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The Segments of Influenza Viral mRNA

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Influenza viral mRNA, i.e., complementary RNA (cRNA), isolated from infected cells, was resolved into six different species by electrophoresis in 2.1% acrylamide gels containing 6 M urea. The cRNA's were grouped into three size classes: L (large), M (medium-size), and S (small). Similarly, when gels were sliced for analysis, the virion RNA (vRNA) also distributed into six peaks because the three largest vRNA segments were closely spaced and were resolved only when the gels were autoradiographed or stained. Because of their attached polyadenylic acid [poly(A)]sequences, the cRNA segments migrated more slowly than did the corresponding vRNA segments during gel electrophoresis. After removal of the poly(A) by RNase H, the cRNA and vRNA segments comigrated, indicating that they were approximately the same size. One of the cRNA segments, S2, was shown by annealing to contain the genetic information in the vRNA segment with which it comigrated, strongly suggesting that each cRNA segment was transcribed from the vRNA segment of the same size. In contrast to the vRNA segments, which when isolated from virions were present in approximately 1:1 molar ratios, the segments of the isolated cRNA were present in unequal amounts, with the segments M2 and S2 predominating, suggesting that different amounts of the cRNA segments were synthesized in the infected cell. The predominant cRNA segments, M2 and S2, and also the S1 segment, were active as mRNA's in wheat germ extracts. The M2 cRNA was the mRNA for the nucleocapsid protein; S1 for the membrane protein; and S2 for the nonstructural protein NS_1.

The single-stranded RNA genome of influenza virus is segmented. Influenza A virus contains eight RNA segments that range in molecular weight from 2.5×10^5 to 1.0×10^6 (2, 20, 22, 24, 27). Each of these segments has a unique oligonucleotide pattern after T1 ribonuclease digestion (9, 20), and genetic recombination studies suggest that each virion RNA (vRNA) segment codes for a different virus-specific protein (22, 26, 27).

The viral mRNA is complementary to vRNA (4-6, 8, 15, 25). Influenza viral complementary RNA (cRNA), like other eucaryotic mRNA's, contains polyadenylic acid [poly(A)] sequences (4-6, 15) and various 5'-terminal 7-methyl guanosine cap structures (16). We report here that, like the vRNA from which it is transcribed, the viral cRNA is segmented. These cRNA segments have been characterized with respect to their number, size, relationship to the vRNA segments, and, in some cases, ability to code for individual virus-specific proteins in vitro.

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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 (12, 13). For the preparation of ^{32}P-labeled virus, carrier-free ^{32}P was present throughout infection.

Preparation of vRNA. ^{32}P-labeled virus was purified, and its RNA was extracted as described previously (12, 13).

Purification of viral cRNA. The procedures for obtaining from infected cells a preparation of viral cRNA in which all of the radiolabel is in viral cRNA have been described (6). Cordycepin (40 μg/ml) was added at 40 min postinfection, cycloheximide (100 μg/ml) and [^3H]adenosine or [^3H]uridine (20 μCi/ml) were added at 2.5 h postinfection, and the infected cells were collected 3 h later. The viral cRNA was purified from the cytoplasmic RNA as described previously (6).

Gel electrophoresis of viral RNA. The viral RNA preparation was heated and fast-cooled and subjected to gel electrophoresis on 2.1% acrylamide gels containing 6 M urea as described previously (15, 23, 24, 28).

Isolation of individual cRNA and vRNA seg-

ments. After electrophoresis, the gels from which RNA segments were to be eluted were cut into 2-mm slices, and one-fourth of each slice was processed for counting to identify the slice(s) containing the desired segment(s). The remaining three-fourths of the gel slices containing the cRNA or vRNA segments was eluted by electrophoresis, and the eluted RNA was extracted with phenol chloroform and ether as described previously (16). After ethanol precipitation, the RNA segments were further purified by column chromatography. For the poly(A)-containing cRNA segments, oligodeoxythymidylic acid [oligo(dT)]cellulose was employed. For the vRNA segments, CF-11 cellulose was employed, and the vRNA was eluted in STE buffer (0.1 M NaCl-0.001 M EDTA-0.05 M Tris-hydrochloride, pH 7.0) containing 15% ethanol (7). The cRNA or vRNA segments obtained by column chromatography were ethanol precipitated.

