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Nucleotide sequences at the 5' termini of influenza virus RNAs and their transcripts

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ABSTRACT

The results of analyses of the 5'-terminal sequences of Fowl Plague virus RNAs are presented. The first 13 residues of each of the eight RNA molecules which constitute the genome are in the identical sequence 5'AGUAGAAAUUAGG- and this conservation of sequence is shown to extend to other influenza viruses. The 5'-terminal sequences of virion RNA transcripts produced in vitro are also reported and again the first 12 nucleotides of these are identical for all influenza type A transcripts examined in the sequence 5'AGCAAAAGCAGG-. In addition the results of attempts to determine the sequence relationship between vRNAs and the two classes of complementary RNA synthesized in influenza infected cells are described which support the conclusion that influenza messenger RNAs are incomplete transcripts.

INTRODUCTION

The genome of Fowl Plague virus, a type A influenza virus, contains eight unique single-stranded RNA molecules with molecular weights of between 3 x 10^5 and 10^6 (1,2,3). These are transcribed during infection, into complementary RNAs (cRNA) which function as messenger RNAs (3,4,5,6) and as templates in genome replication (3). Both functions, however, appear not to be performed by the same molecules and the results of several experiments indicate that in infected cells there are two populations of cRNA. The members of one of these, which are polyadenylated, are associated with polysomes and variations in their relative abundance at different times during infection appear to correlate with the varying levels of production of virus-specific polypeptides. They are, therefore, considered to be messenger RNAs. The members of the other population of cRNA share none of these properties and probably function as templates in genome replication (3,7).

In further distinction, the results of comparative RNAase T_1 oligonucleotide analyses of representatives of the two classes of cRNA

have indicated that messenger RNAs are not complete transcripts in that they lack sequences complementary to the 5'-terminal regions of the genome RNAs (8). These observations prompted the experiments reported here which involve nucleotide sequence analyses of the 5'-terminal regions of virion RNAs and attempts to determine precisely the differences in primary structure between messenger and template RNA molecules. In addition the results of initial analyses of the 5'-terminal sequences of virus-specific messenger RNAs are presented.

MATERIALS AND METHODS

Fowl Plague (FPV), X-31 and B/Hong Kong/8/73 influenza viruses were produced in 10 day old fertile eggs and purified as described before (9).

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 as described in Hay et al. (3).

Preparation of 5' terminal ^{32}P-labelled virus RNAs. Virus RNAs (1 mg/ml) were incubated at 37° for 30 min in Tris-Cl pH 8.0, 20 mM, containing 20 units/ml bacterial alkaline phosphatase. The reaction mixtures were extracted 3 times with phenol and the RNAs precipitated in 70% ethanol at -20°. After washing in 70% ethanol the RNAs were dissolved in Tris-Cl pH 8.0, 50 mM, containing $MgCl_2$, 10 mM; 2-mercaptoethanol, 10 mM; 100 µC γ-^{32}P-ATP; and 2 units of polynucleotide kinase and incubated for 30 min at 37°. The mixtures were adjusted to 7 M urea, 0.5% SDS and 5×10^{-3} M EDTA and the labelled components were separated by electrophoresis for 14 hr at 6.5 volts/cm in polyacrylamide gels containing 3% acrylamide; 0.15% N,N' methylene bis-acrylamide; 0.4% N,N,N'N' tetramethyl ethylene diamine (TEMED); 2×10^{-3} M EDTA; 2.5×10^{-2} M sodium citrate pH 3.5; 0.1% SDS and ammonium persulphate 1 mg/ml. The radioactive RNAs were eluted from the polyacrylamide gel by homogenizing gel slices in a solution containing ammonium acetate, 0.5 M; magnesium acetate, 1mM; and SDS, 1%, and incubating at 37° for 15 min. The eluted RNAs were precipitated in 70% ethanol after adding tRNA, 10 µg/ml, as carrier and the precipitated RNA was further purified by precipitation as the cetyl trimethyl ammonium bromide salt (10).

