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# Purification of Influenza Viral Complementary RNA: Its Genetic Content and Activity in Wheat Germ Cell-Free Extracts

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Influenza viral complementary RNA (cRNA) was purified free from any detectable virion-type RNA (vRNA), and its genetic content and activity in wheat germ cell-free extracts were examined. After phenol-chloroform extraction of cytoplasmic fractions from infected cells, poly(A)-containing viral cRNA is found in two forms: in single-stranded RNA and associated with vRNA in partially and fully double-stranded RNA. To purify single-stranded cRNA free of these double-stranded forms, it was necessary to employ, as starting material, RNA fractions in which cRNA was predominantly single stranded. Two RNA fractions were successfully employed as starting material: polyribosomal RNA and the total cytoplasmic RNA from infected cells treated with 100 $\mu$g of cycloheximide (CM) per ml at 3 h after infection. In WSN virus-infected canine kidney (MDCK) cells, the addition of CM at 3 h after infection stimulates the production of cRNA threefold and causes a very large increase in the proportion of the cytoplasmic cRNA which is single stranded; double-stranded RNA forms are greatly reduced in amount. Total cRNA was obtained by oligo(dT)-cellulose chromatography, and single-stranded cRNA was separated from double-stranded forms by Sepharose 4B chromatography. The cRNA preparation purified from polyribosomes consists of 95% single-stranded cRNA, with the remaining 5% apparently being double-stranded RNA forms. The cRNA preparation purified from CM-treated cells (CM cRNA) is even more pure: 100% of the radiolabeled RNA is single-stranded cRNA. Annealing experiments, in which a limited amount of $^{32}$P-labeled genome RNA was annealed to the cRNA, indicate that the purified cRNA contains at least 84 to 90% of the genetic information in the vRNA genome. Purified viral cRNA (CM cRNA) is very active in directing the synthesis of virus-specific proteins in wheat germ cell-free extracts. Four of the proteins synthesized in vitro co-migrate during gel electrophoresis with the four major nonglycosylated virus-specific proteins, and three of these proteins have been identified after specific immunoprecipitation. The protein product also contains candidates for the protein moieties of the two virion glycoproteins. These results thus provide further strong evidence that cRNA constitutes the entirety of influenza virus-specific mRNA. On this basis, the activity of viral RNA preparations in in vitro protein synthesis was re-examined. At relatively low potassium chloride concentrations, viral RNA preparations direct the synthesis of very small polypeptides. This activity is most likely due to small cRNA transcripts incorporated into virus.

Previous work from this laboratory strongly suggests that influenza viral complementary RNA (RNA complementary to virion RNA) constitutes the entirety of viral mRNA. Complementary RNA (cRNA) is essentially the only virus-specific RNA found on infected cell polyribosomes; and cRNA contains polyadenylate [poly(A)], whereas virion RNA (vRNA) does not (9). This is consistent with the fact that the virion contains a transcriptase (3, 7, 21, 32). Conclusive proof that cRNA constitutes the entirety of viral mRNA requires the demonstration that cRNA directs the synthesis of all virus-specific proteins in an in vitro protein-synthesizing system.

Previous studies of the in vitro translation of influenza virus-specific RNA have been ambiguous. Kingsbury and Webster (11) found that infected cell RNA was active in cell-free rabbit reticulocyte extracts for the synthesis of the viral membrane protein (MP) and of another protein which was immunoprecipitated but did

not co-migrate with known virus-specific proteins during polyacrylamide gel electrophoresis (PAGE). Because these workers found that vRNA was inactive, they postulated that cRNA was the active mRNA but did not directly prove this. Another group of workers also found vRNA to be inactive in cell-free extracts, in this case obtained from mouse ascites cells (10). However, Siegert et al. (30) reported that vRNA was active in an *Escherichia coli* cell-free system and presented some immunological evidence that one of the proteins synthesized was the viral nucleocapsid protein (NP). Whether the vRNA preparation was pure and did not contain cRNA was not investigated.

In the present study, our approach was to purify relatively large amounts of cRNA free from any detectable vRNA. Once the purity of the cRNA was established, we determined (i) whether this cRNA contains all the genetic information in vRNA and (ii) whether this cRNA directs the synthesis of virus-specific proteins in a wheat germ cell-free protein-synthesizing system. Our results provide further strong evidence that cRNA constitutes the entirety of the viral mRNA. In light of these results, we re-examined the activity of vRNA preparations in vitro; our observations will be discussed.

## MATERIALS AND METHODS

**Cells and virus.** The procedures for culture of the MDCK (canine kidney) cell line, and for growth and purification of WSN virus, have been described (12, 13).

**Preparation of infected cell extracts.** The procedure for infection of MDCK cells at a multiplicity of 30 to 60 plaque-forming units/cell was described previously (12–14). Approximately $8 \times 10^8$ cells grown on roller bottles were used for each experiment. Forty micrograms of cordycepin per ml was added after viral adsorption (40 min after infection at 37 C) (9). At 1.0 h after infection, [$^3$H]adenosine or [$^3$H]uridine was added to a final concentration of 20 $\mu$Ci/ml. At the times of infection indicated, the cells were collected, and cytoplasmic extracts were prepared as previously described (12–14). Where indicated, polyribosomes were isolated from cytoplasmic extracts as previously described (9).

In many experiments, infected cells were treated with cycloheximide (CM). At 3 h after infection, CM at a final concentration of 100 $\mu$g/ml was added. The cells were incubated for an additional 2.5 to 3.0 h at 37 C before collection.

**Purification of viral cRNA.** The cytoplasmic extract or polyribosome preparation was made 0.02 M in EDTA, 2% in sodium dodecyl sulfate, and 0.1 M in Tris-hydrochloride, pH 9.0. The RNA was deproteinized by extracting three times with a 1:1 mixture of phenol and chloroform equilibrated with 0.1 M Tris-hydrochloride, pH 9.0 (9), and the extracted RNA was precipitated with three volumes of (−25 C) ethanol. After 3 h at −25 C, the RNA was collected by centrifugation for 20 min at 30,000 × *g* in the Sorvall RC2-B centrifuge. The pellet was taken up in 2.5 ml of 0.5 M KCl and 0.01 M Tris-hydrochloride, pH 7.4 (buffer A) and was applied to an oligodeoxythymidylic acid [oligo(dT)]-cellulose column (4 by 1.5 cm) at room temperature. Two-milliliter fractions were collected. To determine the amount of radioactivity in each fraction, a 25-$\mu$l aliquot was spotted onto a membrane filter (Millipore Corp.) and the filter was dried and counted in a toluene scintillation mixture. The column was washed with buffer A until no further radiolabeled RNA was detectable in the effluent. The RNA bound to the column was eluted with 0.01 M Tris-hydrochloride, pH 7.4. The fractions containing the eluted RNA were pooled and were made 0.01 M in $MgCl_2$; rRNA was added as carrier, and the RNA was precipitated with four volumes of (−25 C) ethanol.

