Volume 4 Number 12 December 1977 Nucleic Acids Research

Influenza virus messenger RNAs are incomplete transcripts of the genome RNAs

A.J.Hay, G.Abraham, J.J.Skehel, J.C.Smith* and P.Fellner*

Division of Virology, National Institute for Medical Research, Mill Hill, London NW7 and *Searle Research Laboratories, High Wycombe, Bucks, UK.

Received 13 October 1977

ABSTRACT

The results of ribonuclease T_1 oligonucleotide fingerprint analyses indicate that influenza virus messenger RNAs are incomplete transcripts of the corresponding genome RNAs and that in this respect they differ from the unpolyadenylated virus specific complementary RNAs obtained from infected cells. From the position of the untranscribed oligonucleotide in the virus RNA sequence and the ability or inability of the different transcripts to protect the 5' terminal nucleotide of virus RNAs against nuclease S_1 digestion, it is concluded that whereas the unpolyadenylated cRNAs are complete transcripts, the polyadenylated messenger RNAs lack sequences complementary to the 5' end of the genome molecules.

INTRODUCTION

The genomes of influenza viruses contain eight unique single-stranded RNAs of molecular weights between 2×10^5 and 10^6 which are transcribed during infection into corresponding molecules of complementary sequence (1-7). Since in the replication of all "negative-strand" RNA viruses complementary RNAs function both as messengers in protein synthesis and as templates in genome replication the question arises of whether or not the same molecules perform both functions. The results of analyses of transcription in influenza virus-infected cells have indicated that for this virus they do not (7). During influenza virus replication two classes of complementary RNA (cRNA) are produced - polyadenylated cRNA and unpolyadenylated cRNA - each of which consists of molecules complementary to all the genome RNAs. From observed differences in their kinetics of synthesis, their relative abundance and the sensitivity of their synthesis to metabolic inhibitors the cRNAs of the two classes appear to be produced and to function independently. Thus, cRNAs which are present in the polysomes of infected cells as messenger RNAs are polyadenylated and their synthesis is controlled throughout infection both with respect to the amount of each transcript produced and to the time at which each is

produced in maximal amount. Unpolyadenylated cRNAs, on the other hand, are produced in similar amounts during infection, are not associated with polysomes and are suggested to be the templates involved in the replication of virion RNA.

In addition, in the course of these analyses of transcription, comparisons of the electrophoretic mobilities of the double-stranded RNAs formed between virus RNA and molecules of the two classes of cRNA indicated that the polyadenylated transcripts are shorter. This communication contains the results of experiments designed to investigate directly the possibility that there are sequence differences between the two classes of cRNA. The results presented involve comparisons of the ribonuclease T_1 oligonucleotide fingerprints of equivalent molecules of the two classes of cRNA and of similar fingerprints of virus RNA hybridized to the different cRNAs and digested with nuclease S_1 before oligonucleotide analysis.

MATERIALS AND METHODS

Viruses and cells. The Rostock strain of fowl plague virus was grown in 12-day old embryonated eggs and purified as described previously (8). Primary chick cells were prepared as described by Porterfield (9) and incubated in Gey's medium containing 10% calf serum.

Growth of ^{32}P-labelled virus. Monolayers of chick cells were incubated for 16 hours in phosphate-free HEPES-buffered Gey's medium containing ^{32}P-orthophosphate (20 mCi/culture). Fowl plague virus (10 PFU per cell) was added and the cultures incubated for a further 24 hours. Virus was precipitated from the culture medium by the addition of polyethylene glycol to 5% at 4° for 2 hours and purified by sucrose density gradient centrifugation as previously described (8).

Infection of cells and radioactive labelling. Chick cell monolayers (5×10^6 or 5×10^7 cells/culture) were infected with fowl plague virus at an added multiplicity of 100 PFU per cell for 30 min at 20°, washed twice and incubated in Tris-buffered Gey's Medium. Cells infected in the presence of cycloheximide were incubated in medium containing cycloheximide (100 µg/ml) from 1 hr prior to infection. Cells were labelled either by incubating in medium containing ^{3}H-uridine (100 µCi/ml) between 1½ and 2½ hr or in phosphate-free medium containing ^{32}P orthophosphate (1 mCi/ml) for 9 hr after infection.

RNA extraction. Cell monolayers were washed three times with cold saline and dissolved in 0.5% SDS, 10 mM sodium acetate pH 5. The solution was extracted twice with an equal volume of phenol and the RNA was precipitated by the addition of two volumes of ethanol. To a suspension of purified virus in 0.1 M NaCl, 10 mM Tris-HCl pH 7.5 was added 1% SDS, 50 mM sodium acetate pH 5, 1 mM EDTA and the solution was extracted three times with an equal volume of phenol. The precipitated RNA was washed three times with 70% ethanol containing 30 mM NaCl.