Cell-free protein synthesis. Assays using wheat germ extracts were carried out as described previously (6), except 0.5 mM spermidine was added, and the Mg^{2+} concentration was reduced from 3.5 to 2.0 mM (1). The [^{35}S]methionine-labeled protein products were analyzed on cylindrical 7.5% polyacrylamide gels as described previously (14), using as internal markers either [*methyl*-^{3}H]methionine-labeled virion proteins or [*methyl*-^{3}H]methionine-labeled NS_1 protein purified infected-cell nucleoli by agarose chromatography in 6 M guanidine hydrochloride (13).

Tryptic peptide analysis. The [^{35}S]methionine-labeled proteins synthesized in vitro were subjected to gel electrophoresis, and the appropriate protein was eluted from the gel, using as eluting buffer 2% sodium dodecyl sulfate-0.01 M Tris-hydrochloride, pH 7.4. The authentic virus-specific proteins, labeled with [*methyl*-^{3}H]methionine, were eluted from gels on which sodium dodecyl sulfate-disrupted virions or sodium dodecyl sulfate-disrupted infected-cell nucleoli were electrophoresed. For the preparation of labeled membrane protein, virus was grown in medium containing 0.2% bovine serum albumin rather than serum to minimize the cleavage of the hemagglutinin protein (HA) to HA_1 and HA_2 (19), since the latter protein migrates close to the membrane protein during gel electrophoresis. After elution, the in vitro and authentic virus-specific protein were mixed and precipitated with ethanol after the addition of 500 μg of bovine serum albumin as carrier. The proteins were digested with trypsin, and the resulting tryptic peptides were subjected to chromatographic analysis on a PA 35 cation exchange resin, using a concave gradient of pH and pyridine-acetate, as described previously (3).

Digestion of viral cRNA by RNase H. The conditions of digestion were essentially as described by Stavrianopoulos et al. (30, 31). Radiolabeled cRNA (unfractionated or individual segments) was hybridized for 15 min at 22°C with 25 μg of oligo(dT) in 0.150 ml containing 0.1 M KCl, 0.010 M $Mg Cl_2$, and 0.05 M Tris-hydrochloride, pH 8.0. A 10-μl amount of a solution containing 0.2 U of RNase H in 0.05 M Tris-hydrochloride, pH 7.5, 0.4 M NaCl, 0.03 M $Mg Cl_2$, 1 mm dithiothreitol, and 50% glycerol was added, and the mixture was incubated for an additional 20 min at 22°C. The reaction mixtures were then extracted three times with phenol chloroform (1:1) at pH 9.0, and the RNA was ethanol precipitated for analysis by gel electrophoresis.

RNA annealing. The procedures were essentially as described previously (12, 23). Annealing was carried out in a volume of 50 μl, overlaid with 0.2 ml of mineral oil, for 14 h at 68°C.

Materials. [2,8-^{3}H]adenosine, [5-^{3}H]uridine, [^{35}S]methionine, L-[*methyl*-^{3}H]methionine, and carrier-free ^{32}P were purchased from New England Nuclear Corp., Boston, Mass. Oligo(dT) cellulose, and CF-11 cellulose were obtained from Collaborative Research, Inc., Waltham, Mass., and Whatman Co., England, respectively. Purified RNase H was generously supplied by Janus Stavrianopoulos.