Preparation of 5' terminal ^{32}P-labelled *in vitro* transcripts. Transcription reactions containing Tris-Cl pH 8.0, 50 mM; NaCl, 150 mM;

$MgCl_2$, 8 mM; nonidet P40, 0.5%; β-mercaptoethanol, 0.2%; ATP, CTP, GTP and UTP, 1 mM; ApG, 0.5 mM; Macaloid, 2 mg/ml and purified influenza virus, 1 mg/ml were incubated at 32° for 2 hours. The reaction was terminated by the addition of SDS, 1%, and EDTA, 10 mM, the mixture extracted twice with phenol and the RNA precipitated in 70% ethanol. The polyadenylated transcripts isolated by oligo (dT)-cellulose chromatography were end-labelled using polynucleotide kinase and γ-^{32}P-ATP, as described above. The reaction mixture was extracted with phenol and the precipitated RNA was incubated at 37° for 30 min with nuclease S_1 (3), 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 components separated by electrophoresis for 16 hr 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 above.

Sequence analysis. Partial nuclease digestion was done as described by Donis-Keller et al. (11). To 20 μl aliquots of 20 mM sodium citrate buffer, pH 5.0 containing EDTA, 1 mM; NaH_2PO_4, 1 mM; Urea, 7 M; tRNA, 500 ug/ml; xylene cyanol and bromophenol blue, 250 ug/ml; and labelled RNA, was added 0.01 units of RNAase T_1 or 2 units of RNAase U_2 or 2 μl of a phy-1 nuclease preparation as indicated. Other aliquots contained no nuclease. All samples were incubated at 50° for 15 min. The products of limited alkaline hydrolysis were obtained by incubating a sample of each RNA at 90° for 10 min in 50 mM $NaHCO_3/Na_2CO_3$ buffer pH 9.0 containing 1 mM EDTA and 250 μg/ml tRNA. The hydrolysates were applied to the gels after addition of urea to 5 M. The oligonucleotides were separated by electrophoresis at 17 volts/cm on gels containing 20% acrylamide; 0.12% N,N' methylene bis-acrylamide; urea, 7 M; Tris-borate pH 8.3, 0.1 M; and EDTA, 2.5 mM.

Materials. γ-^{32}P-ATP 3000-5000 C/mmole was obtained from the Radiochemical Centre, Amersham; bacterial alkaline phosphatase, BAPF, from Worthington; polynucleotide kinase from PL Biochemicals; T_1 and U_2 nucleases from Calbiochem and nuclease S_1 from Sigma. The phy-1 nuclease preparation from Physarum Polycephalum which hydrolyses the bonds between 3' AMP, 3' UMP and 3' GMP and the 5'-OH groups of adjacent nucleotides (19) was given by Helen Donis-Keller of the Department of Biochemistry and Molecular Biology, Harvard University.

RESULTS

5'-terminal sequence analyses of influenza genome RNAs. The bacterial alkaline phosphatase-treated RNA components of FPV were labelled using polynucleotide kinase and γ-^{32}P-ATP and following electrophoretic separation (Figure 1), the 5'-terminal nucleotide sequences were determined by the method of Donis-Keller et al. (11). The results obtained are presented in Table 1 and an example of the oligonucleotide separation patterns for RNAs 5 and 6 is shown in Figure 2. In all cases residues 1 to 13 and 17 to 22 were identical. In addition to the eight RNA components mentioned in the Introduction some influenza viruses contain additional RNA molecules of molecular weights about 100,000 and FPV has two such components (3). 5'-terminal sequence analyses of these indicated that