Preparation of 5'-terminal ^{32}P-labelled virus RNA. Virus RNA (10 µg) dissolved in 20 mM Tris-HCl pH 8 was incubated at 37° for 20 min with 3 units of bacterial alkaline phosphatase. The reaction mixture was extracted 3 times with phenol and three times with ether and the RNA was precipitated and redissolved in 50 µl 50 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 15 mM 2-mercaptoethanol containing 15-50 µCi γ-^{32}P-ATP and 1 unit of polynucleotide kinase. After incubation at 37° for 20 min the reaction was terminated by the addition of 0.1% SDS and the reaction mixture extracted 3 times with phenol and 3 times with ether and the RNA again precipitated with ethanol.

Isolation of polyadenylated RNA and double-stranded RNA. RNA dissolved in 0.5 M LiCl, 0.5% lithium dodecyl sulphate, 10 mM Tris-HCl pH 7.5 was applied to a column of oligo (dT)-cellulose equilibrated with the same buffer. The eluate was reapplied to the column and the column was washed with the buffer till all unbound radioactivity had been removed. The bound RNA was then eluted with 10 mM Tris-HCl pH 7.5 and the RNA in both fractions was recovered by precipitation with ethanol. The unbound fraction was dissolved in 10 mM sodium acetate pH 5 and single-stranded and partially double-stranded RNA were precipitated by the addition of 2 M LiCl at 4° for 12 hrs. The completely double-stranded RNA which did not precipitate under these conditions was recovered by ethanol precipitation.

RNA-RNA hybridization was carried out essentially as described by Ito and Joklik (10). RNA mixtures in 10 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA were denatured by adding 9 volumes of dimethyl sulphoxide and incubating at 45° for 30 min. NaCl, Tris-HCl pH 7.5 and EDTA were added to give final concentrations of 30 mM, 10 mM and 1.5 mM respectively, the dimethyl sulphoxide concentration was reduced to 63% and incubation was continued at 37° for 20 hrs. The RNA was precipitated and washed twice with 70% ethanol containing 20 mM NaCl.

Nuclease S_1 digestion. RNA samples dissolved in 0.1 M NaCl, 10 mM sodium acetate pH 4.5, 0.5 mM $ZnSO_4$ were incubated at 37° for 4 hours with nuclease S_1 (10-20 units/µg RNA). Nuclease digestion was stopped by the addition of 1% SDS, 1 mM EDTA and the RNA was precipitated with ethanol.

Gel electrophoresis and autoradiography. Radioactive double-stranded RNAs dissolved in 7 M urea, 10 mM Tris-acetate pH 7.8, 5 mM EDTA were separated by electrophoresis at 90 volts for 18 hrs in polyacrylamide slab gels (16 cm) containing 4% acrylamide, 0.2% N,N^1 methylene bis-acrylamide (BIS), 0.4% N,N,N^1,N^1 tetramethyl ethylene diamine (TEMED), 0.1% SDS, 10 mM EDTA, 40 mM Tris-acetate pH 7.8, and ammonium persulphate (1mg/ml). Gels containing ^{32}P-labelled RNA were exposed to film either wet or after drying. Gels containing ^{3}H-labelled RNA were processed for fluorography as described by Bonner and Laskey (11) and exposed to pre-exposed film (12).

Analysis of ribonuclease T_1 oligonucleotides. The individual ^{32}P-labelled RNAs were cut from the gel and eluted by incubating the crushed gel in 0.5 M NaCl, 0.1% SDS, 10 mM EDTA, 20 mM Tris-HCl pH 7.5 at 37° for 16 hours. Each RNA dissolved in 10 mM Tris-HCl pH 7.5, 1 mM EDTA (50 µl) containing 80 µg carrier RNA was denatured by heating at 100° for 3 min and incubated at 37° for 30 min with 4 µg ribonuclease T_1. The digests were fractionated by two-dimensional polyacrylamide gel electrophoresis (13). The first dimension gel contained 10% acrylamide, 0.3% BIS, 6 M urea, 25 mM citric acid pH 3.5 and electrophoresis at 400 volts was for 16 hours. Electrophoresis in the second dimension gel which contained 20% polyacrylamide, 0.6% BIS, 0.1 M Tris-borate pH 8.3, 2.5 mM EDTA was at 500 volts for 16 hours.

Materials. 5,6 - ^{3}H uridine (49 Ci/mmol), γ^{32}P-ATP (3000 Ci/mmol) and ^{32}P orthophosphate (carrier free) were obtained from The Radiochemical Centre, Amersham. Nuclease S_1 and cycloheximide were obtained from the Sigma Chemical Co. Ribonuclease T_1 was obtained from Calbiochem Ltd. Alkaline phosphatase was obtained from the Worthington Biochemical Corpn. Polynucleotide kinase and oligo (dT)-cellulose were obtained from P-L Biochemicals, Inc. The constituents used to make polyacrylamide gels were obtained from Eastman Kodak Ltd. and recrystallised.

