + ApG (0.5 mM).

gent concentration from 0.1 to 0.5% increased synthesis in the presence of ApG or GpG by two- to threefold without affecting the level of synthesis in their absence. The rate of synthesis in the presence of ApG or GpG was initially more than 20 times that in their absence; but whereas the 'primed' synthesis gradually decreased and ceased after about 2 h, in the absence of dinucleoside monophosphates synthesis continued linearly for 4 to 5 h and attained a level greater than half that in their presence (Fig. 1).

Characteristics of the reaction product

The product of the in vitro reaction is extracted as double-stranded molecules containing both genome and transcript RNAs. Hybridization analyses of 3H-labelled reaction product with a greater than 20-fold excess of unlabelled virion RNA and of 3H-labelled virion RNA with a similar excess of unlabelled product showed that the reaction products were complementary to the genome and that after 2 h of reaction in the presence of ApG or GpG they were equivalent in amount to approx. 50% of the virus template. A substantial proportion of the product was polyadenylated as indicated by its binding to oligo(dT)-cellulose and this varied between 30 and 70% depending on the particular virus preparation used but was similar in the presence and absence of dinucleoside monophosphate.

Polyacrylamide gel electrophoresis of the 3H-labelled reaction product following hybridization with excess unlabelled virion RNA indicated that both fractions obtained by oligo(dT)-cellulose chromatography contained intact transcripts present in similar relative proportions, although the fraction eluted by high salt buffer contained a higher proportion of shorter molecules as indicated by the background in the gel (Fig. 2a). The results of the experiment depicted in Fig. 2(a) also show that the proportion of polyadenylated product was not significantly affected by the time of reaction or the presence or absence of dinucleoside monophosphate. It is quite clear, however, that the relative proportions of the transcripts produced is influenced by the presence of ApG or GpG and in particular it was consistently observed that, relative to RNA 7, there is an increase in the proportions of
RNAs 1, 2, 3 and 4 and a decrease in the proportion of RNA 8 in the absence of 'primer' (Fig. 2b). In this connection it was also noted that the relative proportions of the transcripts synthesized in vitro by different influenza A viruses varied.

Size of in vitro transcripts

Comparisons of the electrophoretic mobilities of double-stranded RNAs, containing in vitro transcripts, with those of the double-stranded RNAs formed between virion RNA, and either polyadenylated or non-polyadenylated cRNA extracted from influenza-infected cells suggested that the in vitro transcripts were incomplete (Hay et al. 1978). To determine more exactly how the sequences of these transcripts correspond to those of their RNA templates the nuclease susceptibility of the termini of hybrid molecules was determined. Since the dinucleoside monophosphate 'primer' is incorporated into the 5' terminus of the transcript (Plotch & Krug, 1977), the terminus is unphosphorylated and can be specifically labelled using γ-32P-ATP and polynucleotide kinase as previously described (Skehel & Hay, 1978). Prior treatment with bacterial alkaline phosphatase did not increase the labelling of the transcript indicating that the majority of the 5' termini possess a 5'-hydroxyl group derived from the dinucleoside monophosphate.

The susceptibility of the 5' termini of the virion RNA strands and the insusceptibility of their 3' termini and the 5' termini of the transcripts to nuclease S1 digestion of the hybrid molecules (Fig. 3a and b) indicates that the transcripts possess exact copies of the 3' termini of their templates but lack sequences complementary to their 5' termini. The extent of the virion RNA sequence not represented in the transcript was investigated by sequencing the unprotected 5' terminal region of the virion RNA strand present in hybrid molecules. 5' Terminal 32P-labelled FPV virion RNAs 4, 5, 6, 7 and 8 were isolated and hybridized individually with polyadenylated in vitro transcript or polyadenylated cRNA extracted from FPV-infected cells and the labelled hybrids were purified by oligo(dT)-cellulose chromatography. Half of each hybrid was heated at 100 °C for 2 min and both double-stranded and denatured samples were incubated with Phy1 nuclease under standard sequencing conditions (Fig. 4). The nuclease-susceptible residues in the native hybrids were identified by comparing these results with previous nucleotide sequence data (Skehel & Hay, 1978) and as indicated for RNA 8 in Fig. 4 it was evident that the nuclease susceptible sequences of the virion RNA strands, residues 1 to 34 of RNA 8 and 1 to 28 of RNAs 5, 6 and 7, were the same in hybrids with in vitro transcripts as in those with in vivo synthesized mRNAs.

