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Cell-Free Translation of Influenza Virus mRNA

J. CONTENT

Department of Virology, Institut Pasteur du Brabant, B-1040 Brussels, Belgium

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Cytoplasmic poly(A)-rich RNA extracted from fowl plague virus-infected cells was found to program efficiently the translation of two major peptides in the wheat germ cell-free system. These peptides have the same electrophoretic mobility, on polyacrylamide gels, as the two major virion proteins M and NP. [^{35}S]methionine tryptic peptide analysis by one-dimensional paper ionophoresis and finger printing by two-dimensional thin-layer ionophoresis and chromatography show a high degree of similarity between the two in vitro products and the authentic viral proteins M and NP. Although virion RNA is devoid of any poly(A) sequence, it is confirmed here that the viral complementary cytoplasmic RNA contains poly(A) stretches of varying lengths. Intact purified virion was found to promote the synthesis of very low amounts of the same NP and M proteins in this cell-free system. Quantitative aspects of the data would indicate that this is due to minute amounts of complementary viral RNA associated with the virion or with the virion RNA itself. In conclusion, it is shown directly by cell-free translation of authentic viral products that the influenza virion is "negative stranded" (Baltimore, 1971), at least for its two major structural proteins.

Influenza viruses are enveloped animal viruses whose main particularity is a segmented, single-stranded RNA genome (12, 36, 37).

Structural studies have shown that all these segments replicate independently (11), are packed into different ribonucleoprotein structures (9), and represent different pieces of genetical information, accounting presumably for discrete monocistronic RNAs or their complementary sequence (8, 39). The coding function of those different RNA components, however, is not known.

Influenza virus contains a transcriptase associated with the virion itself (4, 7, 30). In addition, Pons (31) and Etkind and Krug (14) showed that viral mRNA isolated from polysomes of infected cells is entirely complementary to virion RNA and contains poly(A) sequences, whereas virion RNA does not. These different facts suggest that influenza virus might be "negative stranded" (3) like the other large enveloped, transcriptase-containing RNA viruses (paramyxoviruses, rhabdoviruses).

Is the virus completely negative stranded or does it contain some RNA segments used directly for the translation of some viral peptides? Recent evidence indicating a temporal control of the expression of the different influenza proteins has been obtained by three groups of investigators (26, 40; S. E. Glass, D. McGeoch, and R. D. Barry, Abstr. 3rd Int. Cong. Virol., p. 177, 1975) and could be explained easier if some translation occurs on the virus strand.

Available observations concerning the in vitro translation of influenza RNA have not settled this question so far. Kerr and Martin (19) using a cell-free system for protein synthesis derived from Krebs ascites cells and, later on, Kingsbury and Webster (21) in a rabbit reticulocyte cell-free system failed to detect any mRNA activity when assaying virion-extracted RNA. However, when the same cell-free system was programmed with total RNA extracted from influenza-infected cells, a specific protein was synthesized with the same electrophoretic mobility and the same antigenicity as the M protein of influenza virus (21). In strong contrast with the preceding groups, Siegert et al. (38) programmed a cell-free system of *Escherichia coli* with RNA from influenza viral particles and observed the synthesis of a peptide antigenically similar to the influenza virus protein NP.

We have reinvestigated this question, using a very sensitive cell-free translation system derived from wheat germ extracts (34) combined with a simple method of purification (or enrichment) of mRNA on the basis of its poly(A) content (2). Similar approaches have been used successfully to translate a wide variety of viral mRNA's in vitro (6, 27, 29, 32).

In this paper we describe the in vitro transla-

tion, in the wheat germ system, of a poly(A)-rich cytoplasmic RNA isolated from fowl plague virus (FPV)-infected cells. We show that the two major peptides of the virion, proteins M and NP (20, 39), can be translated by this in vitro system. Their identity with the two authentic virion proteins has been shown by tryptic peptide analysis.

MATERIALS AND METHODS

Cells and virus. FP Virus (Rostock strain) was kindly given by H. D. Klenk, Giessen, Germany.

Preparations of nonradioactive virus were grown in the allantoic cavity of embryonated eggs and purified as described earlier. Radioactive virus, cytoplasmic RNA, and proteins were obtained from infected primary cultures of chicken embryo fibroblasts cultivated as described elsewhere (11). Labeling was performed by adding [^{35}S]methionine (6 μCi/ml, 140 to 210 Ci/mmol) to the tissue culture medium, from 10 to 30 h after infection in the case of ^{35}S-labeled FPV and as indicated in the text for the cytoplasmic proteins.