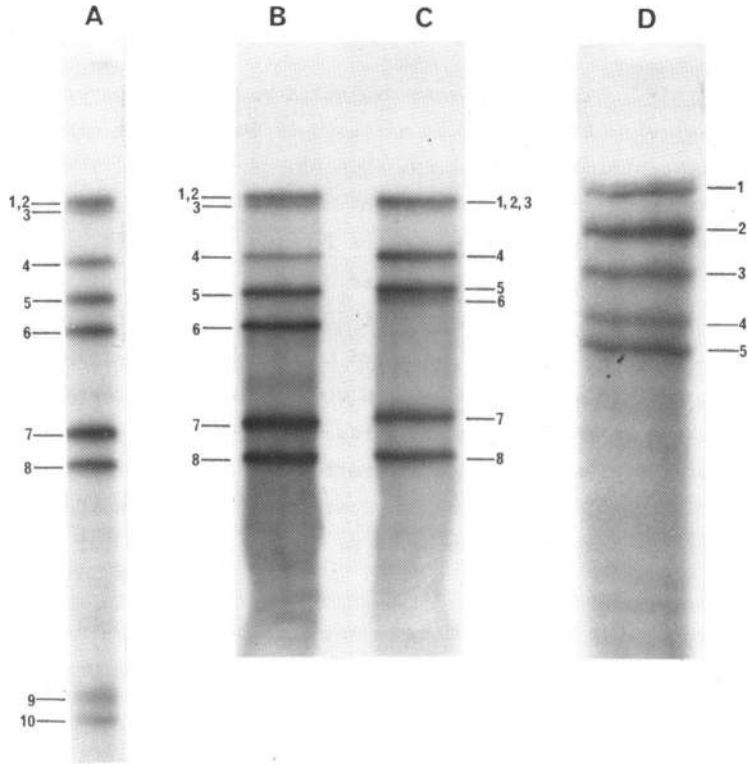

Figure 1. Autoradiograms of 5' terminal ^{32}P-labelled influenza virus RNAs. A. fowl plague virus RNA electrophoresed at 4.5 volts/cm for 14 hr. B. and C. fowl plague and X-31 virus RNAs, respectively, electrophoresed at 6.5 volts/cm for 14 hr. D. B/Hong Kong/8/73 virus RNA electrophoresed at 3.5 volts/cm for 14 hr.

TABLE 1. 5'-terminal nucleotide sequences of RNAs of Fowl Plague, X-31 and B/Hong Kong/8/73 influenza viruses.

	1 5 10 15 20 23
FPV 1-3	A G U A G A A A U U A G G U A G U U U U U U A
4	A G U A G A A A U U A G G G A G U U U U U U U
5	A G U A G A A A U U A G G G U A U U U U U U U
6	A G U A G A A A U U A G G A G A U U U U U U U
7	A G U A G A A A U U A G G U A G U U U U U U A
8	A G U A G A A A U U A G G G U G U U U U U U A
9	A G U A G A A A U U A G G U A G U U U U U U A
10	A G U A G A A A U U A G G U A G U U U U U U A
X-31 1-3	A G U A G A A A U U A G G U A G U U U U U U A
4	A G U A G A A A U U A G G G U G U U U U U U A
5+6	A G U A G A A A U U A G G G/A U/G A U U U U U U A
7	A G U A G A A A U U A G G U A G U U U U U U A
8	A G U A G A A A U U A G G G U G U U U U U U A
B/HK 1	A G U A G A A A C A C G A G C A U U U U U U A A
2	A G U A G A A A C A A G A G C A U U U U U U A
3	A G U A G A A A C A A G A G C A U U U U U U A
4	A G U A G A A A C A A G A/G G A A U U U U U U A
5	A G U A G A A A C A A G A G G A U U U U U A A

they were indistinguishable at least for 50 residues and that they were not related to RNAs 4, 5, 6, 7 or 8. On the other hand, clear similarities between their oligonucleotide patterns and those obtained using mixtures of RNAs 1, 2 and 3 indicated that they were related to one of these components and experiments are in progress to determine the precise relationship.

Following these observations the RNAs of an antigenically unrelated type A influenza virus of human origin, X-31 (H_3N_2), were also analyzed and found to have identical properties (Table 1). In addition the eight RNA components of a type B influenza virus (12) were separated into five size classes (Figure 1) and similar anlyses were made of these RNAs. The results which are also presented in Table 1 indicate that again these molecules have nearly the same 5'-terminal sequences which are clearly analogous to those of the type A virus RNAs.