RESULTS

Gel pattern of influenza viral cRNA (mRNA). ^{32}P-labeled vRNA and ^{3}H-labeled cRNA were coelectrophoresed on a cylindrical 2.1% acrylamide gel containing 6 M urea (Fig. 1). The vRNA was resolved into six distinct RNA peaks, ranging in estimated molecular weight from 2.5×10^5 to 1.0×10^6 (15, 23). In gels that were stained or autoradiographed rather than sliced, the vRNA peak of largest size was resolved into three bands, indicating the presence of eight vRNA segments as shown previously (2, 20, 22-24, 27). The vRNA segments were numbered from 1 to 8 as shown in Fig. 1. Based on the amount of radioactivity in each peak, the vRNA segments were present in approximately a 1:1 molar ratio to each other.

Influenza viral cRNA migrated in the same molecular weight range as vRNA and was also distributed into six peaks. The cRNA segments were designated L for the largest segment(s), M1, M2, and M3 for the three middle-size segments, and S1 and S2 for the two smallest segments. Each cRNA peak migrated more slowly

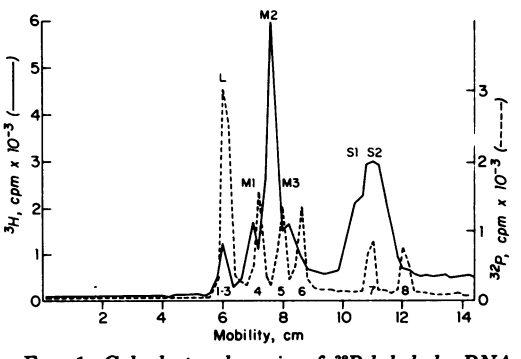

FIG. 1. *Gel electrophoresis of ^{32}P-labeled vRNA and ^{3}H-labeled cRNA. The preparation of ^{32}P-labeled vRNA and [^{3}H]adenosine-labeled cRNA and the gel electrophoresis were as described in the text.*

than did the presumed corresponding vRNA segment. In Fig. 1, cRNA peak L and vRNA peak (1→3) appeared to comigrate but, when the gels were run for longer times, cRNA peak L migrated about 2 mm slower than did vRNA peak (1→3). The cRNA peaks M1, M2, and M3 migrated 3 to 4 mm slower than did vRNA peaks 4, 5, and 6, respectively. The cRNA peaks of lowest molecular weight, S1 and S2, exhibited the largest difference in mobility from their presumed corresponding vRNA segments, 7 and 8. In addition, these two cRNA peaks, particularly S2, were not as sharp as the other cRNA or vRNA peaks, suggesting some heterogeneity in the S1 and S2 cRNA species. As will be shown later, the difference in mobility between the cRNA and vRNA segments and the apparent heterogeneity in the smallest cRNA segments was due to the poly(A) sequences in cRNA that were not found in vRNA.

It should also be noted that the cRNA segments were not present in a 1:1 molar ratio to each other. With the labeling conditions employed, the M2 and S2 cRNA segments were the predominant radiolabeled species.

Relationship between the cRNA and vRNA segments. To determine whether the difference in mobility in gels between the cRNA and vRNA segments is due to the poly(A) sequences in cRNA, the poly(A) in viral cRNA was hybridized to oligo(dT) and was then removed by digestion with RNase H from calf thymus (33, 34). Control experiments indicated that this treatment removed most, if not all, of the poly(A) and caused little, or no, hydrolysis of nonpoly(A) regions of the cRNA. Thus, as a result of the RNase H digestion, 20 to 30% of [^{3}H]adenosine-labeled cRNA was rendered acid soluble, and its resistance to pancreatic and T1 RNases was reduced from 30 to 5%. In contrast, with [^{3}H]uridine-labeled cRNA, no detectable radiolabeled RNA was rendered acid-soluble as a result of RNase H digestion. After removal of poly(A) by RNase H, the cRNA and vRNA segments comigrated during gel electrophoresis (Fig. 2), indicating that the cRNA segments without their poly(A) sequences were approximately the same size as the vRNA segments.