Preparation of poly(A)-containing RNA. Sixteen hours after infection with FPV at a multiplicity of infection of 0.5 PFU/cell (or differently when specified), the chicken embryo fibroblasts were washed two times in ice-cold phosphate-buffered saline, scraped off the plates with a rubber policeman, washed again, swollen 5 min in Tris-hydrochloride (10 mM, pH 7.5), 5 mM KCl, and 2 mM $MgCl_2$, and disrupted with 25 strokes of a tight-fitting Dounce glass homogenizer. Nuclei were spun down for 15 min at 30,000 $\times$ g (or 2 min at 1,000 $\times$ g) (see Results), and the cytoplasmic supernatant was then extracted by phenol-chloroform-isoamyl alcohol and chromatographed on oligodeoxythymidylate [oligo-(dT)]-cellulose as described by Aviv and Leder (2). The eluted RNA was ethanol precipitated after addition of NaCl to 0.4 M and Krebs ascites cells-tRNA to 1 optical density unit at 260 nm per ml.

RNA from parallel uninfected cultures was extracted and processed in the same way.

Cell-free translation in extracts of wheat germ. Extracts were prepared from wheat germ obtained from "Bar-Rav" Mill, Tel-Aviv (a kind gift from C. Prives, Rehovot, Israël) as described by Roberts and Paterson (34), except for the following modifications. Preincubation of the lysate was omitted, and desalting was performed rapidly by centrifugation over a G-25 fine Sephadex column.

Protein synthesis was assayed as described by Roberts and Paterson (34). A 10-μCI portion of [^{35}S]methionine (140 to 210 Ci/mmol; The Radiochemical Centre, Amersham, England) was added per 50-μl reaction volume. Potassium acetate and spermidine-hydrochloride were optimized to 88 and 0.35 mM, respectively, whereas magnesium acetate was kept constant at 2 mM.

Product identification. Samples of the in vitro reaction product or from a 1% Nonidet P-40 (Shell Co.) cytoplasmic extract were mixed with 0.5 volume of a solution containing 0.15 M Tris-hydrochloride, pH 6.8, 3% sodium dodecyl sulfate, 3% 2-mercaptoethanol, and 30% glycerol and heated for 3 min at 100 C. The treated samples (5 to 20 μl) were subjected to electrophoresis for 5 h at 125 V through slab gels containing a 10 to 20% polyacrylamide gradient (41). After staining with Coomassie brilliant blue, the gels were either dried and exposed to an X-ray film (Kodak RP, Royal X, O-mat) at room temperature or impregnated first with 20% (wt/vol) 2,5-diphenyloxazole in dimethyl sulfoxyde (5), dried, and exposed to the same X-ray film but at −70 C. Scanning of the autoradiograms was done with a Beckman Analytrol film densitometer.

Peptide analysis. FPV proteins were resolved on 6 to 20% polyacrylamide gradient slab gels. Whereas the virion proteins could be located after staining with Coomassie brilliant blue, the in vitro products were determined after autoradiography of the fixed dried gels. Trypsin digestion of the individual FPV proteins was performed directly on the gel slice exactly as described by Morrison and Lodish (J. Biol. Chem., in press).

The [^{35}S]methionine tryptic peptides were analyzed by paper ionophoresis at pH 6.5 on 3MM Whatman filter paper in a Shandon L24 high-voltage electrophoresis apparatus for 3 h at 2,500 V. The dried paper was sliced in 1-cm strips and counted in toluene-based scintillation fluid in a Packard scintillation counter.

For two-dimensional finger printing, the same [^{35}S]methionine tryptic peptides were first analyzed by ionophoresis at pH 3.5 for 150 min at 400 V on silica gel-coated, thin-layer plates (20 by 20 cm, Merck, no. 5748) in a Desaga thin-layer electrophoresis apparatus (no. 121221) cooled with tap water at about 18 C. After drying, the thin-layer plates were submitted to ascending chromatography in n-butanol-pyridine-acetic acid-water (15:12:3:12) for 7 h. The plates were dried again, treated with 7% (wt/vol) 2,5-diphenyloxazole in ethyl ether, and exposed to the X-ray film at −70 C (33).

RESULTS

FPV structural proteins. Polyacrylamide gel electrophoresis of a purified [^{35}S]-methionine-labeled virion shows a distribution of seven different structural peptides. Their approximative molecular weight was calculated by comparison with known protein markers as shown in Fig. 2. When purified [^{3}H]fucose-labeled FPV was run in a parallel lane of the gel, only three viral components were found to share both [^{35}S]methionine and [^{3}H]fucose radioactivity.