After 24 h at −25 C, the RNA was collected by centrifugation for 1 h at 25,000 rpm in the SW41 rotor. The pellet was taken up in 0.5 ml of Sepharose buffer (0.1 M NaCl; 0.002 M EDTA; 0.5% sodium dodceyl sulfate; 0.01 M Tris-hydrochloride, pH 7.4), and the solubilized RNA was applied to a Sepharose 4B (70 by 2 cm) column at room temperature (2). The column was run with a 25-cm pressure head, resulting in a flow rate of 4 ml/h. Fractions of 1.2 to 1.5 ml were collected, and the amount of radioactivity in each fraction was determined by counting an aliquot (100 $\mu$l) of each fraction in a Triton-toluene scintillation mixture. Fractions were pooled as indicated in each figure, and the RNA in the pooled fractions was collected after ethanol precipitation as described above.

**Annealing experiments.** RNA annealing was carried out as described previously (13). For the determination of the total cRNA produced during infection, infected cell cytoplasmic RNA was annealed to an excess of [$^3$H]adenosine-labeled vRNA, and the amount of vRNA rendered RNase resistant was determined (13, 16).

**Cell-free extract.** Wheat germ cell-free extracts were prepared essentially according to Roberts and Paterson (25). Extracts were not preincubated and were stored at −90 C. Aliquots were thawed just before use.

**Cell-free protein synthesis.** Assays contained in a final volume of 50 $\mu$l: 20 $\mu$l of non-preincubated wheat germ S30 fraction; 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2′-ethanesulfonic acid buffer, pH 7.3; 2 mM dithiothreitol; 1 mM ATP (K salt); 20 $\mu$M GTP (Tris salt); 4.5 mM creatine phosphate (Tris salt); 40 $\mu$g of creatine phosphokinase per ml; 80 $\mu$M of unlabeled amino acids; 3 mM $MgAc_2$; KCl as indicated; 250 $\mu$Ci of [$^{35}$S]methionine; and 5 to 7 $\mu$l of the RNA being tested for activity. Assays were incubated for 1 h at 25 C. To determine the amount of protein synthesized, a 10-$\mu$l aliquot was added to 5 ml of 5% trichloroacetic acid containing 3% Casamino Acids. After boiling for 15 min, the mixture was filtered through a membrane filter (Millipore Corp.). These filters were then counted in a toluene-based scintillation fluid.

**PAGE.** Reaction mixtures which were to be directly analyzed by PAGE were incubated with 0.1 M KOH for 15 min at 37 C and were precipitated with an equal volume of 10% trichloroacetic acid containing 3% Casamino Acids. The precipitates were washed three times with 5% trichloroacetic acid containing 3% Casamino Acids and two times with acetone (19, 20). The washed pellets were taken up in 0.01 M sodium phosphate, pH 7.1, 1% sodium dodecyl sulfate, 2% β-mercaptoethanol, 0.5 M urea, and $10^{-2}$ M EDTA and were boiled for 3 min. The samples were analyzed on cylindrical sodium dodecyl sulfate-containing 7.5% polyacrylamide gels as described previously (14). To serve as internal protein markers, $^3$H-amino acid-labeled virion proteins, or $^3$H-amino acid-labeled infected cell nucleolar proteins, were always coelectrophoresed with the samples from the reaction mixture. The nucleolar preparation contains the virion proteins P and NP, the putative nonstructural protein $NS_2$, and a virus-specific protein of approximately 25,000 daltons. Because the latter protein consistently migrates ahead of the virion MP protein during PAGE (14, 15), it has been designated $NS_1$ (nonstructural protein 1) distinct from MP. Little or no protein comigrating with MP is detectable in the nucleolar preparation (14, 15). As shown in the present study, MP and $NS_1$ can also be distinguished immunologically. For the preparation of infected cell nucleoli, infected MDCK cells were exposed to a mixture of $^3$H-labeled amino acids from 3.75 to 5 h postinfection, and the crude nucleolar pellet was obtained as previously described (14, 15).

**Antisera.** For the preparation of antiserum against viral structural proteins, purified virus was pelleted by centrifugation for 1 h at 35,000 rpm in the SW41 rotor. The pellet was taken up in 8 M guanidine hydrochloride (GuHCl), 2% β-mercaptoethanol, 0.1 M Tris-hydrochloride, pH 8.5, and was boiled for 5 min. The GuHCl was removed by dialysis against 0.0025 M Tris-hydrochloride, pH 7.4, and the mixture was lyophilized to dryness. The lyophilized protein was taken up in 8 ml of water for injection into rabbits. One-half milliliter was injected intravenously, and 1.5 ml was mixed with an equal volume of complete Freund adjuvant and was injected into the footpads and subcutaneously into four sites along the back of the animal. Boosters, consisting of 2 ml of the antigen diluted with an equal volume of Freund adjuvant, were injected subcutaneously 4 and 8 weeks after the initial injections. The animals were bled from the ear 10 to 14 days after the second booster injection. In immunodiffusion, the antiserum formed multiple precipitin lines when tested against GuHCl-disrupted virus.

For the preparation of an antiserum which would be directed against nonstructural virus-specific proteins (in particular $NS_1$), infected cell nucleoli were used as the antigen. Crude nucleoli were isolated from infected MDCK cells at 5 h after infection as previously described (14, 15). The nucleolar pellet was disrupted with GuHCl and injected into rabbits as described above, except that an intraperitoneal injection was substituted for the intravenous injection because of the particulate nature of the antigen. In immunodiffusion, this antiserum formed several precipitin lines when tested against GuHCl-disrupted infected cell nucleoli and against GuHCl-disrupted virus.

Preimmune serum was obtained from the rabbits prior to inoculation with GuHCl-disrupted virus or GuHCl-disrupted infected cell nucleoli. This serum, which did not form any precipitin lines against either GuHCl-disrupted virus or GuHCl-disrupted infected cell nucleoli, was used as a control for nonspecific precipitation.

The virus-specific proteins detected by the two antisera were determined by immunoprecipitation as described below.