RESULTS

Ribonuclease T_1-oligonucleotide fingerprint analysis of polyadenylated and unpolyadenylated cRNAs. ^{3}H or ^{32}P-labelled virus-specific cRNA synthesised

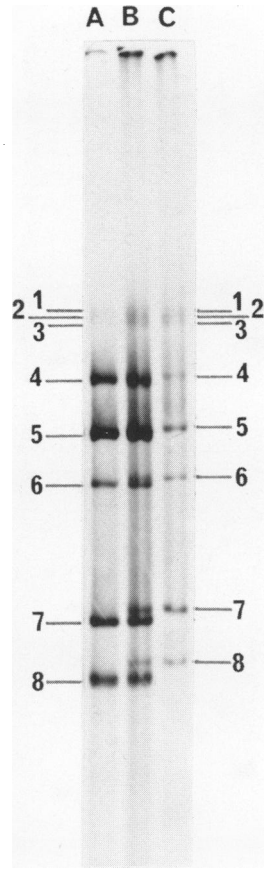

Figure 1. Analysis of double-stranded RNAs formed between virus RNA and ^{3}H-polyadenylated or unpolyadenylated cRNAs. Equivalent amounts of radioactive double-stranded RNAs from polyadenylated (A) and unpolyadenylated (C) hybrids were analysed separately or as a mixture (B). The direction of migration was from top to bottom.

in fowl plague virus-infected chick cells was isolated by hybridizing with an excess of unlabelled virus RNA (7). The hybrid molecules formed between virus RNA and either polyadenylated or unpolyadenylated cRNAs were separated using oligo (dT)-cellulose chromatography and LiCl fractionation and treated with nuclease S_1 to remove any unhybridized, non-complementary sequences. Analysis by polyacrylamide gel electrophoresis showed that the double-stranded molecules formed between virus RNA and the polyadenylated cRNAs migrated faster than the corresponding molecules formed between virus RNA and the unpolyadenylated cRNAs (Fig. 1). The differences in electrophoretic mobility were most marked for the smaller RNAs 6, 7 and 8 and corresponded to differences in molecular weights, estimated using reovirus double-stranded RNAs as markers (15), of approximately 20-30,000, i.e. of the order of

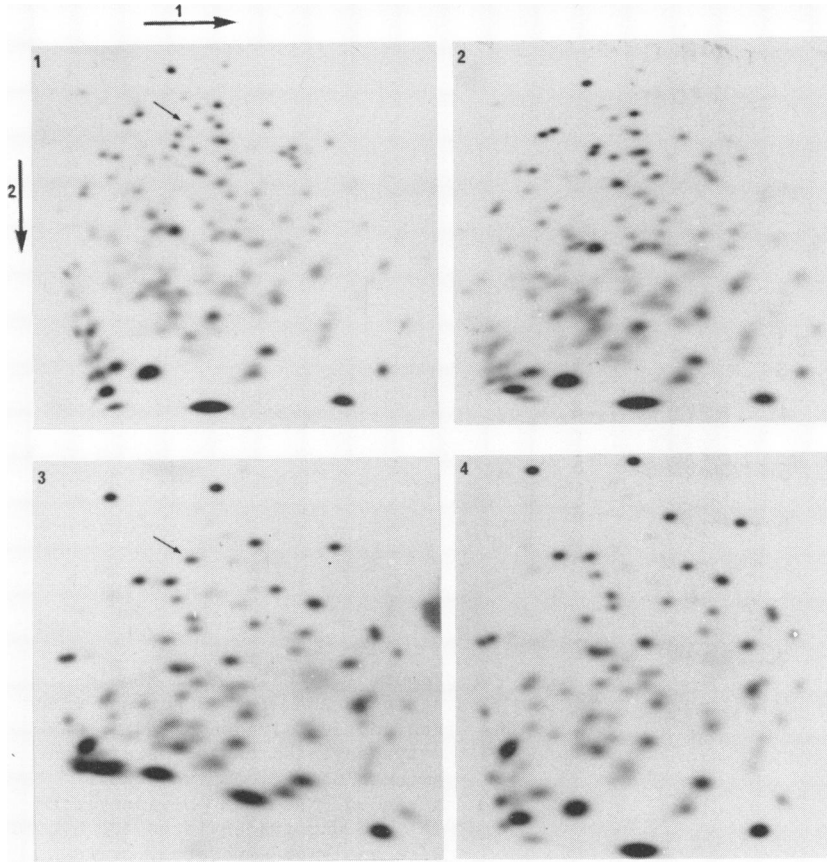

Figure 2. Ribonuclease T_1 oligonucleotide fingerprints of polyadenylated and unpolyadenylated cRNAs. The fingerprints are of 1. unpolyadenylated cRNA 6; 2. polyadenylated cRNA6; 3. unpolyadenylated cRNA 7; 4. polyadenylated cRNA 7. The arrows indicate the observed differences between the polyadenylated and unpolyadenylated cRNAs. Migration in the first dimension was from left to right and in the second dimension from top to bottom, as indicated.