These data, together with previously published observations on influenza A glycoproteins (10, 35) and, more specifically, on FPV glycoproteins (22, 39), allow the identification of the different non-glycosylated proteins on the tracing as P1 (mol wt, 89,000), P2 (mol wt, 80,000), NP (mol wt, 59,000), and M (mol wt, 28,000). For the nomenclature we follow the convention generally used (20). Among the

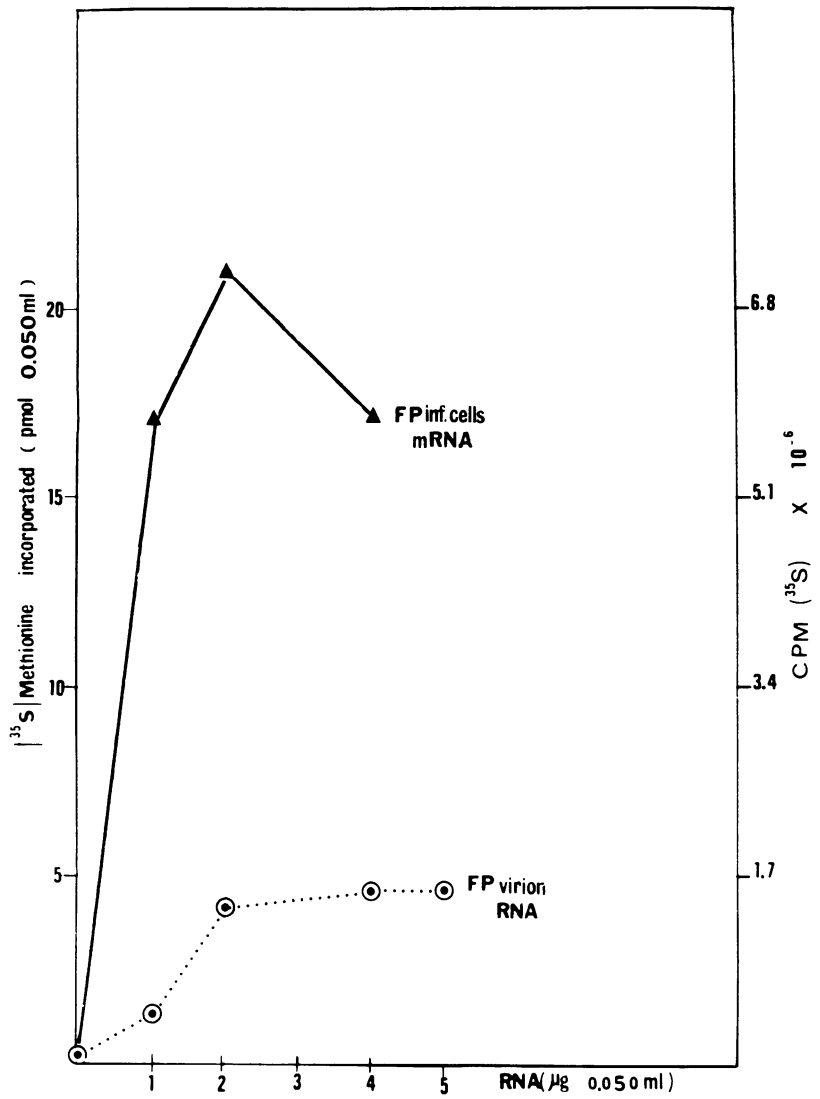


FIG. 1. *Protein synthesis directed by RNA from FPV-infected cells and FP virion RNA in wheat germ extracts. Conditions for cell-free translation were as described in Materials and Methods. The specific activity of the [35S]methionine was 340,000 counts/min per pmol. Incubation was for 90 min at 25 C. FP virion RNA is the same preparation as described in Fig. 6.*

three detected glycoproteins the fastest partially overlaps with protein M from which it is incompletely resolved, as is usually the case for HA2 (8). The two other glycoproteins probably represent HA1 and NA. These seven polypeptides are quantitatively very unevenly represented inside the virion. Compans and Choppin (8) have estimated for an average influenza virion an approximative content of 30 to 60 molecules of P1 or P2, 500 to 940 molecules of NP, and 2,500 to 3,120 molecules of M. Skehel (39) found also that proteins M and NP accounted for 64% of the total [35S]methionine incorporated in the FP virion.

Assay for mRNA activity of poly(A)-containing RNA extracted from FPV-infected chicken embryo fibroblasts. Chicken embryo fibroblast primary cells (3×10^9) were infected with FPV at a multiplicity of infection of 0.5 PFU/cell. After 16 h at 37 C, a cytoplasmic extract was prepared, deproteinized, and chromatographed on oligo(dT)-cellulose as described in Materials and Methods. Under the best conditions, 150 to 200 µg of poly(A)-containing RNA was obtained from such a preparation. When this RNA was first assayed in the wheat germ system to check its ability to promote [35S]methionine incorporation into hot

trichloroacetic acid-insoluble radioactive material, it was found to be very active: its activity as mRNA was about the same as that obtained with tobacco mosaic virus RNA under similar conditions. Figure 1 shows that its optimal activity was reached with 1 to 2 μg/50-μl reaction. Similar or even five times higher concentrations of purified virion-extracted RNA only yielded a fifth of the [^{35}S]methionine incorporation. Moreover, poly(A)-rich RNA extracted from noninfected cells was up to nine times less active at the optimal RNA concentration than the same RNA extracted from infected cells. Table 1 shows also that RNA extracted from cells, 3 and 6 h after infection, showed intermediate activities. This seems to indicate that some viral-specified RNA or RNAs are better translated in the in vitro wheat germ system than is the bulk of host cell mRNA.