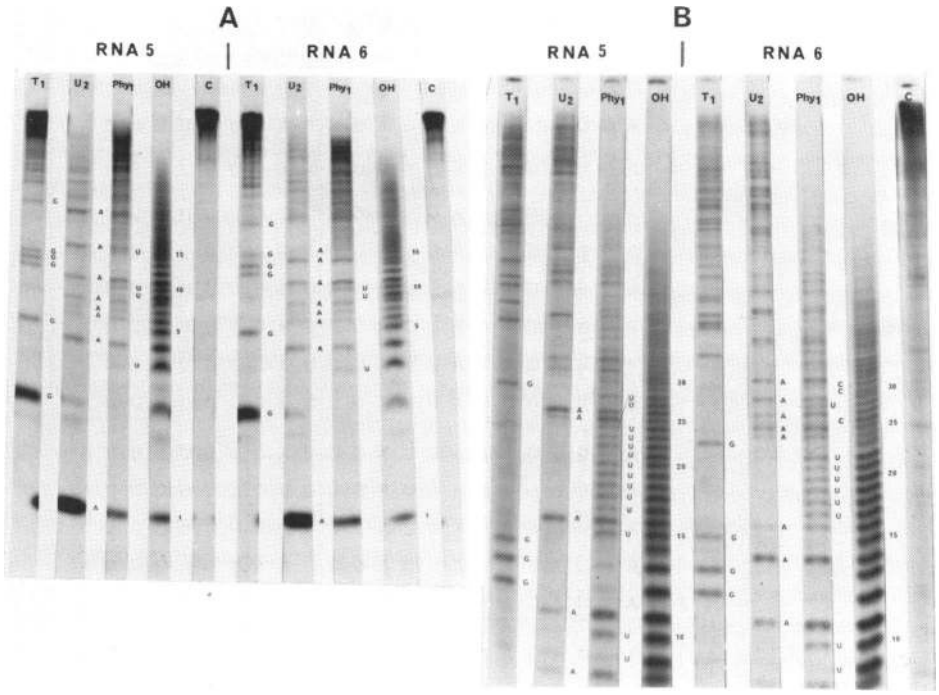

Figure 2. Autoradiograms of partial nuclease digestion products derived from ^{32}P end-labelled RNAs 5 and 6 of fowl plague virus. Electrophoresis was for A. 5 hr and B. 20 hr at 17 volts/cm. The lanes are labelled according to treatment of the samples: T_1, U_2 or Phy_1 nuclease; C, no nuclease; OH, alkaline hydrolysis, which indicates the length of the product in nucleotides.

In vivo transcription - indirect analyses of the 3'-terminal sequences of virus messenger RNAs. Determinations of the nuclease susceptibility of virus RNAs (vRNAs) in hybrids formed with either messenger RNA or non-polyadenylated cRNA indicated that protection of the labelled 5'-termini of vRNAs from nuclease S_1 digestion was only obtained in the latter (8). These observations together with estimates of the size of nuclease S_1 digested hybrids and the results of comparative analyses of the oligonucleotides obtained from them following ribonuclease T_1 digestion suggested that messenger RNAs were about 30 nucleotides shorter than complete transcripts (7, 13). Attempts were therefore made to obtain more precise estimates of the 5'-terminal vRNA sequences not represented in messenger RNAs. In these experiments 5'-terminally labelled virion RNAs 5, 6, 7 and 8 were separately hybridized with polyadenylated cRNA extracted from FPV-

infected cells and the hybrids formed were purified by chromatography on oligo (dT)-cellulose. Half of each hybrid was heated at 100° for 2 min and both double-stranded and denatured aliquots were then incubated with Phy_1 nuclease under standard sequencing conditions (Fig. 3A and 3B). From the known electrophoretic mobilities of the 5'-terminally-labelled oligonucleotides together with the differential susceptibilities of G and A residues to Phy_1 digestion, identification of the nuclease-susceptible residues in the native hybrids was made. The results indicate (Table 2) that after residues 28 in the cases of RNAs 5, 6, 7 and 34 in the case of