Initiation of transcription

To compare the initiation of transcription in the presence of ApG with that in its absence or in the presence of GpG the 5' terminal nucleotide sequences of the transcripts synthesized under the different conditions were determined. Polyadenylated reaction product synthesized in the absence of dinucleoside monophosphate was treated with bacterial alkaline phosphatase and labelled using γ-32P-ATP and polynucleotide kinase. The 5' terminal 32P-labelled transcripts were purified by two cycles of hybridization with excess unlabelled virion RNA followed by chromatography on oligo(dT)-cellulose. The nucleotide sequence at the 5' terminus of these molecules, 5' AGCAAAAGCAGG, was the same as that of transcripts synthesized in the presence of ApG. Comparative sequence analyses of 5' terminally labelled transcripts synthesized in vitro in the presence of either ApG or GpG showed, as indicated for RNA 7 (Fig. 5), that the sequences of all the transcripts differed only in the terminal nucleotide, the sequence of the 5' dodecanucleotide of the GpG primed transcripts being 5' GGCAAAAGCAGG. A small amount of 5' terminal A was detectable in the transcript synthesized in the presence of GpG which may have resulted from
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Fig. 2. (a) Polyacrylamide gel electrophoresis of in vitro transcripts synthesized in the presence or absence of ApG. Two reactions, A - 1 ml containing ApG (0.5 mM) and Nonidet P40 (0.5 %) and B - 2 ml containing Nonidet P40 (0.2 %), were as described in Methods. RNA was extracted from half of the reaction mixture at 1 and 2 h (reaction A) and 2 and 4 h (reaction B) and was denatured and re-annealed with a greater than fivefold excess of virion RNA in 63 % dimethyl sulphoxide at 37 °C for 16 h. The RNA was then fractionated by chromatography on oligo(dT)-cellulose and the two fractions eluting in 0.5 M-LiCl, 0.5 % LiDS, 10 mM-tris-HCl, pH 7.4, or in 10 mM-tris-HCl, pH 7.4, were incubated with nuclease S1 and analysed by electrophoresis on a 4 % polyacrylamide gel followed by fluorography as described by Hay et al. (1977). Lanes 1, 3, 5 and 7, 0.5 M-LiCl eluates; 2, 4, 6 and 8, 10 mM-tris-HCl, pH 7.4, eluates. Reaction A (+ ApG): lanes 1 and 2 (1 h), 3 and 4 (2 h); reaction B: lanes 5 and 6 (2 h), 7 and 8 (4 h). (b) Microdensitometer tracings of fluorographs (as shown in a) of polyadenylated transcripts: (A) Synthesized in the absence of ApG (lane 6, part a); (B) synthesized in the presence of ApG (lane 2, part a).
(a) Nuclease-susceptibility of the 5' termini of hybrid molecules containing virion RNAs and polyadenylated *in vitro* transcripts. Hybrids prepared by two methods: (i) 5' terminal ³²P-labelled virion RNA was hybridized with a 10-fold excess of unlabelled polyadenylated *in vitro* reaction product in 63% dimethyl sulphoxide at 37 °C for 16 h and the polyadenylated hybrid molecules were isolated by oligo(dT)-cellulose chromatography; (ii) polyadenylated product of an *in vitro* reaction containing ApG was end-labelled as described in Methods. Both RNA samples were dissolved in 0.6 ml 0.3 M-NaCl, 10 mM-sodium acetate, pH 4.5, 1 mM-ZnSO₄ containing tRNA (100 µg/ml) and divided into three equal aliquots. To two, nuclease S₁ (20 units/µg RNA) was added and they were incubated at 37 °C for 30 min or 90 min. The third aliquot had no nuclease treatment. Nuclease digestion was stopped by the addition of 1% SDS, 2 mM-EDTA and the RNA precipitated and analysed by electrophoresis on a 4% polyacrylamide gel followed by autoradiography. Lanes 1 to 3, hybrids from (i) containing 5' terminal ³²P-labelled virion RNA; 4 to 6, hybrids from (ii) containing 5' terminal ³²P-labelled transcript; 1 and 4, no nuclease; 2 and 5, 30 min incubation; 3 and 6, 90 min incubation.