Analysis of the wheat germ in vitro translation products obtained with different mRNA preparations. Wheat germ translation products were compared by polyacrylamide gel electrophoresis to the viral structural peptides and to pulse-labeled cytoplasmic extracts from infected and uninfected chicken embryo fibroblasts. Figure 2A shows, in a cytoplasmic extract from infected cells, four major peptides matching exactly those of the purified virion and not detectable in the uninfected cell. They apparently correspond to all viral protein components except for P1 and P2, which could not be detected. Several investigators (24, 39) have described one or two additional nonstructural peptides in the cytoplasm or in a nucleolar fraction (NS_1; 23) of influenza-infected cells. It is likely that NS_1 was not resolved from M, the most predominant peptide under the experimental conditions.

TABLE 1. *Translation efficiency of RNA extracted from chicken embryo fibroblasts at different times after infection with FPV in the wheat germ cell-free system*[a]

Poly(A)-rich RNA	pmol of [^{35}S]methionine incorporated after 90 min at 25 C in the wheat germ system (per 50-μl reaction vol)
Uninfected chicken embryo fibroblasts	
1 μg	0.92
2 μg	0.44
Chicken embryo fibroblasts 3 h after infection with FPV	
1 μg	1.96
2 μg	2.00
4 μg	0.65
Chicken embryo fibroblasts 6 h after infection with FPV	
1 μg	4.63
2 μg	4.70
4 μg	3.87
Chicken embryo fibroblasts 16 h after infection with FPV	
1 μg	8.39
No RNA	0.15

[a] Reaction mixtures containing magnesium acetate (2 mM), potassium acetate (88 mM), spermidine hydrochloride (0.35 mM), and 10 μCi of [^{35}S]methionine (140 Ci/mmol) were otherwise as indicated by Roberts and Paterson (34). Hot trichloroacetic acid-insoluble radioactivity was determined as described in reference 34.

In vitro products obtained in the wheat germ system programmed with FPV-infected-cell, poly(A)-rich cytoplasmic RNA consist of two major bands with the same electrophoretic mobility as proteins M and NP, in addition to some low-molecular-weight heterodisperse products, which represent either some host cell products or early quitting peptides.

Figure 2A shows also that neither of these two major products is detectable when the in vitro system is programmed with uninfected-cell poly(A)-rich RNA. It can be concluded that the poly(A)-rich RNA extracted from infected cells contains one or several mRNA's responsible for the synthesis of two major non-glycosylated proteins indistinguishable in polyacrylamide gels from the two major virion proteins M and NP.

Since none of the three viral glycoproteins could be detected in the in vitro translation product, poly(A)-rich RNA was extracted and purified similarly from a low-speed supernatant (1,000 × *g*) of an infected cell homogenate. This should obviate elimination of most of the membrane-bound polysomes, where the viral glycoproteins are predominantly or exclusively synthesized (18; in the case of vesicular stomatitis virus, see Morrison and Lodish, in press).

Under these conditions, however, no additional viral peptides could be detected: the pattern was identical with that of Fig. 2A. One could also argue that RNA extracted at 16 h postinfection might be deprived of some mRNA components, since at this time some cytopathogenic effect exists and degradation of some of the most susceptible mRNA's could occur. RNA extracted under similar conditions, but at 3 and 6 h after the onset of infection, promotes as well the translation of the two major peptides coinciding in the gel with NP and M; no additional viral peptide appears (Fig. 2B).