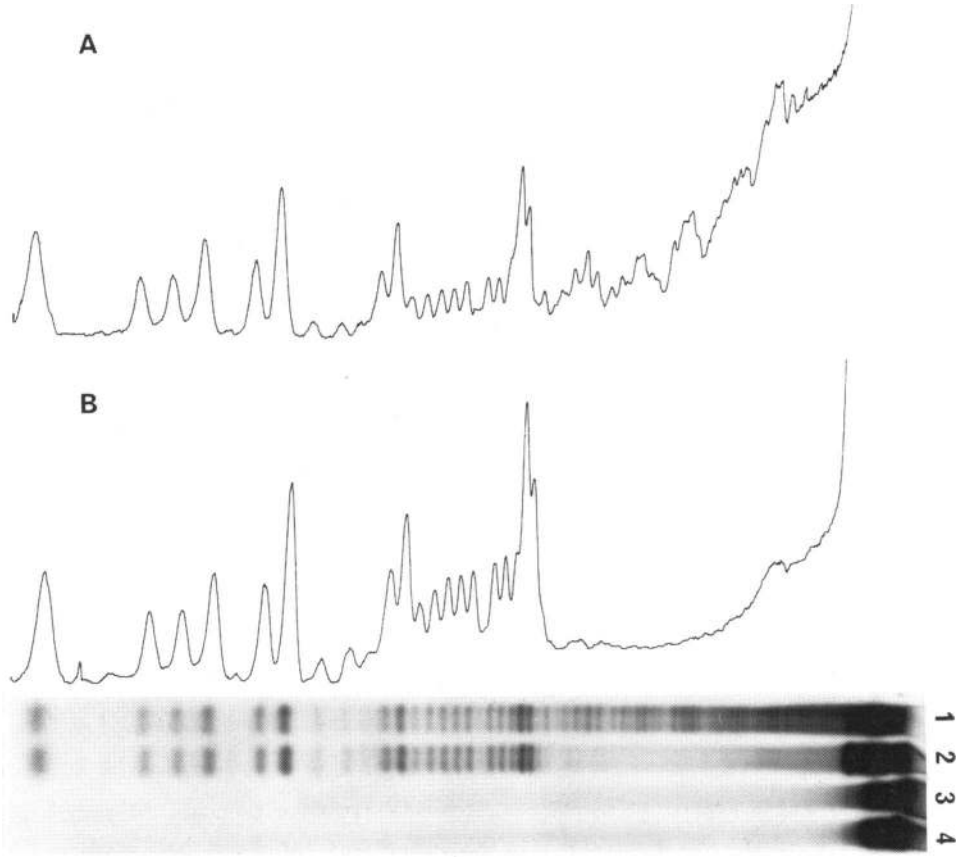

Figure 3A. Autoradiograms and microdensitometer tracings of partial Phy_1 nuclease digestion products of native and denatured RNA hybrids containing polyadenylated cRNA and end-labelled vRNA 5. The labelled oligonucleotides were separated by electrophoresis at 17 volts/cm for 16 hr. The gel lanes 1, 2, 3 and 4 contained in order: denatured and native hybrids + nuclease and denatured and native hybrids without nuclease. A. and B. are microdensitometer tracings of lanes 1 and 2 respectively.