**Immunoprecipitation.** The procedure for immunoprecipitation was essentially as described by Oberg et al. (20) and was employed for two purposes: (i) to determine which virus-specific proteins the antisera are directed against and (ii) to demonstrate the viral specificity of the in vitro synthesized proteins. To characterize the antisera, infected cell cytoplasmic extracts containing radiolabeled virus-specific proteins were precipitated with the two antisera. The cytoplasmic extracts were prepared from infected cells labeled with [$^{35}$S]methionine from 3.75 to 5.0 h after infection.

Cytoplasmic extracts or cell-free translation mixtures were incubated at 37 C for 30 min with 100 μg of pancreatic RNase per ml in the presence of 0.1 M EDTA and were dialyzed overnight at 4 C against 7 M urea-1 mM dithiothreitol in phosphate-buffered saline lacking $Mg^{2+}$ and $Ca^{2+}$ (phosphate-buffered saline deficient). After dialysis, the solution was centrifuged at 16,000 × *g* for 10 min. The resulting supernatant was diluted with phosphate-buffered saline deficient to 1 M urea, was made 1% in Triton X-100, and was centrifuged again at 16,000 × *g* for 10 min. To 1 ml of this supernatant was added 20 μl of the appropriate antiserum or preimmune serum, and the mixture was incubated at 30 C for 1 h and overnight at 4 C. All samples then received 300 μl of goat anti-rabbit immunoglobulin G, and the mixture was incubated for 1 h at 30 C and 2 to 3 h at 4 C. The immunoprecipitates were collected and washed as described by Oberg et al. (20) and were analyzed by PAGE.

**Materials.** For the preparation of ribosomal carrier RNA, cytoplasmic extracts of uninfected MDCK cells were phenol extracted, and the 28*S* and 18*S* rRNA was collected after sucrose density gradient centrifugation. [2,8-$^3$H]adenosine, [5-$^3$H]uridine, and [$^{35}$S]methionine (100 to 400 Ci/mmol) were purchased from New England Nuclear Corporation, Boston, Mass. Oligo(dT)-cellulose was obtained from Collaborative Research Inc., Waltham, Mass. Sepharose 4B was purchased from Pharmacia Fine Chemicals Inc., Piscataway, N.J. Goat anti-rabbit immunoglobulin G was obtained from Hyland Laboratories, Costa Mesa, Calif., and was clarified by centrifugation at 1,000 × *g* for 30 min.

## RESULTS

**Purification of viral cRNA.** Influenza viral cRNA contains poly(A), and the synthesis of

this poly(A) is resistant to the drug cordycepin (9), which inhibits the synthesis of host-specific RNA containing poly(A) (1). In contrast to cRNA, influenza vRNA does not contain poly(A) (9). Consequently, oligo(dT)-cellulose chromatography or other methods which select RNAs by virtue of their poly(A) segments should separate single-stranded cRNA from single-stranded vRNA and from all host cytoplasmic RNAs made in the presence of cordycepin.

However, oligo(dT)-cellulose chromatography of total infected cell cytoplasmic RNA does not yield a preparation of viral cRNA free of vRNA, for after phenol-chloroform extraction of the cytoplasmic extract most of the cRNA is found associated with vRNA in partially and fully double-stranded RNA forms. The extent to which the cRNA in cytoplasmic extracts is in double-stranded forms is revealed by Sepharose 4B chromatography, a procedure known to separate single-stranded RNA from RNA with double-stranded regions (2, 8). Figure 1 shows the Sepharose 4B chromatography of the oligo(dT)-cellulose-bound RNA obtained from the cytoplasmic extract of infected cells labeled with [$^3$H]adenosine from 1 to 3.75 h after infection. The majority of the radiolabeled RNA elutes close to the excluded volume (region A). Consistent with the behavior of other RNAs subjected to Sepharose 4B chromatography (2, 8), the RNA eluting in region A is enriched in double-stranded forms: it has a high (50 to 60%) RNase resistance, and annealing experiments indicate the presence of both vRNA and cRNA. Most significantly for the present study, only a small fraction of the radiolabeled RNA elutes in the included volume (region B), where single-stranded RNA is expected to elute (2, 8). Annealing experiments indicate that the RNA in region B consists of single-stranded cRNA contaminated with partially and fully double-stranded RNA (Table 1). The presence of double-stranded regions is indicated by the 10% decrease in RNase resistance after heating and fast-cooling. After annealing to vRNA, the RNase resistance increases to 88%, or 54% higher than the RNase resistance of the untreated sample. Thus, at least 54% of the radiolabel is in single-stranded cRNA. Actually, the percentage of single-stranded cRNA is greater than 54%, as the 24% of the radiolabel in poly(A) (heated and fast-cooled sample) can be presumed to be part of the cRNA molecules (9; see below). Clearly, however, this cRNA is not free of double-stranded forms. Our results indicate that a small fraction of the double-stranded forms trails into the included volume during Sepharose 4B chromatography. Since these double-stranded forms predominate over single-stranded cRNA in the cytoplasmic extract, they constitute a significant fraction of the RNA in the single-stranded region from Sepharose 4B.

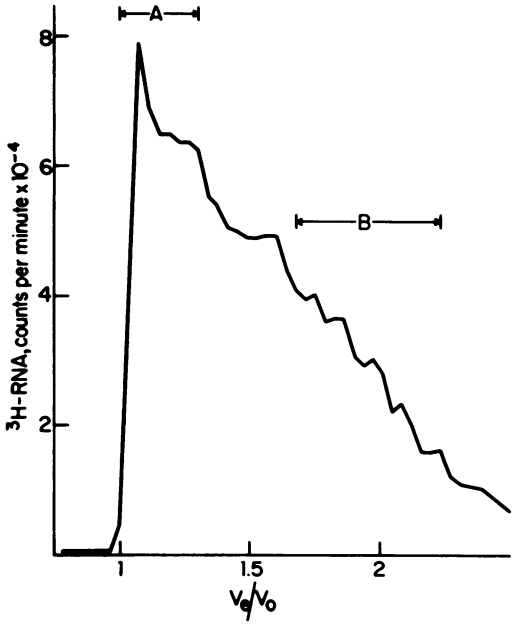


FIG. 1. *Sepharose 4B chromatography of the oligo(dT)-cellulose-bound RNA obtained from the cytoplasm of cordycepin-treated infected cells. Cells were labeled with [$^3$H]adenosine from 1 to 3.75 h after infection. The procedures for obtaining the cytoplasmic RNA and for oligo(dT)-cellulose and Sepharose 4B chromatography were described.*

To overcome this difficulty, different RNA fractions were employed as starting materials for cRNA purification. These RNA fractions were enriched in single-stranded cRNA and were deficient in partially and fully double-stranded RNAs. Two different starting materials were employed. Initially, we isolated cRNA from the polyribosomes. As shown previously, the putative mRNA released from polyri-