Tryptic peptide analysis. We then compared

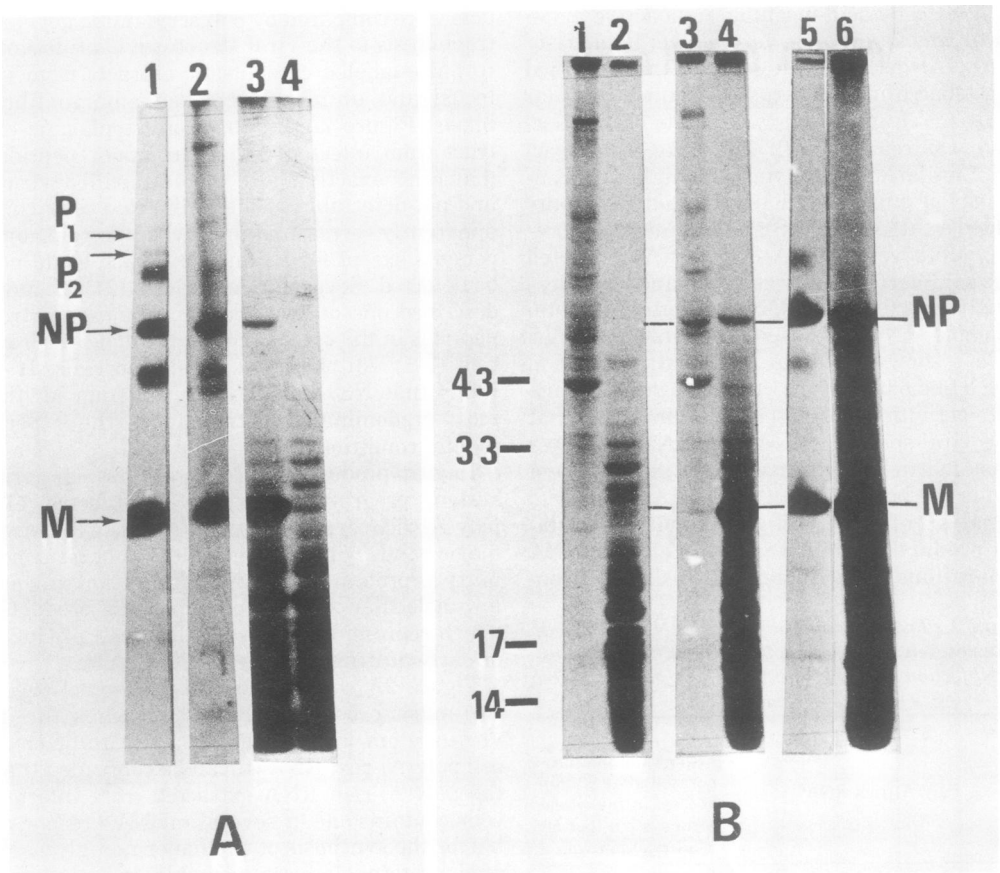


FIG. 2. *(A) Autoradiograms of sodium dodecyl sulfate-polyacrylamide gel electrophoretic slabs of polypeptides directed by poly(A)-rich RNA from uninfected and FPV-infected cells in the wheat germ system. In vitro translation in wheat germ extracts, polyacrylamide gel electrophoresis, and autoradiography were as described in Materials and Methods. (1) ^{35}S-labeled proteins from purified FP virion were used as a marker. (2) Polypeptides from FPV-infected cells: 16 h after onset of infection, chicken embryo fibroblasts infected with 0.5 PFU of FPV were labeled for 1 h with 6 μCi of [^{35}S]methionine per ml (140 Ci/mmol), washed three times with ice-cold phosphate-buffered saline, and disrupted in phosphate-buffered saline containing 1% Nonidet P-40. The homogenate was centrifuged for 5 min at 10,000 × g, and the supernatant was prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in Materials and Methods. (3) In vitro [^{35}S]methionine products directed by 2 μg of poly(A)-rich RNA per 50-μl reaction from FPV-infected cells 16 h after infection. (4) The same as (3) with RNA from uninfected cells. (B) Autoradiograms of sodium dodecyl sulfate-polyacrylamide gel electrophoretic slabs of polypeptides directed by poly(A)-rich RNA from FPV-infected cells in comparison with in vivo pulse-labeled proteins, at different times after infection. (2) Translation products obtained with RNA from uninfected cells. (4) Translation products obtained with RNA from chicken embryo fibroblasts, 3 h after infection with FPV. (6) Translation products obtained with RNA from chicken embryo fibroblasts, 6 h after infection with FPV. Under the same conditions a sample of the 1-h [^{35}S]methionine, pulse-labeled cytoplasmic protein was analyzed: (1) uninfected cells; (3) 3 h after infection; (5) 6 h after infection. Ovalbumin (mol wt, 43,000), asparaginase (mol wt, 33,000), tobacco mosaic virus protein (mol wt, 17,000), and lysozyme (mol wt, 14,400) were used as known molecular weight protein markers.*

the two major translation products obtained in vitro in the wheat germ system programmed with infected cell mRNA to the corresponding proteins M and NP derived from purified virus. These proteins were obtained by preparative polyacrylamide gel electrophoresis, digested with trypsin, and analyzed by paper ionophoresis at pH 6.5, as described in Materials and Methods. Figure 3 shows that the [^{35}S]methionine-labeled tryptic peptide patterns obtained from the authentic FPV, M, and NP proteins coincide almost entirely with their presumptive counterparts obtained from the in vitro reaction. However, in the case of protein M two