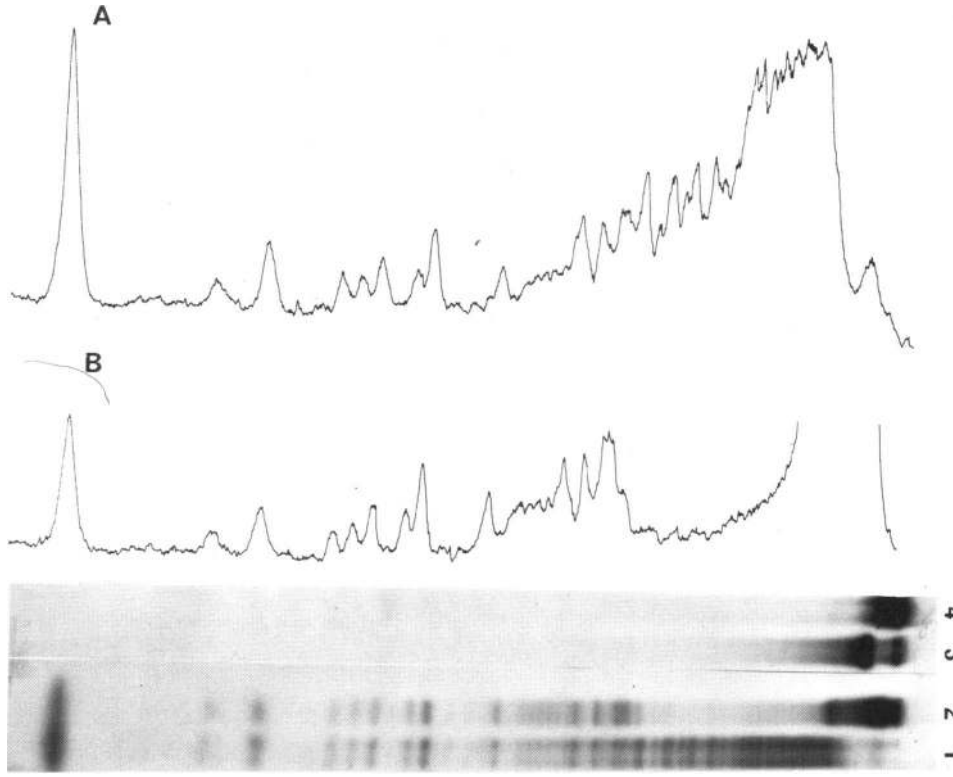

Figure 3B. Autoradiograms and microdensitometer tracings of partial Phy_1 nuclease digestion products of native and denatured RNA hybrids containing polyadenylated cRNA and end-labelled vRNA 8. The labelled oligonucleotides were separated by electrophoresis at 17 volts/cm for 14 hr. The gel lanes 1, 2, 3 and 4 contained in order: denatured and native hybrids + nuclease and denatured and native hybrids without nuclease. A. and B. are microdensitometer tracings of lanes 1 and 2 respectively.

RNA 8, complete protection from nuclease digestion is obtained in the hybrid molecules. Similar results were also obtained using RNAase T_1 and RNAase U_2.

Analyses of the 5'-terminal sequences of messenger RNAs transcribed in vitro. The activity of the virion transcriptase enzymes of influenza viruses are stimulated in vitro by dinucleoside phosphates such as GpG and ApG which are incorporated into the 5'-termini of the transcripts (14, 15). As a consequence the products of transcription present 5'-OH groups accessible to phosphorylation by polynucleotide kinase. The 5'-terminally-labelled transcripts prepared in this way using FPV and X-31 viruses are nuclease resistant since double-stranded molecules containing both genome and

TABLE 2. Nucleotide sequences near the 5'-termini of Fowl Plague virus RNAs 5, 6, 7 and 8 following which nuclease protection is observed in hybrids containing polyadenylated cRNA.

	23	25				30					35		
RNA 5	U	U	U	A	A	↓ U	U	G	U	C	A	C	A
RNA 6	A	A	C	A	U	↓ A	C	C	A	C	C	U	G
RNA 7	A	C	U	A	C	↓ A	G	C	U	A	U	A	
RNA 8	A	U	C	A	A	U	A	A	A	U	A	↓ A	G	C	U

The sequence data was obtained from experiments such as that shown in Fig. 3A and 3B. The arrows indicate the last nucleotide sensitive to nuclease in the hybrids and the numbers denote the residue number from the 5'-termini of the virion RNAs.

transcript RNAs are extracted from transcription reaction mixtures. These were purified by polyacrylamide gel electrophoresis (Figure 4) following nuclease S_1 digestion to remove 3'-terminal polyadenylic acid sequences

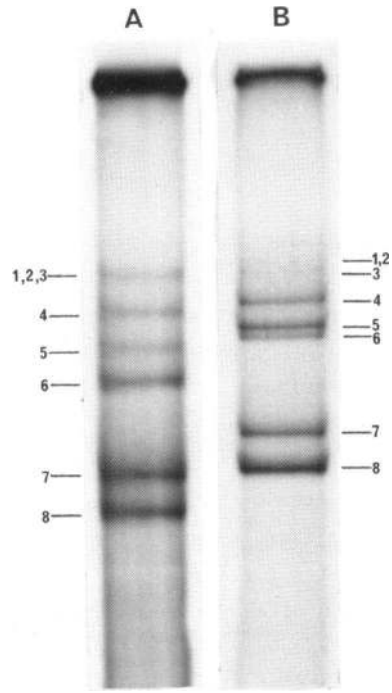

Figure 4. Autoradiogram of RNA hybrids containing 5'-terminal ^{32}P-labelled *in vitro* transcripts of fowl plague virus (A) and X-31 virus (B).

TABLE 3. 5'-terminal nucleotide sequences of in vitro transcripts of Fowl Plague and X-31 virus RNAs.

	1 5 10 15 20 25 30
FPV 1-3	A G C A A A A G C A G G
4	A G C A A A A G C A G G
5	A G C A A A A G C A G G
6	A G C A A A A G C A G G A G A A C A A A A U G A
7	A G C A A A A G C A G G U A G A U A U U G A A A G A U G A
8	A G C A A A A G C A G G G U G A C A A A A A C A U A A U G G
X-31 1+2	A G C A A A A G C A G G
3	A G C A A A A G C A G G
4	A G C A A A A G C A G G
5	A G C A A A A G C A G G
6	A G C A A A A G C A G G
7	A G C A A A A G C A G G
8	A G C A A A A G C A G G

and in addition the 5'-termini of vRNAs (3,8). Before sequence analysis the purified hybrids were heated at 100° for 2 min. The results obtained are presented in Table 3. Again conservation of sequence at the 5'-termini of these molecules was observed and residues 1-12 appear to be similar for all FPV and X-31 messenger RNAs.