Based on the amount of radiolabeled RNA in each of the cRNA peaks before and after RNase H digestion, it is evident that the M2, S1, and S2 cRNA segments, after removal of their poly(A) sequences, comigrated with vRNA segments 5, 7, and 8, respectively. This was confirmed by isolating these three cRNA segments and treating them individually with RNase H (Fig. 3). These results indicate that the M2, S1, and S2 cRNA corresponded in mobility and size

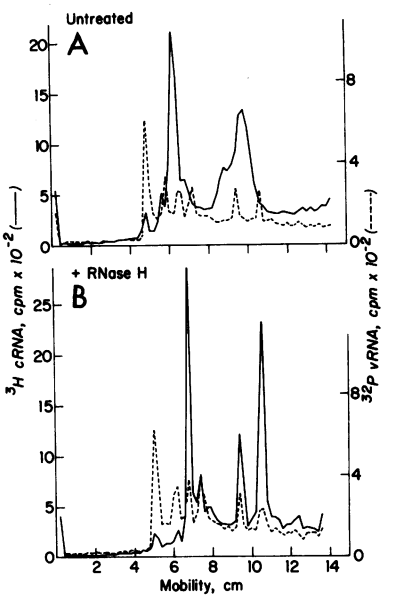

FIG. 2. *Gel electrophoresis of ^{3}H-labeled cRNA before (A) and after (B) RNase H treatment. The RNase H treatment and gel electrophoresis were as described in the text. The untreated and RNase H-treated ^{3}H-labeled cRNA were mixed with marker ^{32}P-labeled vRNA just prior to the electrophoresis.*

to the vRNA segments 5, 7, and 8, respectively.

As further evidence of the correspondence between comigrating cRNA and vRNA segments, a large amount of the S2 cRNA segment (not treated with RNase H) was annealed to small amounts of ^{32}P-labeled vRNA segments 7 and 8 (Table 1). Although the amount (in micrograms) of the S2 cRNA is unknown because of the presence of unlabeled host RNA in the cRNA preparation (6), the annealing results indicate that the cRNA was present in excess relative to the vRNA. The S2 cRNA segment protected vRNA segment 8 almost completely. Conceivably, even greater protection of this vRNA would have been obtained with larger amounts of the cRNA and/or longer annealing times. The small amount of protection of vRNA segment 7 was presumably due to the small amount of S1 cRNA contaminating the S2 cRNA preparation (Fig. 3). These results indicate that the S2 cRNA segment has the genetic information in vRNA segment 8.

Activity of the cRNA segments in wheat germ extracts. Individual cRNA segments (not RNase H treated) were tested for their ability to code for virus-specific proteins in wheat germ extracts. The largest stimulation of [^{35}S]-methionine incorporation, 10- to 15-fold, was obtained with the M2 and S2 segments, the

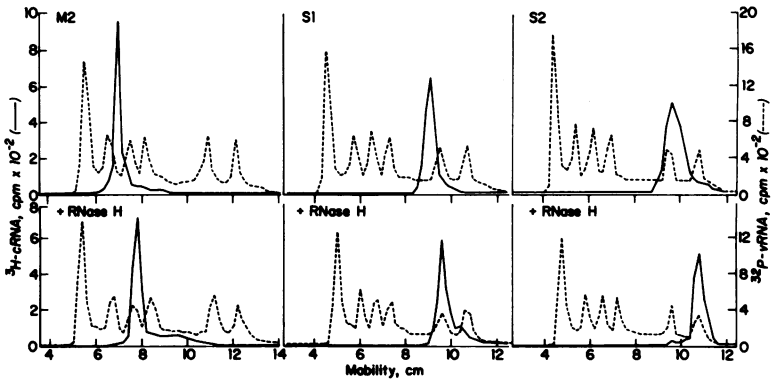

FIG. 3. *Gel electrophoresis of the M2, S1, and S2 cRNA segments before and after RNase H treatment.* ^{32}P-*labeled vRNA was added as marker just prior to electrophoresis.*

TABLE 1. *Hybridization between cRNA and vRNA segments*[a]

^{32}P-labeled vRNA segment	^{3}H-labeled cRNA segment	% RNase resistance after annealing with:	
		^{32}P-labeled vRNA	^{3}H-labeled cRNA
8	–	6	–
–	S2	–	7
8	S2	88	11
7	–	8	–
7	S2	18	7

[a] Where indicated, the amount of RNA added to an annealing mixture was: [^{3}H]uridine-labeled cRNA segment S2, 5,000 cpm; ^{32}P-labeled vRNA segment 7, 6,000 cpm (50,000 cpm/μg); ^{32}P-labeled vRNA segment 8, 4,500 cpm (40,000 cpm/μg).

ones present in largest amount in the cRNA preparation.