30-40 bases for the single-stranded cRNAs.

^{32}P-labelled polyadenylated and unpolyadenylated cRNAs prepared in this way were denatured and digested with ribonuclease T_1 and the resulting oligonucleotides were analysed by 2-dimensional polyacrylamide gel electrophoresis (13). In the majority of comparisons no differences were detected. However for RNAs 6 and 7 the fingerprints of the polyadenylated and unpolyadenylated cRNAs differed (Fig. 2) and in each case a unique

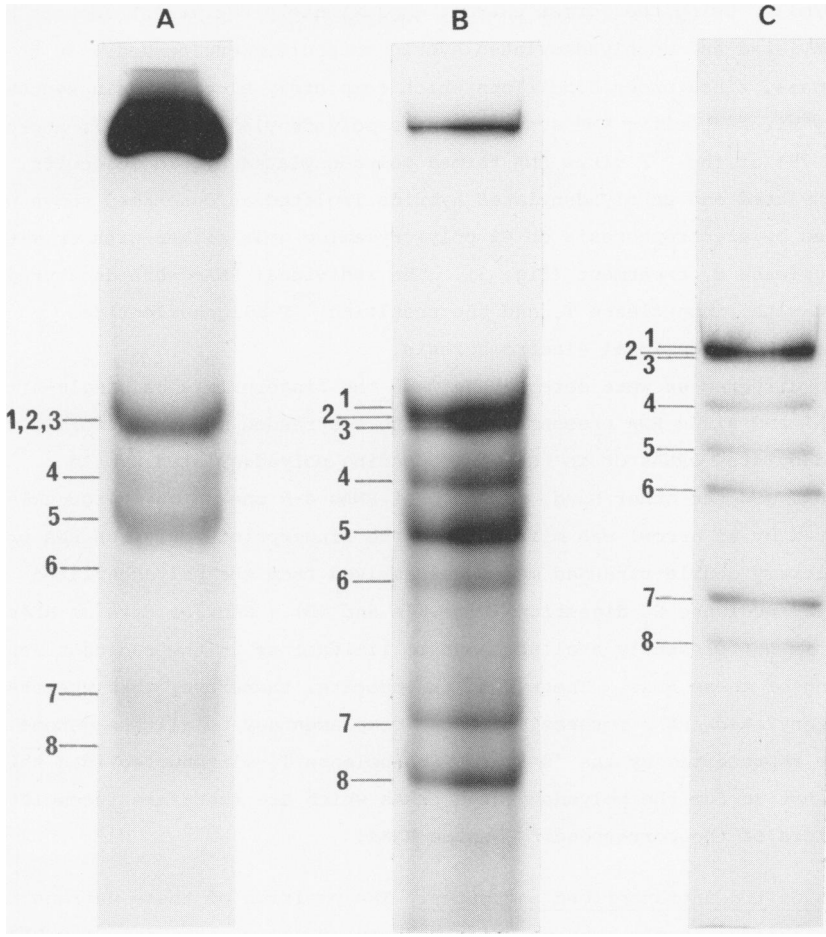

Figure 3. Separation of double-stranded RNAs formed between ^{32}P virus RNA and unpolyadenylated or polyadenylated cRNA. The results shown are of A. polyadenylated hybrids - no nuclease S_1 treatment; B. polyadenylated hybrids after nuclease S_1 treatment; C. unpolyadenylated hybrids after nuclease S_1 treatment. The direction of migration was from top to bottom.

oligonucleotide of the unpolyadenylated cRNA (indicated by an arrow) was absent from the polyadenylated cRNA.

Ribonuclease T_1-oligonucleotide fingerprints of double-stranded RNAs containing ^{32}P-labelled virus RNA. ^{32}P virus RNA was hybridized with an excess of unlabelled cRNA extracted from cells either 3 hours after a normal infection or 5 hours after infection in the presence of cycloheximide

(100 µg/ml). Using the former extract approximately equivalent amounts of polyadenylated and unpolyadenylated hybrid molecules were formed. In the latter case, since under conditions which completely block protein synthesis the only virus-specific RNA synthesised is polyadenylated cRNA (7), approximately 75% of the ^{32}P virus RNA formed polyadenylated hybrid molecules. The polyadenylated and unpolyadenylated hybrids isolated as described above were separated by electrophoresis on 4% polyacrylamide gels either with or without prior nuclease S_1 treatment (Fig. 3). The individual RNAs were denatured and digested with ribonuclease T_1 and the resulting ^{32}P-oligonucleotides analysed by 2-dimensional electrophoresis.