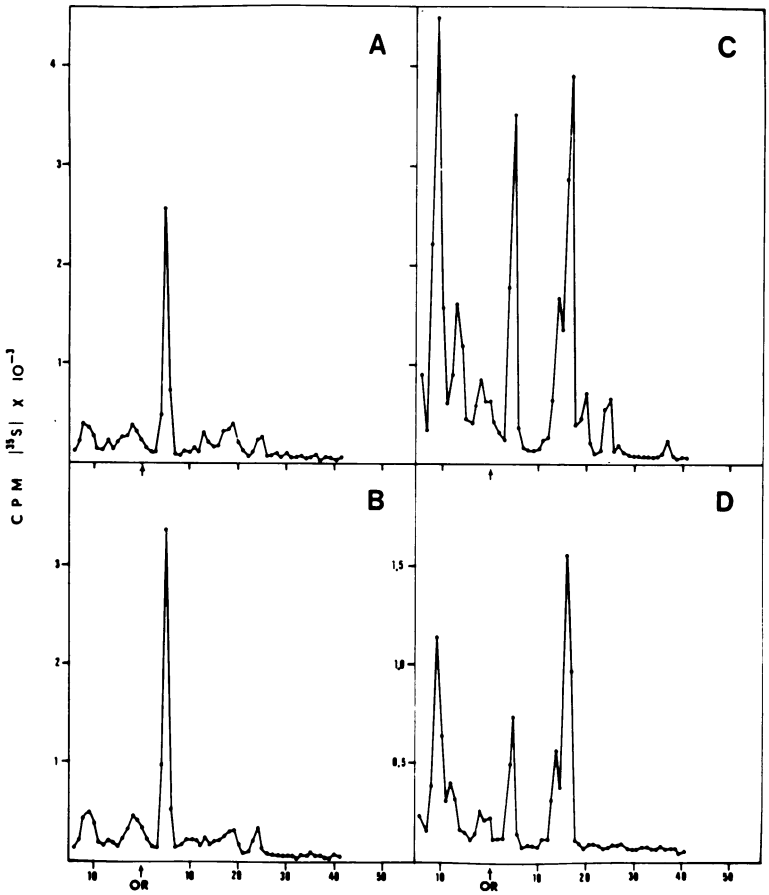


FIG. 3. *Paper ionophoresis of [35S]methionine-labeled tryptic peptides derived from FPV proteins NP and M. (A) Tryptic peptides derived from the putative NP protein made in the cell-free system. (B) Tryptic peptides derived from the authentic NP virion protein. (C) Tryptic peptides derived from the putative M protein made in the cell-free system. (D) Tryptic peptides derived from the authentic M virion protein. Proteins M and NP were obtained by preparative slab gel electrophoresis either from the purified virus or from the cell-free system translation products, as indicated in Fig. 2. Trypsin digestion was as indicated in Materials and Methods. Paper ionophoresis was for 3 h at 2,500 V. Arrows indicate the origin. Cathode was to the right in each panel.*

small additional peptides were detected in the in vitro product (Fig. 3C and D).

The tryptic peptide patterns were then further compared by two-dimensional mapping on silica gel thin-layer plates as described in Materials and Methods. Autoradiograms of the four patterns as well as schematic tracings are shown in Fig. 4. The peptide map derived from authentic protein NP shows at least 22 spots having their identical counterpart in the in vitro presumptive NP product (Fig. 4A and B). This latter, however, shows at least two distinct additional [35S]-labeled spots.

The patterns obtained for protein M are distinct and more complex. At least 21 spots have the same migration in the authentic viral protein and in the presumptive M in vitro product. However, the latter again shows a few additional spots (Fig. 4C and D).

Thus, two independent analyses of the tryptic peptides derived from proteins M and NP show that the two major in vitro translation products contain at least all the [35S]methionine peptides of the two major structural proteins obtained from the virus itself.

Poly(A) content of the FPV mRNA. Although it is well established that influenza virion RNA is devoid of poly(A) stretches (14, 17), there has been some controversy in the literature concerning the poly(A) content of influenza mRNA (1, 14).

In our system we could directly assay the poly(A) content of the active viral mRNA preparation described so far. The oligo(dT)-cellu-