The predominant species of protein synthesized in vitro in response to M2 cRNA comigrated with authentic NP protein during gel electrophoresis (Fig. 4). The heterogeneous in vitro products of smaller size presumably resulted from premature termination which occurred in the wheat germ system. The tryptic peptides of the [^{35}S]methionine-labeled, in vitro protein comigrating with NP were identical to those of [*methyl*-^{3}H]methionine-labeled NP isolated from virus (Fig. 5). Thus, we conclude that the M2 cRNA segment codes for the NP protein.

The S2 cRNA segment coded for a protein that comigrated with authentic NS_1 protein during gel electrophoresis (Fig. 6B) and that migrated ahead of the virion MP protein (Fig. 6A). Tryptic peptide analysis confirmed that this in vitro protein is NS_1 and not MP (Fig. 7). Thus, the tryptic peptides of the in vitro protein and authentic NS_1 protein co-chromatograph on the PA 35 cation-exchange resin (Fig. 7A).

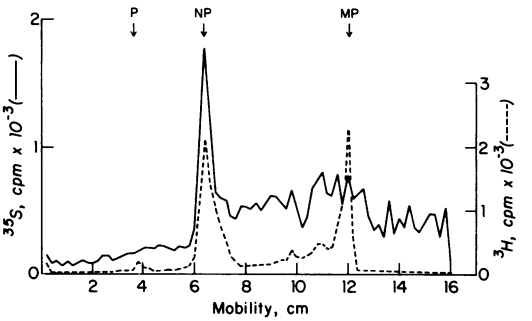

FIG. 4. *Polyacrylamide gel electrophoresis analysis of the proteins synthesized in a wheat germ extract programmed by the M2 cRNA segment. The in vitro product is labeled with [*^{35}S*]methionine and the marker virion proteins are labeled with [methyl-*^{3}H*]methionine (the HA and NA virion proteins are only minimally labeled with this precursor). Experimental details were as described in the text.*

Some variations in the chromatographic pattern of NS_1 were observed: the peak eluting around fraction 20 at times broke into two peaks, and the ratios among other peaks varied from run to run (cf. the NS_1 pattern in Fig. 7A and B). These variations probably reflect the unique properties of the NS_1 protein: it is very "sticky" and tends to aggregate (14). It is to be emphasized, however, that regardless of these variations, the chromatographic profiles of the tryptic peptides of this in vitro protein and of authentic NS_1 protein were always the same within a given column run. In contrast, no discernible correspondence between the chromatographic profile of the tryptic peptides of this in vitro protein and those of authentic MP protein was evident (Fig. 7B). Therefore, the S2 cRNA segment codes for the NS_1 protein.

Only relatively small amounts of the S1 cRNA segment free of the S2 segment could be purified and, with these amounts of S1 cRNA,