No differences were detected between the fingerprints of single-stranded virus RNA and virus RNA present in the double-stranded molecules formed with unpolyadenylated cRNAs or in the corresponding polyadenylated hybrid molecules. On the other hand, for each of RNAs 4-8 one unique oligonucleotide (indicated by an arrow) was missing from the fingerprints of virus RNA present in completely double-stranded molecules derived from the polyadenylated hybrids by nuclease S_1 digestion (Figs. 4A and 4B). Similar data on RNAs 1, 2 and 3 is not presently available due to limitations in the radioactive labelling of these RNAs. These results indicate, therefore, that whereas the unpolyadenylated cRNAs possess sequences complementary to all the genome sequence represented by the 'unique' ribonuclease T_1-oligonucleotides this is clearly not so for the polyadenylated cRNAs which are therefore incomplete transcripts of the corresponding genome RNAs.

Position of the untranscribed sequences. The position of these untranscribed oligonucleotides in the sequence of the genome RNAs is known only for RNAs 7 and 8 (15). The oligonucleotide untranscribed in RNA7 has the sequence UUUUUUAC (C,U)CAG and is located within 30 nucleotides from the 5' end of the RNA. That in RNA8 has the sequence UUUUUU(AAAU,AUU,AUC)AAG and is present in a partial ribonuclease T_1 oligonucleotide of approximately 100 nucleotides which contains the 5' end of the molecule. Additional information which suggests that there are sequences at the 5' termini of all genome RNA molecules which are not transcribed into mRNA has been obtained from experiments which examined the ability of the two types of cRNA to protect the 5' terminal nucleotide of virus RNA in hybrid molecules against digestion with nuclease S_1.

Virus RNA was treated with alkaline phosphatase and labelled using $\gamma^{32}P$-ATP and polynucleotide kinase (16). The 5' terminal ^{32}P-labelled RNA and uniformly labelled ^{32}P virus RNA were hybridized with an excess of