DISCUSSION

The data presented here allow the conclusion that the 5'-terminal nucleotide seqeunces of influenza A and B virion RNAs are similar for the first twenty-three nucleotides. They also show that the 5'-termini of the RNA transcripts produced in vitro by the virion polymerases of FPV and X-31 virus are similar for the first twelve residues. The significance of these findings is not yet clear but since these sequences appear to be conserved throughout the influenza A viruses and similar sequences were detected in the influenza B virus it may be assumed that they have some important function in virus replication. For example, since they are on the one hand complements of the initiation sites for genome replication and on the other complements of the initiation sites for transcription, it may be that they or their complements are recognized specifically by replicase or transcriptase molecules. Observations of conservation of

sequence have been made in equivalent regions of the genome RNAs of brome mosaic virus although in this case conservation at the 3' termini is much more extensive, involving 161 nucleotides (16). In addition similarities in sequence of the first 11 nucleotides at the 5' termini of the N and M mRNAs of vesicular stomatitis virus have been reported (17) and six of the reovirus mRNAs also contain identical 5' terminal tetranucleotides (18).

Among the in vitro transcripts slightly more extensive analyses have so far been made of RNAs 6, 7 and 8 (Table 3) in which the first AUG triplets have been detected at residues 21-23 in transcript 6, 26-28 in transcript 7 and 27-29 in transcript 8. Further experiments are required to ascertain whether or not these are the sites of initiation of polypeptide synthesis. In comparisons of the sequences of these transcripts with the terminal sequences of their corresponding virus RNAs it is noteworthy (Table 4) that sequences complementary to the 5'-terminal variable regions between residues 14 and 16 of the vRNAs are present between residues 13 and 15 of the deduced 3'-terminal sequences of the homologous RNAs. The possibility, therefore, exists that interactions occur between the 5'- and 3'- terminal regions of vRNAs or their cRNA templates and that these are of significance in virus replication.

Concerning the relationship between the different complementary RNAs which are formed in virus-infected cells, the results of experiments involving treatment of RNA-RNA hybrids with nuclease S_1 have indicated

TABLE 4. Comparisons of the 5'-terminal and 3'-terminal nucleotide sequences of virus RNAs 6, 7 and 8.

		1				5					10					15	
RNA 6	5' -	A	G	U	A	G	A	A	A	U	U	A	G	G	A	G	A
	3' -	U	C	G	U	U	U	U	C	G	U	C	C	U	C	U	
RNA 7	5' -	A	G	U	A	G	A	A	A	U	U	A	G	G	U	A	G
	3' -	U	C	G	U	U	U	U	C	G	U	C	C	A	U	C	
RNA 8	5' -	A	G	U	A	G	A	A	A	U	U	A	G	G	G	U	G
	3' -	U	C	G	U	U	U	U	C	G	U	C	C	C	A	C	

The 3'-terminal sequences presented are simply the complements of the sequences presented in Table 3. Possible sites of base pairing are underlined.

that in hybrids containing non-polyadenylated cRNA both 5'- and 3'-terminal nucleotides of vRNA are insensitive to digestion but that in those which contain polyadenylated cRNA only the 3'-terminal nucleotides of vRNAs are protected (8, and unpublished). The results reported here in Figure 3 and Table 2 clearly indicate that messenger RNAs 5, 6 and 7 contain sequences complementary to their vRNAs down to residue 28 and similarly for mRNA 8 down to residue 34. Whether or not these results indicate the exact 3'-termini of the transcripts is not yet known but they suggest that the conserved sequences at the 5'-termini of virion RNAs are not represented in messenger RNA. More direct analysis are required to ascertain the precise site of termination, to determine whether or not the U-rich sequence between residues 17 and 23 in all influenza vRNAs is in fact transcribed and possibly to indicate details of the mechanism of complete and incomplete transcript formation.

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