TABLE 1. *Hybridization analysis of the poly(A)containing RNA from the cytoplasm which elutes in the included volume during Sepharose 4B chromatography*[a]

| Treatment of RNA | RNase resistance (%) |
|---|---|
| Untreated | 34 |
| Heated and fast-cooled | 24 |
| Self-annealed | 42 |
| Annealed with 10 μg of viral RNA | 88 |

[a] The RNA fractions from the Sepharose 4B chromatography of Fig. 1 eluting in region B were pooled for this analysis. The amount of radioactivity used in the annealing experiment was 46,000 counts/min.

bosomes by puromycin and high salt consists of essentially pure single-stranded cRNA (9). However, the puromycin-high salt-released cRNA, after subsequent phenol-chloroform extraction, was found to be partially degraded and hence not suitable for the present study. Consequently, polyribosomes were directly extracted with phenol-chloroform, and the extracted RNA was subjected to sequential oligo(dT)-cellulose and Sepharose 4B chromatography. Of the polyribosomal RNA labeled with [$^3$H]-adenosine from 1 to 4.5 h, 35 to 60% binds to oligo(dT)-cellulose. The bound RNA consists of predominantly single-stranded RNA, as shown by subsequent Sepharose 4B chromatography (Fig. 2). Approximately 80% of the oligo(dT)-cellulose-bound RNA elutes from Sepharose 4B in the single-stranded region, suggesting that this RNA should be free of most, if not all, double-stranded forms. Annealing experiments indicate that this is the case (Table 2). After annealing to virion RNA, this RNA is rendered 95% RNase resistant, indicating that 95% of the RNA is comprised of cRNA. The small decrease in RNase resistance after heating and fast-cooling (3%) and the small increase after self-annealing (4%) indicate that only small amounts of partially and fully double-stranded RNAs are present. Consequently, this RNA purified from the polyribosomes is single-stranded cRNA with a maximum possible 5% contamination with double-stranded forms.

Both the purity and the amount of cRNA recovered were increased by employing a different RNA fraction as starting material. These

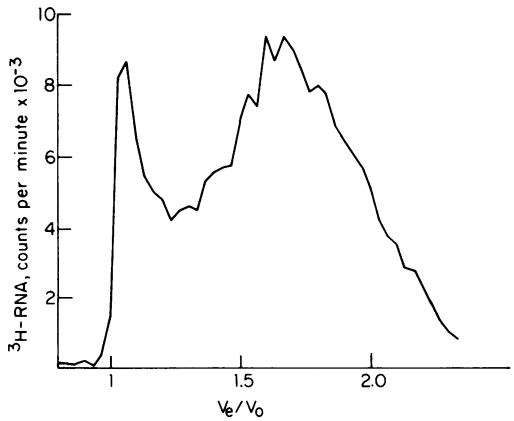


FIG. 2. *Sepharose 4B chromatography of the oligo(dT)-cellulose-bound RNA obtained from the polyribosomes of infected, cordycepin-treated cells. Cells were labeled with [$^3$H]adenosine from 1 to 4.5 h after infection. The procedures for obtaining the polyribosomal RNA and for oligo(dT)-cellulose and Sepharose 4B chromatography were as described.*

experiments involve the use of CM, which inhibits both protein synthesis and influenza vRNA synthesis (23, 29). When added at a time (approximately 3 h after infection) at which substantial amplification of cRNA synthesis has already occurred, CM reportedly inhibits subsequent cRNA synthesis not at all (29) or slightly (23). In the WSN virus-MDCK cell system studied in the present work, the addition of CM (100 $\mu$g/ml) at 3 h stimulates the production of cRNA approximately threefold (Table 3). Even more significantly, CM addition causes a very large increase in the proportion of the cytoplasmic RNA which is single stranded; double-stranded forms are greatly reduced in amount. Figure 3 shows the Sepharose 4B chromatography of the oligo(dT)-cellulose-bound RNA obtained from the cytoplasmic extract of infected cells treated with 100 $\mu$g of CM per ml at 3 h after infection. It is to be emphasized that the [$^3$H]adenosine precursor was added at 1 h after infection or 2 h before the addition of CM, so that the double-stranded forms synthesized from 1 to 3 h (see Fig. 1) would be radiolabeled. Approximately 90 to 95% of the radiolabeled

TABLE 2. *Hybridization analysis of the single-stranded, poly(A)containing RNA purified from polyribosomes*[a]

| Treatment of RNA | RNase resistance (%) |
|---|---|
| Untreated | 33 |
| Heated and fast-cooled | 30 |
| Self-annealed | 37 |
| Annealed with 10 $\mu$g of viral RNA | 95 |

[a] The RNA fractions from the Sepharose 4B chromatography of Fig. 2 eluting from $V_e/V_o$ of 1.45 to $V_e/V_o$ of 2.2 were pooled for this analysis. The amount of radioactivity used in the annealing experiment was 10,800 counts/min.

TABLE 3. *Effect of the addition of CM at 3 h on the production of viral cRNA*[a]

| CM added | cRNA produced ($\mu$g) |
|---|---|
| − | 0.057 |
| + | 0.161 |

[a] Two monolayer (60 by 15 mm) cultures of MDCK cells were infected at a multiplicity of 30 plaque-forming units/cell as previously described (10). To one culture, CM at 100 $\mu$g/ml was added at 3 h after infection. At 5 h after infection, the cells from both cultures were collected. Cytoplasmic RNA was extracted and was annealed to 3 $\mu$g of [$^3$H]adenosine-labeled viral RNA (11,000 counts/min per $\mu$g) as previously described (13, 16). The amount of viral RNA rendered RNase resistant after annealing represents the amount of cRNA present in the infected cell cytoplasmic RNA.

RNA elutes from Sepharose 4B in the single-stranded region, suggesting that this RNA should be free of any detectable double-stranded RNA forms. Annealing experiments verify the purity of this preparation of single-stranded cRNA (Table 4). After annealing to vRNA, this RNA is rendered 100% RNase resistant, indicating that all of it is cRNA. The absence of any significant change in RNase resistance after heating and fast-cooling or after self-annealing indicates that this cRNA is completely in the form of single-stranded RNA. This result has been verified with three different preparations. The 33% RNase resistance of the heated and fast-cooled sample represents the poly(A) in viral cRNA. [$^3$H]uridine-labeled cRNA purified by the same procedure has only a 2 to 3% RNase resistance after heating and fast-cooling. As with the [$^3$H]adenosine-labeled RNA, the [$^3$H]uridine-labeled RNA anneals completely to vRNA and exhibits little, or no, change in RNase resistance after heating and fast-cooling or after self-annealing. This further confirms that this RNA is single-stranded viral cRNA containing poly(A). The amount of radiolabeled cRNA recovered is 5- to 30-fold greater than the amount recovered from polyribosomes (compare Fig. 2 and 3).