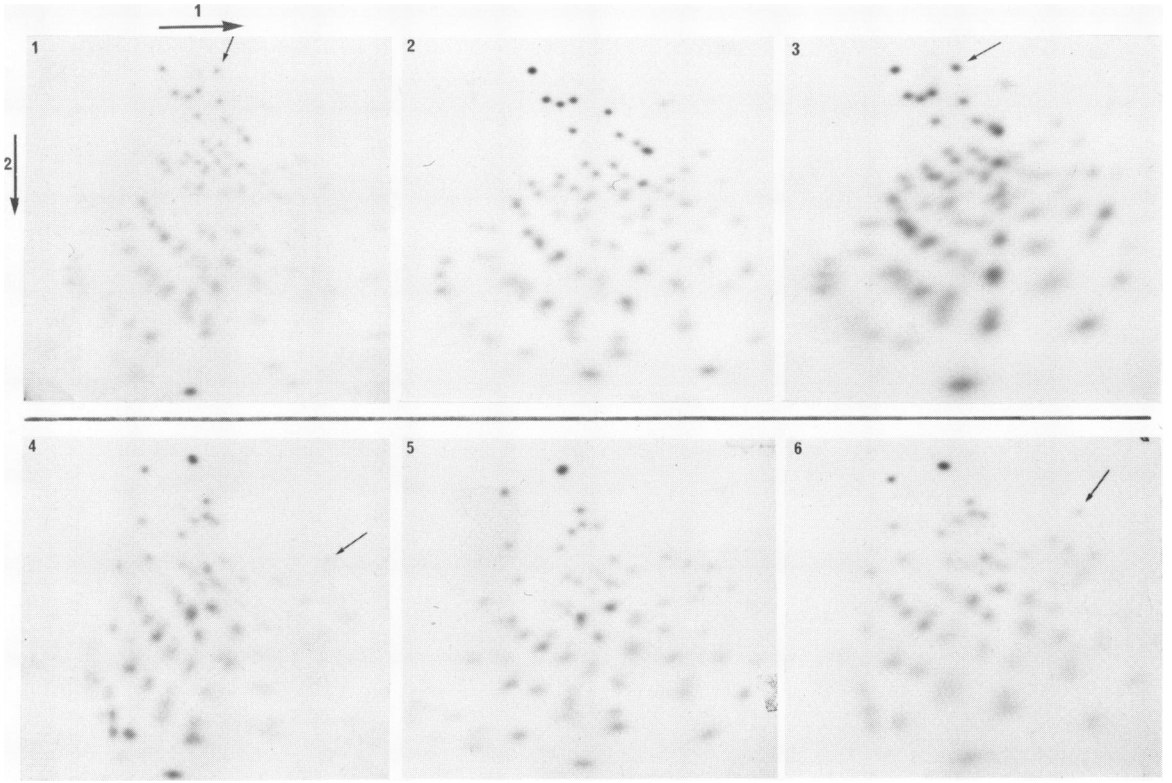

Figure 4A. Ribonuclease T_1 oligonucleotide fingerprints of ^{32}P virus RNAs 4 and 5 in hybrid molecules formed with unpolyadenylated or polyadenylated cRNA. The fingerprints are of ^{32}P virus RNAs 4 (1-3) and 5 (4-6) present in a) unpolyadenylated hybrids after nuclease S_1 treatment (1 and 4), b) polyadenylated hybrids after nuclease S_1 treatment (2 and 5) and c) polyadenylated hybrids - no nuclease treatment. The arrows indicate the observed differences between fingerprints of b) and fingerprints of a) and c). Migration in the first dimension was from left to right and in the second dimension from top to bottom, as indicated.