(b) Analysis of double-stranded RNAs formed between either 3' terminally labelled and uniformly labelled ³²P-virion RNAs and polyadenylated *in vitro* transcripts. 3' Terminal ³²P-labelled virion RNA and uniformly ³²P-labelled virion RNA were separately hybridized with polyadenylated *in vitro* reaction product and the polyadenylated hybrids were isolated by oligo(dT)-cellulose chromatography. Equal aliquots were analysed before and after nuclease S₁ digestion as described in (a); 1 to 3, hybrids containing 3' terminal ³²P-virion RNA; 4 to 6, hybrids containing uniformly labelled ³²P-virion RNA; 1 and 4, no nuclease; 2 and 5, 30 min incubation; 3 and 6, 90 min incubation.
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Fig. 4. Analysis of the partial Phy₁ nuclease digestion products of native and denatured RNA hybrids containing 5' terminal 32P-labelled virion RNA 8 and either \textit{in vitro} transcript or mRNA. End-labelled virion RNAs were separately hybridized with either polyadenylated \textit{in vitro} reaction product or polyadenylated complementary RNA isolated from FPV-infected chick cells and the labelled polyadenylated hybrids purified by two successive adsorptions on to oligo(dT)-cellulose. Each sample was dissolved in 80 µl 20 mM-sodium citrate, pH 5·6, 1 mM-EDTA, 1 mM-NaH₂PO₄, 7 M-urea; 500 µg/ml tRNA and divided into four equal aliquots. Two were heated at 100 °C for 2 min and then one denatured and one undenatured aliquot were incubated at 50 °C for 15 min with 2 µl of a Phy₁ nuclease preparation or without nuclease; 5 M-urea was added to the hydrolysates and the oligonucleotides were separated by electrophoresis at 17 V/cm on polyacrylamide gels containing 20% acrylamide, 0·12% N, N'-methylene bisacrylamide, 7 M-urea, 2·5 mM-EDTA and 0·1 M-tris-borate, pH 8·3, and detected by autoradiography. The sizes of the oligonucleotides are indicated by the numbering on the left hand side. Lanes 1 to 4, hybrids containing \textit{in vitro} transcript; 5–8, hybrids containing mRNA; 1 and 6, native + nuclease; 2 and 5, denatured + nuclease; 3 and 8, native, no nuclease; 4 and 7, denatured, no nuclease.
Fig. 5. Analysis of the partial nuclease digestion products derived from 5' terminal 32P-labelled transcript 7 synthesized in vitro in the presence of ApG or GpG. Aliquots of end-labelled transcript 7 were digested with RNase T1 (1 and 8), U2 (2 and 7) or PhyI (3 and 6) or incubated at 90 °C for 10 min in 50 mM-NaHCO3/Na2CO3, pH 9.0, 1 mM EDTA (4 and 5), as described by Skehel & Hay, 1978, and the hydrolysates were analysed as indicated in Fig. 4. Transcript 7 was isolated from the products of reactions containing either ApG (1 to 4) or GpG (5 to 8). Numbers down the centre indicate the sizes of the oligonucleotides and the letters the identity of the 3' terminal nucleotide.
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‘unprimed’ synthesis. In addition to the specificity of nuclease digestion the difference in the terminal sequence is also reflected in the differing migrations of the mono-, di-, tri- and tetranucleotides.

**DISCUSSION**

The data presented allow the conclusion that the polyadenylated transcripts synthesized *in vitro* by detergent-disrupted influenza virions are similar to mRNAs synthesized in influenza virus-infected cells in that they both possess sequences complementary to the 3’ end of the genome and lack sequences complementary to the same 5’ terminal region which includes the homologous sequence of nucleotides 1 to 22 (Skehel & Hay, 1978). Although, as previously discussed (Skehel & Hay, 1978), it is not possible to distinguish between alternative mechanisms for the production of polyadenylated incomplete transcripts, the lack of evidence for a complete transcript intermediate in the *in vitro* reaction and the detection of ‘unpolyadenylated’ incomplete transcripts is considered to be more consistent with a mechanism involving premature termination of transcription. It is certainly evident that the process of polyadenylated incomplete transcript production can occur independently of host cell enzyme action. The nucleotide sequences at the 5’ termini of the transcripts synthesized *in vitro* in the presence or absence of ApG are identical. In addition to the results of the nuclease protection experiments, data from sequence analyses of the 3’ ends of the genome RNAs (J. W. McCauley, unpublished) confirms that transcription starts at the 3’ terminus. This is also the case when GpG is used as primer even though interaction of this dinucleotide with nucleotides 2 and 3 would be expected to be stronger than with nucleotides 1 and 2 and might explain the less efficient priming by GpC, the complement of nucleotides 2 and 3 (McGeoch & Kitron, 1975; Plotch & Krug, 1977; A. J. Hay, unpublished observations).

There was no detectable incorporation (using reovirus transcriptase as a positive control) of ³H-methyl label from S-methyl-³H-adenosyl methionine into transcripts synthesized *in vitro* in the absence of primer. In addition, the availability of the 5’ termini of these transcripts to labelling by polynucleotide kinase following bacterial alkaline phosphatase treatment indicates that under these conditions there is no incorporation of ‘cap structures’ such as those present in mRNA, m⁷GpppA⁷m and m⁷GpppG⁷m (Krug et al. 1976). Similar conclusions concerning the absence from the virus of enzymes to carry out these functions have been made by Plotch et al. (1978). The equal efficiencies of ApG and GpG in initiating transcription *in vitro* is of interest with regard to the nature of the termini on mRNA produced in infected cells and the mechanism of initiation of transcription. However, the significance of these results obtained in the *in vitro* system and other observations, such as those which indicate that ribosomes or cellular RNA (Horisberger, 1976) or cellular mRNA (Bouloy et al. 1978) also stimulate *in vitro* transcription, is not understood.

Finally, the observation that by varying the conditions of incubation, (by, for example, inclusion of dinucleoside monophosphate primers) different relative amounts of the transcripts can be produced suggests that transcription *in vitro* of the genome RNAs does not occur sequentially. This also appears to be the case *in vivo* since, following infection of different cells by the same virus, the relative amounts of the ‘primary’ transcripts differ (Bosch et al. 1978) and, following infection by partially u.v.-inactivated virus, inhibition of transcription is simply related to the size of the individual RNAs(unpublished observations). Consequently, since transcription of the different genome RNAs appears to be initiated independently it is possible that the relative efficiencies of initiation are determined by factors such as the availability of primers which may account in part for the observed regulation of transcription throughout infection.
REFERENCES


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