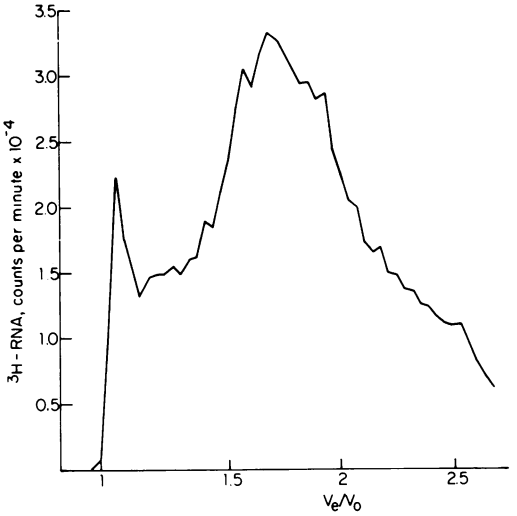


FIG. 3. *Sepharose 4B chromatography of the oligo(dT)-cellulose-bound RNA obtained from the cytoplasm of infected cells treated with 100 μg of CM per ml at 3 h after infection. [$^3$H]adenosine was added at 1 h after infection, and the cells were collected 3 h after the addition of CM. The procedures for obtaining the cytoplasmic RNA and for oligo(dT)-cellulose and Sepharose 4B chromatography were as described.*

TABLE 4. *Hybridization analysis of the single-stranded, poly(A)containing RNA purified from the cytoplasm of CM-treated cells*[a]

| Treatment of RNA | RNase resistance (%) |
|---|---|
| Untreated | 34 |
| Heated and fast-cooled | 33 |
| Self-annealed | 34 |
| Annealed with 10 μg of viral RNA | 103 |

[a] The RNA fractions from the Sepharose 4B chromatography of Fig. 3 eluting from $V_e/V_o$ of 1.45 to $V_e/V_o$ of 2.35 were pooled for this analysis. The amount of radioactivity used in the annealing experiment was 193,000 counts/min.

**Genetic content of purified viral cRNA.** To determine whether the purified cRNA contains all the genetic information in the vRNA genome, cRNA purified from polyribosomes or from cytoplasmic extracts of CM-treated cells (CM cRNA) was annealed to a limited amount of $^{32}$P-labeled viral genome RNA. Under these hybridization conditions, 84 or 90% of the vRNA genome is protected from RNase digestion by polyribosomal cRNA or by CM cRNA, respectively (Table 5). It is not certain whether saturating amounts of cRNA were used in this experiment. The actual amount, in micrograms, of cRNA cannot be determined, for unlabeled ribosomal RNA was added as a carrier during cRNA purification. A 50% reduction in the amount of radiolabeled CM cRNA in the annealing results in a 15% reduction in the protection of the vRNA genome, suggesting that the annealing experiment described in Table 5 was not carried out with completely saturating levels of cRNA. Consequently, 84 to 90% can be considered to be a minimum estimate of the percentage of the genome protected by the purified cRNA, and the poly(A)-containing cRNA, both polyribosomal cRNA and CM cRNA, can be considered to contain essentially

TABLE 5. *Protection of the virion genome RNA against RNase digestion after hybridization with purified cRNA*[a]

| RNA added | $^{32}$P-labeled virion RNA RNase resistance after annealing | |
|---|---|---|
| | Counts/min | % |
| None | 80 | 1 |
| Polyribosomal cRNA | 6,085 | 84 |
| CM cRNA | 6,518 | 90 |

[a] Polyribosomal cRNA and CM cRNA were purified as described in the text and in Tables 2 and 4. The amount of radioactivity used in the annealing experiment was $^3$H-labeled polyribosomal cRNA, 54,000 counts/min; $^3$H-labeled cRNA, 204,000 counts/min; $^{32}$P-labeled virion RNA (specific activity = 72,450 counts/min per μg), 7,242 counts/min. The RNase resistance shown is that of the $^{32}$P-labeled virion RNA.

all the genetic information in the vRNA genome.

**Activity of purified viral cRNA in wheat germ cell-free extracts.** To determine whether viral cRNA directs the synthesis of virus-specific proteins, polyribosomal cRNA or CM cRNA was added to wheat germ cell-free extracts. Both cRNA preparations act similarly. Because CM cRNA could be obtained in larger amounts and appeared to be more pure, most experiments, including all those reported here, were carried out with CM cRNA.

The addition of CM cRNA to wheat germ extracts stimulates [$^{35}$S]methionine incorporation 20- to 40-fold at the optimal potassium chloride concentration of 90 mM (3 mM magnesium) (Table 6). The proteins synthesized in vitro were analyzed by PAGE. Without the addition of CM cRNA (Fig. 4, lower panel), the wheat germ extract synthesizes no discernible proteins migrating in the region of virus-specific proteins. As shown by the two representative PAGE analyses of Fig. 4 and 5, CM cRNA programs the synthesis of several proteins which migrate at the same position as known virus-specific proteins, structural and nonstructural. More of the smaller than the larger proteins are synthesized, and the relative amount of radioactivity in the different peaks has shown variation with different RNA preparations, particularly for the proteins of higher molecular weight. The in vitro product shown in Fig. 4 contains proteins which co-migrate with the virion proteins NP and MP and with the nonstructural protein $NS_1$. Other proteins do not clearly co-migrate with known virus-specific proteins. The protein peak migrating slightly slower than MP was consistently found, as were the several peaks which migrate slower than this peak but faster than NP. Some of the latter in vitro product migrates similarly to the glycosylated virion protein neuramini-

TABLE 6. *Stimulation by CM cRNA of protein synthesis catalyzed by wheat germ cell-free extracts*

| Additions | [$^{35}$S]methionine incorporated (counts/min)[a] |
|---|---|
| None | 9,010 |
| CM cRNA[b] | 301,000 |

[a] Amount incorporated in 10 μl of the reaction mixture.

[b] 7 μl (100,000 counts/min) of CM cRNA, purified as described in the text and in Table 4, was added to a wheat germ cell-free translation reaction containing 90 mM potassium chloride.