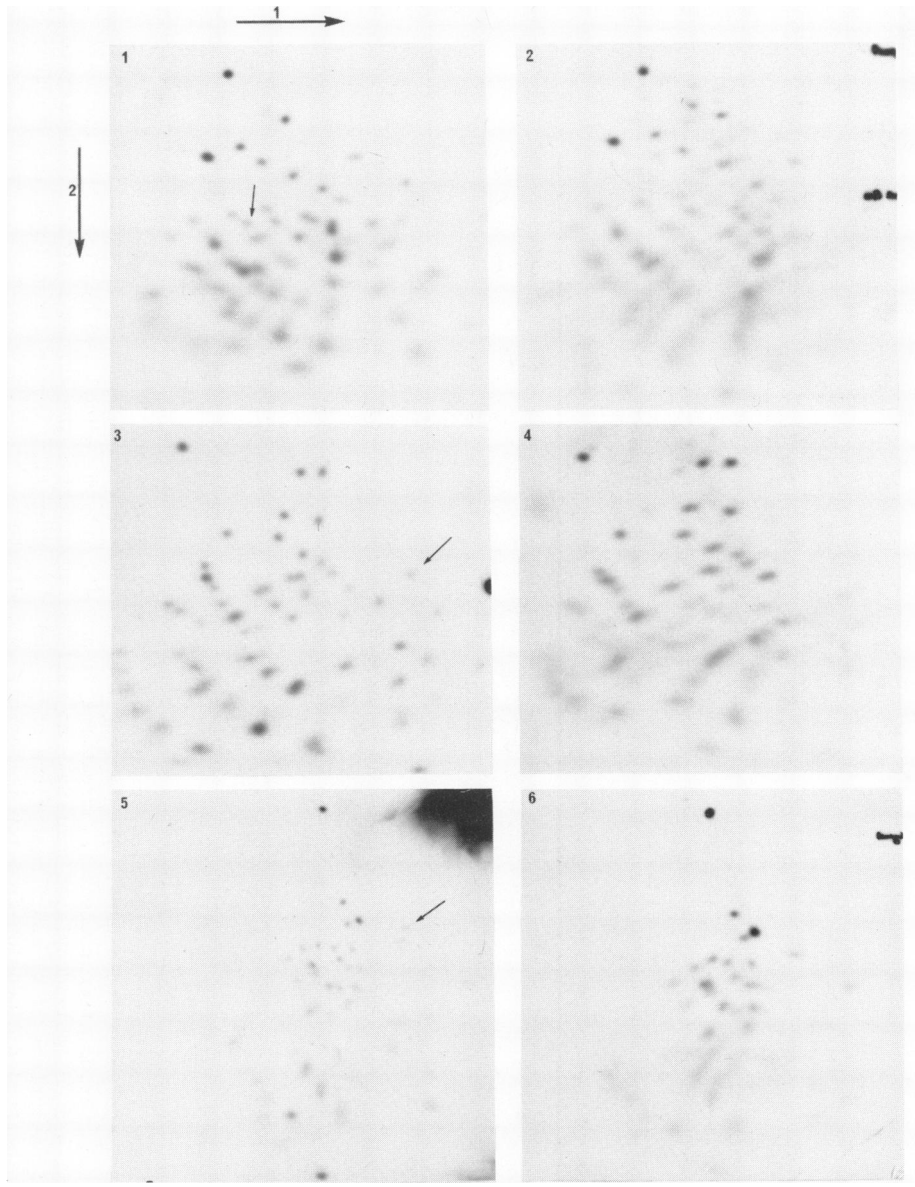

Figure 4B. Ribonuclease T_1 oligonucleotide fingerprints of ^{32}P virus RNAs 6, 7 and 8 in double-stranded molecules formed with unpolyadenylated or polyadenylated cRNA. The fingerprints are of ^{32}P virus RNAs 6 (1 and 2); 7 (3 and 4) and 8 (5 and 6) present in double-stranded RNAs obtained from unpolyadenylated hybrids (3,5) or polyadenylated hybrids (2,4,6) after nuclease S_1 treatment. Fingerprint 1 is of ^{32}P single-stranded virus RNA 6. The arrows indicate the observed differences between the two fingerprints of each RNA. Migration in the first dimension was from left to right and in the second dimension from top to bottom.

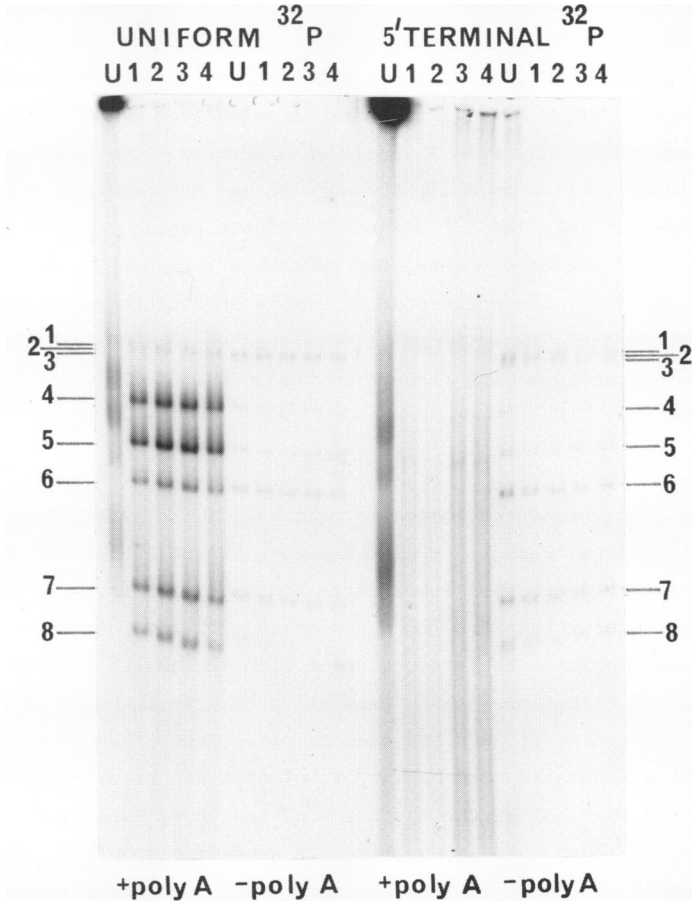

Figure 5. Analysis of double-stranded RNAs formed between either 5' terminally labelled or uniformly labelled ^{32}P virus RNA and polyadenylated or unpolyadenylated cRNA. The polyadenylated and unpolyadenylated hybrid molecules were dissolved in 0.3 M NaCl, 10 mM sodium acetate pH 4.5, 0.5 mM $ZnSO_4$ and equal aliquots were incubated at 37° with nuclease S_1 (10 units/µg RNA) for ½ hr (1), 1 hr (2) or 2 hr (3), respectively. To a fourth aliquot (4) 5 mM $MgCl_2$ was added and the sample incubated with nuclease S_1 for 1 hour. The fifth aliquot (U) was not incubated with nuclease S_1.

unlabelled cRNA extracted from chick cells 3 hours after infection with fowl plague virus. The polyadenylated and unpolyadenylated hybrid molecules were separated by oligo (dT)-cellulose chromatography and LiCl fractionation and digested with nuclease S_1 for different periods of time. From the results shown in Fig. 5 it is clear that whereas the 5' terminal ^{32}P-labelled

nucleotides of virus RNAs present in double-stranded molecules formed by hybridization with unpolyadenylated cRNAs were relatively resistant to nuclease S_1 attack, no radioactive double-stranded molecules were obtained from the hybrids formed between 5' terminally-labelled virus RNAs and polyadenylated cRNAs following S_1 nuclease digestion. On the other hand, radioactive double-stranded molecules were formed between both classes of cRNA and uniformly labelled ^{32}P virus RNA. These result, therefore, indicate that each of the unpolyadenylated cRNAs has a sequence complementary to the 5' terminus of the corresponding genome RNA which the polyadenylated cRNAs clearly do not. Experiments to determine whether or not these cRNAs are complete transcripts with respect to the 3' end of the genome RNAs are still in progress.