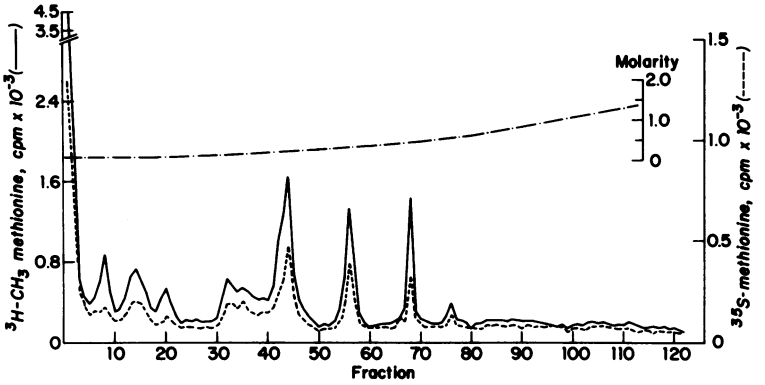

FIG. 5. *Comparison of the tryptic peptide map of authentic NP protein (labeled with [methyl-^{3}H]methionine) with the map of the protein labeled with [^{35}S]methionine which is synthesized in response to M2 cRNA and which co-migrates with NP protein (cf. Fig. 4). The mixture of these two proteins was digested with trypsin and analyzed on a PA 35 cation exchanger as described in the text.*

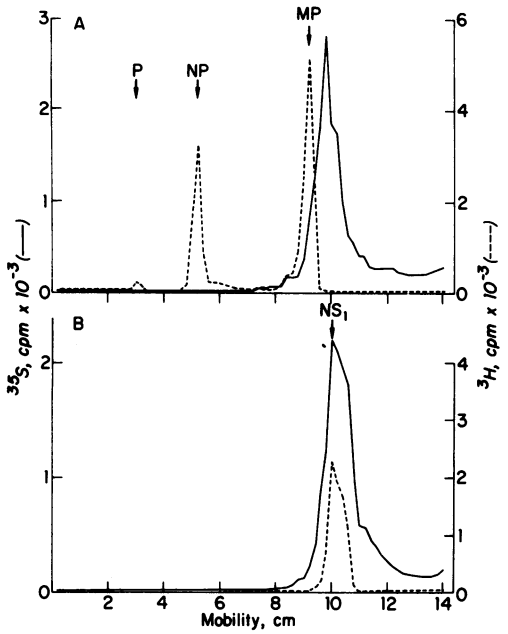

FIG. 6. *Polyacrylamide gel electrophoresis analysis of the proteins synthesized in a wheat germ extract programmed by the S2 cRNA segment. Experimental details were as described in the text. (A) Marker [^{3}H]methionine-labeled virion proteins. (B) Marker [^{3}H]methionine-labeled NS_1 protein.*

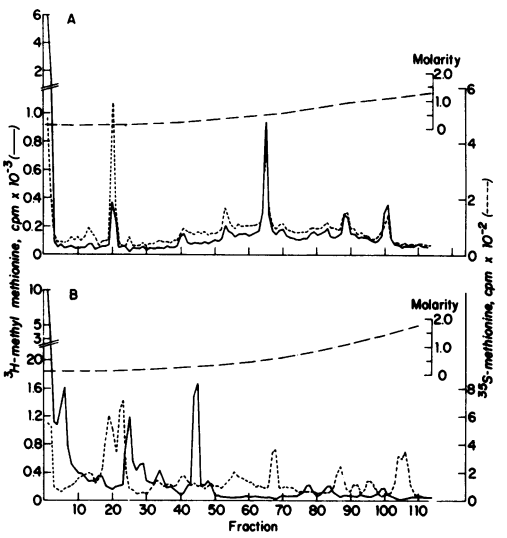

FIG. 7. *Comparison of the tryptic peptide map of the protein ([^{35}S]methionine-labeled) synthesized in vitro in response to S2 cRNA (cf. Fig. 6) with the map of [^{3}H]methionine-labeled authentic NS_1 protein (A) or authentic MP protein (B). Experimental details were as described in the text.*

the stimulation of [^{35}S]methionine incorporation catalyzed by wheat germ extracts was only two- to threefold. The product made in response to S1 cRNA consisted predominantly of a protein that comigrated with the virion MP protein during gel electrophoresis (Fig. 8). Also present were lesser amounts of heterogeneous proteins that migrated similarly to the proteins synthesized by the wheat germ system without the addition of exogenous mRNA. We were not able to obtain sufficient amounts of the in vitro protein comigrating with MP protein for tryptic peptide analysis. These results strongly suggest that the S1 cRNA segment codes for the MP protein.