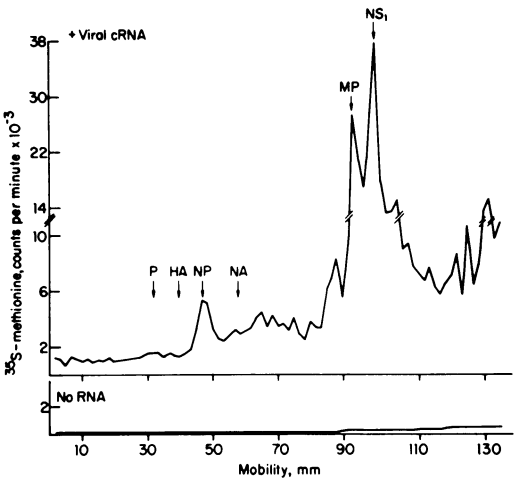


FIG. 4. *PAGE analysis of the proteins synthesized in a wheat germ cell-free reaction with and without the addition of CM cRNA. The reaction mixture contained 90 mM potassium chloride. The reaction products were processed for sodium dodecyl sulfate-gel electrophoresis as described. One-half of the product programmed by CM cRNA was analyzed on a gel with $^{3}$H-amino acid-labeled, infected cell nucleolar proteins serving as internal markers; the other half of this product was analyzed on a gel with $^{3}$H-amino acid-labeled virion proteins serving as internal markers. The arrows indicate the position of the marker virus-specific proteins.*

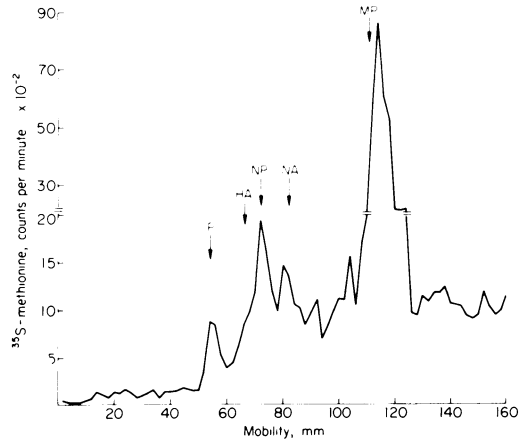


FIG. 5. *PAGE analysis of the proteins synthesized in a wheat germ cell-free reaction programmed by CM cRNA. The reaction mixture contained 90 mM potassium chloride. The reaction products were processed for sodium dodecyl sulfate-gel electrophoresis as described. The product was analyzed on a gel with $^{3}$H-amino acid-labeled virion proteins serving as internal markers. The arrows indicate the position of the marker virus-specific proteins.*

dase (NA), but a definite peak in the NA area of the gel is not clearly resolved. The product analyzed in Fig. 5 contains relatively more of the larger proteins. A protein peak co-migrating with the largest virion protein, protein P, is clearly evident. There is a shoulder on the slower-migrating side of the NP peak at the position where the glycosylated virion protein hemagglutinin (HA) migrates. In addition, the material in the NA region of the gel is clearly resolved into a peak which migrates slightly slower than NA. In this PAGE analysis, MP and $NS_1$ are not resolved into two peaks. The fastest-migrating protein peaks at the end of the gels are presumed to be premature termination products (5).

As the initial step in the characterization of the proteins synthesized in vitro, these proteins were subjected to immunoprecipitation using two different antisera: one prepared against GuHCl-disrupted virus (virion antiserum) and one prepared against GuHCl-disrupted infected cell nucleoli (nucleolar antiserum). Preimmune serum was used as a control for nonspecific precipitation. First, each of these antisera was characterized by its ability to precipitate the virus-specific proteins found in cytoplasmic extracts from infected cells. A cytoplasmic extract was prepared from cells labeled with [$^{35}$S]-methionine from 3.75 to 5.0 h after infection. The proteins in this extract were solubilized in urea and were subjected to immunoprecipitation. As shown in Fig. 6a, virion antiserum definitely precipitates virion proteins P, NP, and MP. The latter protein was identified as MP because it co-migrates with authentic marker virion MP protein and migrates slower than the nucleolar $NS_1$ protein. The amount of the P, NP, and MP proteins precipitated using this technique has varied between 10 to 20% of the amount present in the cytoplasmic extract as estimated from the PAGE analysis of the extract. Whether any of the glycosylated virion polypeptides (HA, NA, $HA_1$ and $HA_2$) are precipitated by the virion antiserum cannot be ascertained from this experiment. These polypeptides constitute only a small fraction of the total [$^{35}$S]methionine-labeled virus-specific proteins in an infected cell cytoplasmic extract (14, 17) and, consequently, might not be detectable after immunoprecipitation. As shown in Fig. 6b, the nucleolar antiserum definitely precipitates virion proteins P and NP and the nonstructural protein $NS_1$. The latter protein was identified as $NS_1$ because it co-migrates with the marker nucleolar $NS_1$ protein and migrates faster than the virion MP protein. Again, 10 to 20% of each of P, NP, and $NS_1$ is immunoprecipitated. With the control preimmune serum (Fig. 6c), only small amounts of virus-specific proteins are precipitated. Small peaks are seen in the NP and MP-$NS_1$ areas of the gel. Ten- to 20-fold more of these three proteins is precipitated with the virus-specific antisera (Fig. 6a and b). Thus, the virion and nucleolar antisera are able to precipitate, at the least, P, NP, MP, and $NS_1$.

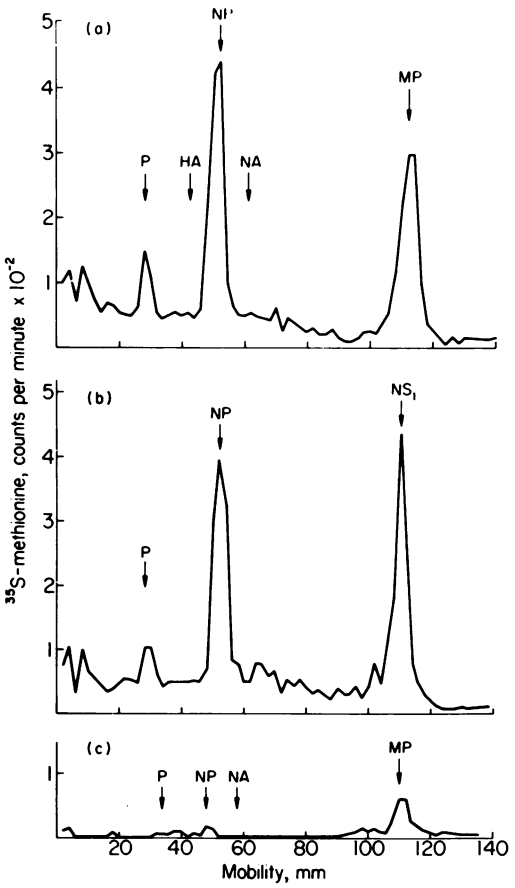


FIG. 6. *PAGE analysis of the cytoplasmic virus-specific proteins precipitated by (a) virion antiserum, (b) nucleolar antiserum, and (c) preimmune serum. A cytoplasmic extract from infected cells labeled with [$^{35}$S]methionine from 3.75 to 5.0 h after infection was treated with RNase and EDTA and was dialyzed overnight against 7 M urea. The procedure for immunoprecipitation was as described. The precipitates were analyzed on 7.5% acrylamide gels with $^3$H-amino acid-labeled virion proteins (a and c) or $^3$H-amino acid-labeled nucleolar proteins (b) as internal markers. The arrows indicate the position of the marker virus-specific proteins.*