DISCUSSION

The evidence presented indicates that all of the mRNAs of influenza virus are incomplete transcripts of the genome RNA molecules and the results of the 5'-terminal protection experiments together with the available sequence data suggest that they do not contain sequences complementary to the 5' terminal region. The production of such incomplete transcripts could be the result of either premature termination of transcription or of degradative processing of complete transcripts. The information presently available which indicates that both types of cRNA can be transcribed by the same enzyme and that unlike the synthesis of messenger RNAs, the production of complete unpolyadenylated transcripts is dependent upon continued virus-specific protein synthesis (7) is consistent with both mechanisms. The transcriptase which normally recognizes a termination signal and produces incomplete transcripts could be modified by virus-specific protein(s) in such a way that it no longer responds to such signals and continues to catalyze the synthesis of a complete transcript. Alternatively, the virus specified protein(s) might affect post-transcriptional modification by, for example, binding to the 3' end of the complete transcript possibly as an initial stage in RNA replication, or by inhibiting the processing enzymes. It is not possible at present to distinguish between these alternatives.

The nucleotide sequence at the site of differentiation is also presently unknown. However for both RNAs 7 and 8 the sequence UUUUUUA is at the end of the T_1 oligonucleotide which is not transcribed into messenger RNA. Although more detailed information is required to establish whether or not this sequence is involved in termination of transcription or processing it may be ncted that similar sequences (although of opposite sense) have been

identified at the 3' ends of a number of bacterial and bacteriophage RNA transcripts (17-20) and implicated as signals for the termination of transcription. In eukaryotic cells also it has been suggested that the sequence AAUAAA detected close to the 3' end of several messenger RNAs may similarly be involved in termination (21).

Finally, the sequences present only at the 3' end of the unpolyadenylated transcripts, the presumed templates in the process of genome replication, may be specifically recognized by the RNA replicase and provide a means of distinguishing between template RNA and messenger RNA molecules. Such a feature may be of significance in the replication of "negative strand" RNA viruses in general.

ACKNOWLEDGEMENTS

A.J.H., G.A. and J.J.S. thank Bernard Precious and David Stevens for excellent assistance. J.C.S. and P.F. thank Dr. N. H. Carey for valuable discussions and Dr. A. J. Hale for provision of research facilities.

REFERENCES

1. Palese, P. and Schulman, J.L. (1976) J. Virol., 17, 876-884.
2. McGeoch, D., Fellner, P. and Newton, C. (1976) Proc. Nat. Acad. Sci. U.S.A., 73, 3045-3049.
3. Ritchey, M.B., Palese, P. and Schulman, J.L. (1976) J. Virol.,20,307-313.
4. Pons, M.W. (1976) Virology, 69, 789-792.
5. Pons, M.W. (1977) Virology, 76, 855-859.
6. Etkind, P.R., Buchhagen, D.L., Hertz, C., Broni, B.B. and Krug, R.M. (1977) J. Virol., 22, 346-352.
7. Hay, A.J., Lomniczi, B., Bellamy, A.R. and Skehel, J.J. (1977) Virology, in press.
8. Hay, A.J. (1974) Virology, 60, 398-418.
9. Porterfield, J.S. (1960) Bull. WHO, 22, 373-380
10. Ito, Y. and Joklik, W.K. (1972) Virology, 50, 202-208.
11. Bonner, W.M. and Laskey, R.A. (1974) Eur. J. Biochem., 46, 83-88.
12. Laskey, R.A. and Mills, A.D. (1975) Eur. J. Biochem., 56, 335-341.
13. DeWachter, R. and Fiers, W. (1972) Analytical Biochemistry, 49, 184-197.
14. Shatkin, A.J., Sipe, J.D. and Loh, P. (1967). J. Virol., 2, 986-991
15. Smith, J.C., Carey, N.H., Fellner, P., McGeoch, D. and Barry, R.D. (1977) In Negative Strand Viruses and The Host Cell, Barry, R.D. and Mahy, B.W.J. Eds. Academic Press, New York. In press.
16. Richardson, C.C. (1965) Proc. Nat. Acad. Sci., 54, 158-165.
17. Pieczenik, G., Barell, B.G. and Gefter, M.L. (1972) Arch. Biochem. Biophys., 152, 152-165.
18. Ikemura, T. and Dahlberg, J.F. (1973) J. Biol. Chem., 248, 5024-5032.
19. Bertrand, K., Korn, L., Lee, F., Platt, T., Squires, C.L., Squires, C. and Yanofsky, C. (1975) Science, 189, 22-26.
20. Rosenberg, M., de Crombrugghe, B. and Musso, R. (1976) Proc. Nat. Acad. Sci., 73, 717-721.
21. Proudfoot, N.J., Cheng, C.C. and Brownlee, G.G. (1976). Progr. in Nuc. Acid Res. and Mol. Biol., 19, 123-134.