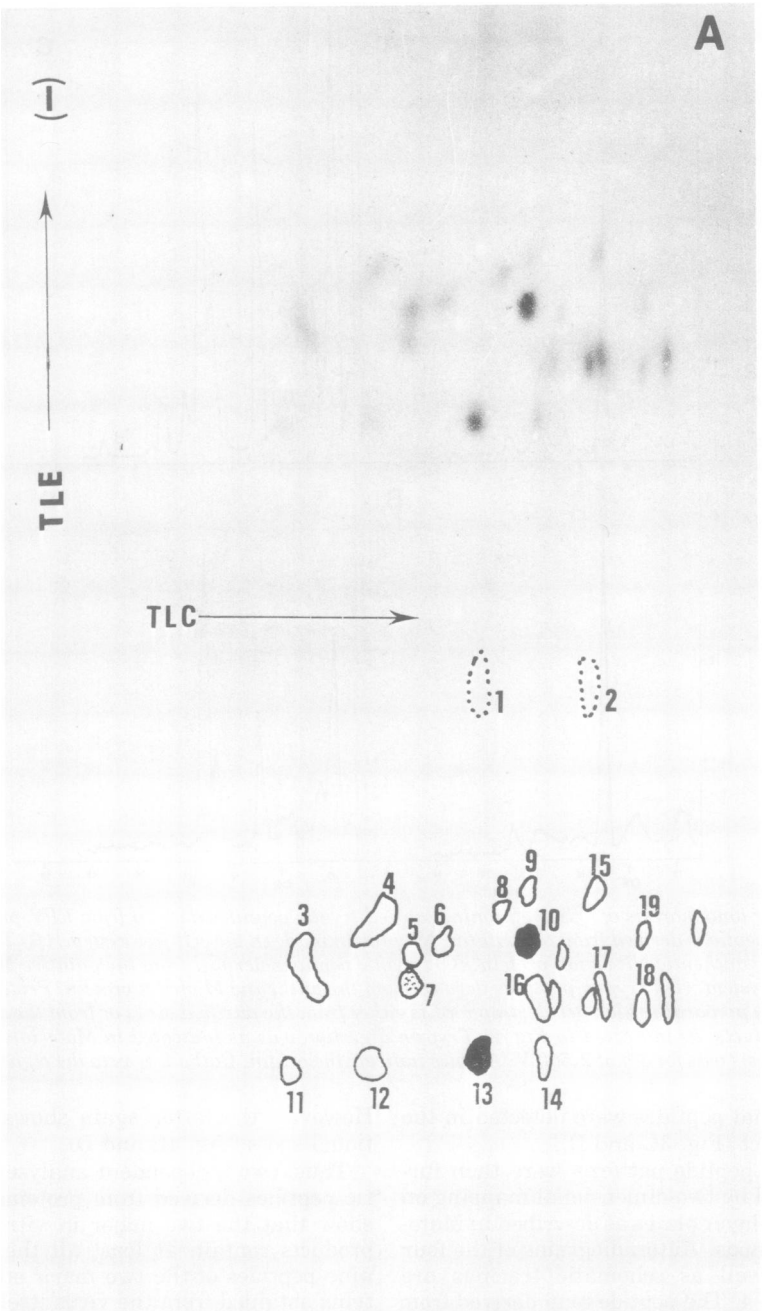


FIG. 4. *Two-dimensional finger printing of the [^{35}S]methionine-labeled tryptic peptides derived from FPV proteins NP and M. (A) Tryptic peptides derived from the authentic NP virion protein. (B) Tryptic peptides derived from the putative NP protein made in the cell-free system. (C) Tryptic peptides derived from the authentic M virion protein. (D) Tryptic peptides derived from the putative M protein made in the cell-free system. Tryptic digests were obtained as described in Fig. 3. Conditions for thin-layer ionophoresis and chromatography on silica gel thin-layer plates were exactly as described in Materials and Methods. A schematic tracing is presented below each autoradiogram to facilitate the comparison of the patterns. All the numbered spots were found in two comparable patterns (A and B, or C and D). In A and B spots 16, 17, and 18 are double.*