Figure 7 shows the results obtained when the in vitro reaction mixture, solubilized with urea,

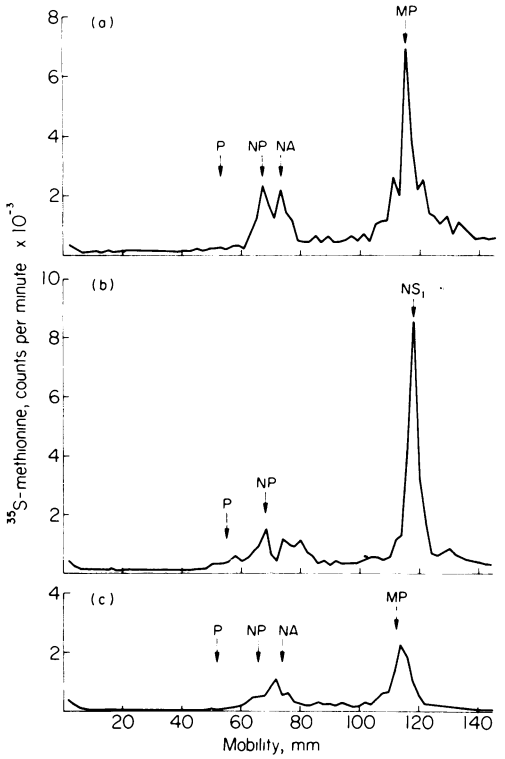


FIG. 7. *PAGE analysis of the CM cRNA-directed proteins precipitated by (a) virion antiserum, (b) nucleolar antiserum, and (c) preimmune serum. The wheat germ reaction mixture contained 90 mM potassium chloride. After incubation at 25 C, the reaction mixture was treated with RNase and EDTA and was dialyzed overnight against 7 M urea. The procedure for immunoprecipitation was as described. The precipitates were analyzed on 7.5% acrylamide gels with $^3$H-amino acid-labeled virion proteins (a and c) or $^3$H-amino acid-labeled nucleolar proteins (b) as internal markers. The arrows indicate the position of the marker virus-specific proteins.*

was subjected to immunoprecipitation. As compared to the results obtained with infected cell cytoplasmic extracts, preimmune serum brings down a larger amount of radiolabeled proteins (Fig. 7c). Nevertheless, the precipitates obtained with the two virus-specific antisera consistently contain subtantially increased amounts of proteins which co-migrate with the marker NP, MP, and $NS_1$ proteins, indicating that these three virus-specific proteins, indeed, are synthesized in vitro. Precipitation with the virion antiserum (Fig. 7a) increases the amount of radiolabeled protein co-migrating with NP and MP by four- to sixfold. Precipitation with the nucleolar antiserum (Fig. 7b) increases the amount of radiolabeled protein co-migrating with $NS_1$ by six- to sevenfold. These increases, observed in several experiments, represent 10 to 20% of the NP, MP, and $NS_1$ in the in vitro product. This efficiency of immunoprecipitation is the same as that obtained with the infected cell cytoplasmic extract and is also the same as that previously obtained by Oberg et al. (20) who designed this immunoprecipitation procedure for the identification of adenovirus-specific proteins synthesized in vitro. The increase in the amount of radiolabeled protein comigrating with NP after precipitation with nucleolar antiserum (Fig. 7b) is about half of that obtained after precipitation with the virion antiserum (Fig. 7a). The approximately twofold increase in the amount of radiolabeled protein in the NA region of the gel after precipitation with virion antiserum (Fig. 7a) has been consistently observed. Thus, these experiments strongly suggest that at least three of the proteins synthesized in vitro are virus specific: NP, MP, and $NS_1$.

**Activity of viral RNA preparations in wheat germ cell-free extracts.** Initial experiments using RNA extracted with phenol-chloroform from purified virus in wheat germ extracts were negative. At the potassium chloride concentration (90 mM) optimal for cRNA translation in vitro, the viral RNA preparations are inactive or stimulate incorporation at most two- to threefold; no discernible protein peaks were observed by PAGE analysis. However, when the potassium chloride concentration was reduced to 60 mM, the viral RNA preparations were very active, stimulating [$^{35}$S]methionine incorporation into proteins 10- to 12-fold (Table 7). Increasing amounts of RNA results in increasing amounts of incorporation. Analysis of the protein products by PAGE (Fig. 8) revealed that almost all of the proteins synthesized are very small, smaller than all of the marker virus-specific proteins. Based on the recovery of radioactivity from the gels, it appears that a

TABLE 7. *Stimulation by viral RNA preparations of protein synthesis catalyzed by wheat germ cell-free extracts*[a]

| Additions | [$^{35}$S]methionine incorporated (counts/min)[b] |
|---|---|
| None | 20,000 |
| Viral RNA | |
| 2 μg | 52,000 |
| 10 μg | 212,300 |

[a] Reaction mixture contained 60 mM potassium chloride.

[b] Amount incorporated in 10 μl of the reaction mixture.

substantial, though undetermined, amount of the product was electrophoresed off the gel and hence is even smaller than those shown in Fig. 8. The small fraction of the protein product migrating in the higher-molecular-weight area of the gel has not consistently co-migrated with any of the virus-specific proteins.