Insufficient amounts of the L, M1, and M3 cRNA segments were obtained so that little, or no, stimulation of [^{35}S]methionine incorporation in wheat germ extracts was observed.

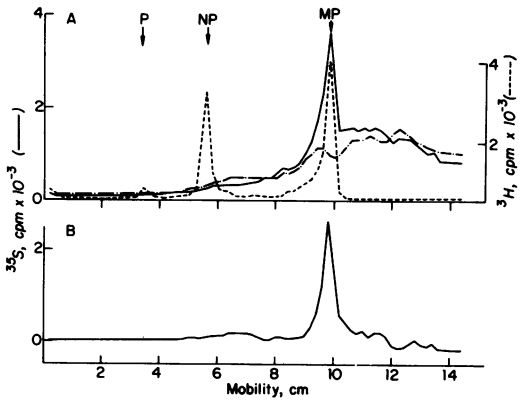

FIG. 8. *Polyacrylamide gel electrophoresis analysis of the proteins synthesized by a wheat germ extract with the S1 cRNA segment as mRNA. (A) Gel pattern of the S1 cRNA-stimulated product (——) and of the product made in the absence of added mRNA (endogenous reaction) (·——·). [³H]methionine-labeled virion proteins serve as markers. (B) Protein pattern obtained when the endogenous pattern is subtracted from the pattern obtained in the S1 cRNA-stimulated reaction.*

DISCUSSION

The present results show that both the vRNA and cRNA of influenza virus distributed into six peaks during electrophoresis in 2.1% acrylamide gels containing 6 M urea. When the gels were stained or autoradiographed, the largest vRNA species was resolved into three bands, resulting in a total of eight vRNA segments (2, 20, 22–24, 27). If the largest cRNA peak were also composed of three closely spaced segments, then cRNA would also be comprised of eight segments. The cRNA segments migrate more slowly than the corresponding vRNA segments. Here we have shown that this slower mobility was due to the poly(A) sequences in cRNA since, after poly(A) removal, the cRNA and vRNA segments comigrated. For one of the cRNA segments, S2, we showed by annealing that it is complementary to its comigrating vRNA segment 8, strongly suggesting that each cRNA segment is transcribed from the vRNA segment of the same size.

The cRNA segments M2, S1, and S2 are the mRNA's for the proteins NP, MP, and NS_1, respectively, in wheat germ extracts. This indicates that the corresponding vRNA segments, namely 5, 7, and 8 code for the NP, MP, and NS_1 proteins, respectively. Only small amounts of the cRNA segments L, M1, and M3 were synthesized in the infected cells, and translation of these cRNA's in wheat germ extracts was not detected. Therefore, the definitive identification of the mRNA's for the P, HA, and neuraminidase (NA) proteins remains open. Evidence obtained by recombination studies with PR8 virus, a virus of the same HA-NA group as WSN, are in line with the coding assignments of vRNA segments 5, 7, and 8 obtained in the present study and also suggest that the vRNA segments (1 → 3), 4, and 6 code for the P, HA, and NA proteins, respectively (22, 26).

The observation in the present study that the isolated cRNA segments are unequally radiolabeled strongly suggests that they are synthesized in unequal amounts and that control at the transcriptional level occurs in the infected cell. It was shown previously that the rate of synthesis of the various virus-specific proteins is controlled during infection (10, 11, 17, 18, 21, 29), but these earlier studies could not definitively distinguish between control at the transcriptional or at the translational level. In the infected cell, NP and NS_1 are the proteins synthesized in largest amounts (10, 11, 14, 17, 18, 21, 29), and we have obtained evidence that the cRNA segments coding for these two proteins, segments M2 and S2, are also synthesized in largest amounts, suggesting that control is at the level of transcription. However, it must be emphasized that the pattern of synthesis of the different cRNA segments observed in the present study may not be completely representative, since the cRNA was obtained from infected cells treated with the protein synthesis inhibitor cycloheximide. Clearly, analysis of the rates of synthesis of the different cRNA segments at various times after infection in the absence of cycloheximide is required.

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