## DISCUSSION

The major emphasis of the present study has been on obtaining large amounts of influenza viral cRNA purified free from any detectable virion (vRNA). The purification methods employed were oligo(dT)-cellulose chromatography, which selects cRNA by virtue of its poly(A) segments, and Sepharose 4B chromatography, which separates single-stranded cRNA from partially and fully double-stranded RNA forms. During Sepharose 4B chromatography, most of the double-stranded forms elute in the void volume, whereas single-stranded cRNA elutes in the included volume. However, as a small fraction of the double-stranded forms trail into the included volume, it is necessary to employ as starting material an RNA fraction in which single-stranded cRNA predominates over the double-stranded forms. The total cytoplasmic RNA from infected cells was found not to be a suitable starting material, whereas polyribosomal RNA and the total cytoplasmic RNA from CM-treated cells were suitable. With the polyribosomal RNA as starting material, 95% of the radiolabeled RNA in the purified cRNA preparation was found to be in single-stranded cRNA with the remaining 5% apparently being partially and fully double-stranded RNA. The cRNA preparation obtained from the cytoplasmic RNA of CM-treated infected cells (CM cRNA) is even more pure. One hundred percent of the radiolabeled RNA in the CM cRNA preparation anneals to vRNA, indicating that all of this RNA is cRNA, and no double-stranded RNA forms are detectable. Thus, the CM cRNA can be considered to be a preparation of single-stranded viral cRNA free of any detectable vRNA.

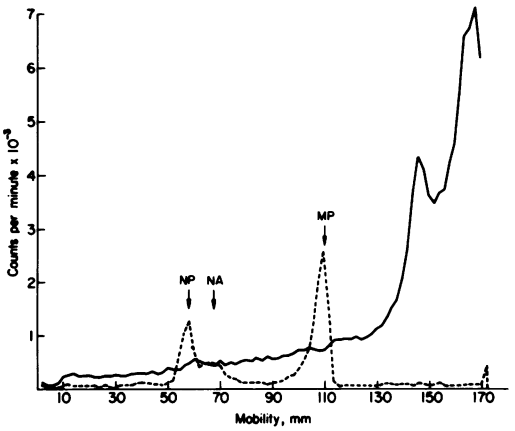


FIG. 8. *PAGE analysis of the proteins synthesized in a wheat germ cell-free reaction programmed by RNA extracted from purified virus. The translation reaction contained 5 μg of viral RNA and 60 mM potassium chloride. The reaction products were processed for sodium dodecyl sulfate-gel electrophoresis as described. The reaction products ([$^{35}$S]methionine labeled) are shown by the solid line, and the $^{3}$H-amino acid-labeled virion proteins are shown by the dotted line.*

Influenza viral cRNA would be expected to be in seven to 10 segments, similar to the vRNA (4, 18, 31) from which it is transcribed. Hybridization experiments indicate that the purified poly(A)-containing cRNA contains at least 84 to 90%, or most probably all, of the genetic information in the genome vRNA. This strongly suggests that all of the cRNA segments contain poly(A) and that all cRNA segments are on the polyribosomes.

The purified viral cRNA is very active in directing the synthesis of virus-specific proteins in wheat germ cell-free extracts. Four of the proteins synthesized in vitro co-migrate during PAGE with the nonglycosylated virus-specific proteins P, NP, MP, and $NS_1$. The latter three proteins have also been identified after immunoprecipitation with virus-specific antisera in the presence of 1 M urea. This method of immunoprecipitation was shown in a previous study of adenovirus-specific proteins synthesized in vitro to eliminate most nonspecific precipitation but to result in a relatively low efficiency of specific immunoprecipitation (20). In the present study, this method also yields low levels of nonspecific precipitation (the precipitation occurring with preimmune serum), though some nonspecific precipitation, primarily with $NS_1$, still persists. This can probably be ascribed, at least in part, to the fact that $NS_1$ is a "sticky" protein which, for example, adsorbs to cytoplasmic ribosomes (14). The more significant finding, however, is that the efficiency of specific immunoprecipitation of NP, MP, and $NS_1$ obtained in the present study is equal to, or greater than, the specific immunoprecipitation obtained in the previous adenovirus study (20). It is extremely unlikely that the portion of the radiolabel in each protein band which is not immunoprecipitated corresponds to a different protein, especially in light of recent tryptic peptide analysis. For example, the methionine-containing tryptic peptides of the NP protein synthesized in vitro are identical to those of authen-

tic virion NP protein (Etkind and Krug, unpublished experiments). The identification of the protein moieties of the glycosylated virion HA and NA is less clear. The protein which migrates similarly to NA during PAGE, and which is found to some extent in the immunoprecipitate obtained with virion antiserum, is a candidate for the protein moiety of NA. The protein detected only as a shoulder on the slower-migrating side of the NP peak is a candidate for the protein moiety of HA. Only by further evidence, specifically including tryptic peptide analysis, can the identity of these proteins with the protein moieties of HA and NA be established. Thus, the initial characterization of the proteins synthesized in vitro in response to influenza viral cRNA indicates that at least three or four virus-specific proteins are present and suggests that further analysis will reveal the presence of the remaining virus-specific proteins.

The results of the present study provide further strong evidence that cRNA constitutes the entirety of infleunza virus-specific mRNA. On this basis, our results obtained in the wheat germ cell-free system with RNA extracted from purified virus suggest an explanation for the previously reported stimulation by influenza viral RNA of protein synthesis catalyzed by *E. coli* cell-free extracts (30). In the wheat germ extracts employed in the present study, viral RNA preparations, at salt concentrations lower than optimal for cRNA, direct the synthesis of essentially only very small polypeptides, smaller than all known virus-specific proteins except possibly the minor nonstructural protein $NS_2$. A possible explanation of this result is that the protein-synthesizing activity in our viral RNA preparations is due to small cRNA transcripts incorporated into virus. Other so-called "negative strand" viruses, parainfluenza viruses and vesicular stomatitis virus, incorporate cRNA strands into virus (24, 26, 27), and it is therefore not unreasonable to suspect that this would also occur with influenza virus. Previous methods of analysis of influenza virus RNA preparations, self-annealing and base composition (13, 22, 28), were probably not sensitive enough to rule out the presence of a small amount of cRNA transcripts. With parainfluenza viruses, the quantity of the cRNA transcripts incorporated into virus varies with different virus preparations (24, 26), and, with vesicular stomatitis virus, cRNA transcripts have only been found in one type of defective virus (27). Perhaps, with influenza virus, the quantity and size of the cRNA transcripts varies with different virus preparations, with more and larger cRNA transcripts being found in defective virus particles. Our virus preparation, WSN virus grown in bovine kidney (MDBK) cells, is probably one of the least defective influenza virus preparations in that it does not exhibit a von Magnus effect (6). This virus preparation presumably contains small cRNA transcripts. Other virus preparations containing more defective particles may contain a large amount of longer, possibly complete, cRNA transcripts; the egg-grown virus employed in the *E. coli* studies (30) may be in this category. Clearly, proof of this explanation for the protein-synthesizing activity associated with viral RNA preparations awaits the direct characterization of the active molecules.

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