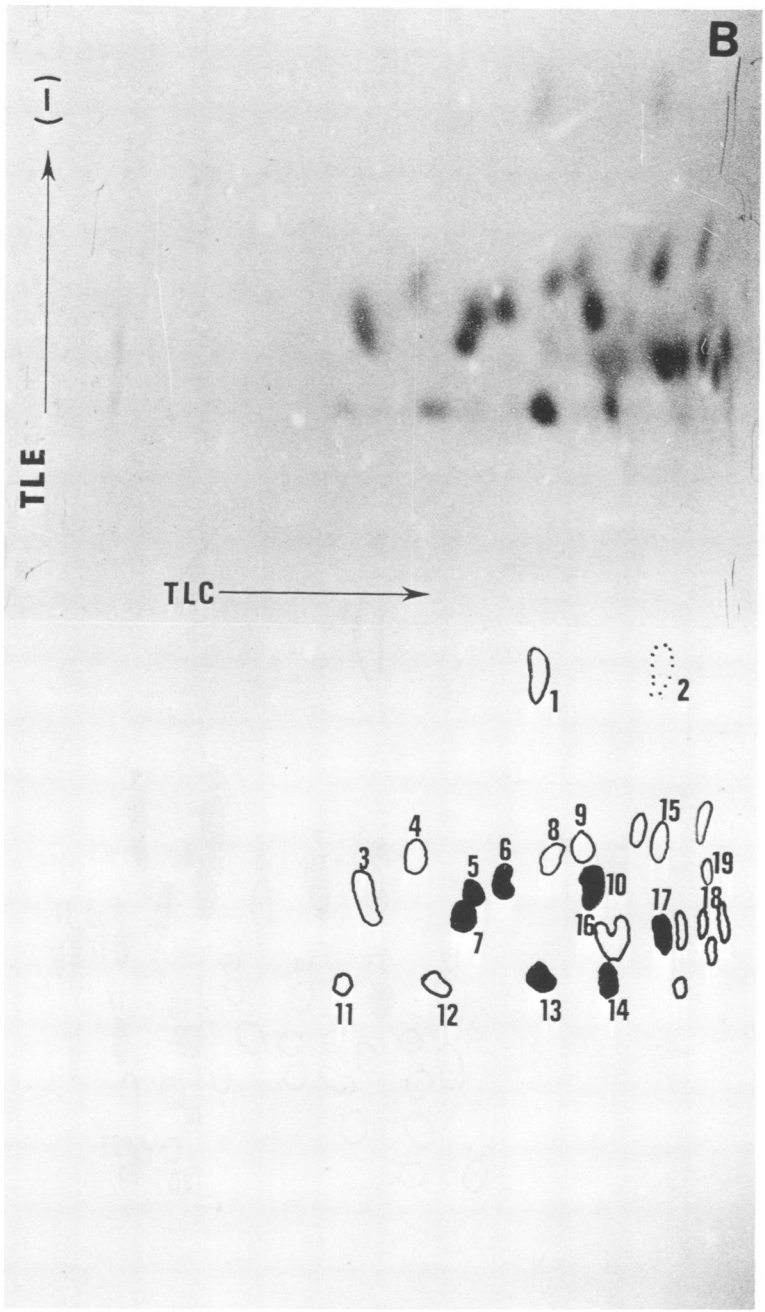


FIG. 4B

lose-retained RNA was further bound to a poly(U)-Sepharose column. Three fractions were obtained: (i) unbound; (ii) bound and eluted in low salt at 20 C; (iii) bound and eluted in low salt at 45 C, according to Nokin et al. (28). After chromatography of the active mRNA preparation on poly(U)-Sepharose, the three fractions obtained were assayed in the wheat germ system and ^{35}S-labeled products were analyzed as before by polyacrylamide gel electrophoresis.

Figure 5 shows that a significant proportion of the M and NP mRNA's was not retained on this column, but that most of the poly(U)-Seph-

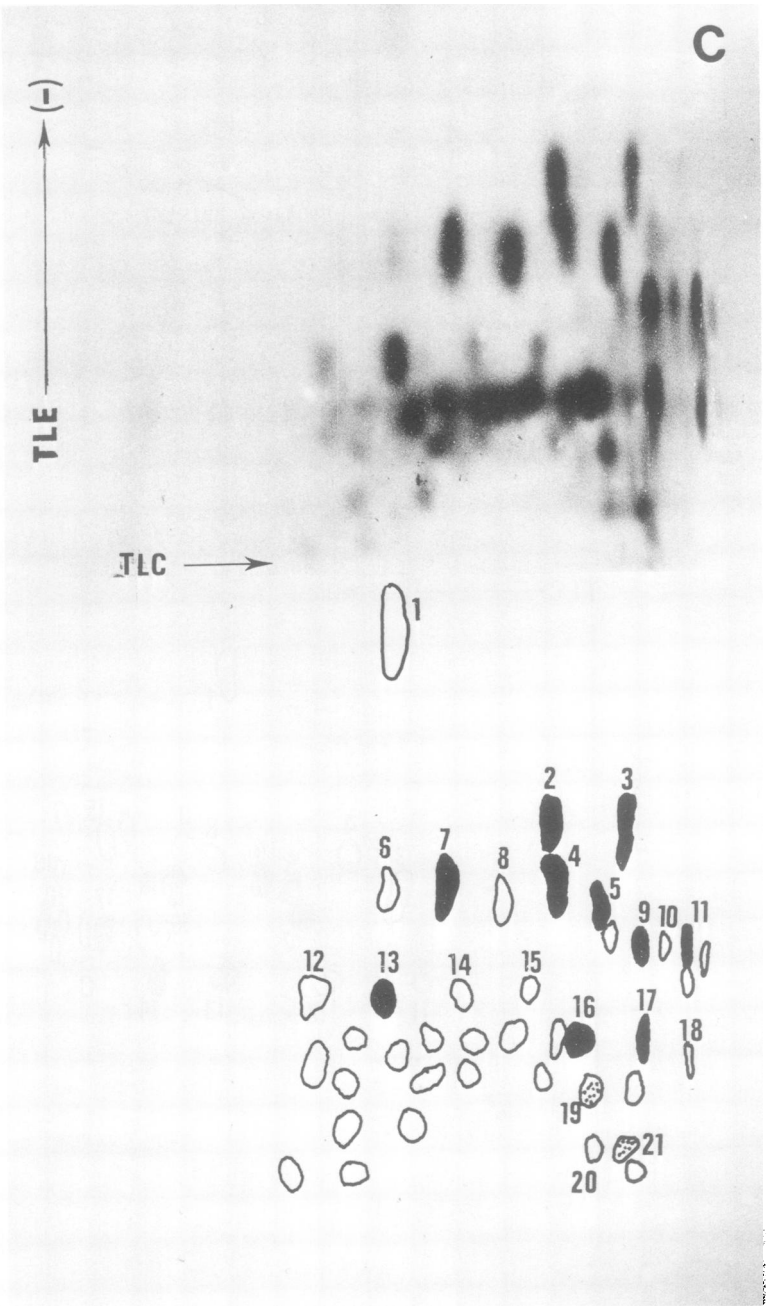


FIG. 4C

arose-selected material eluted from the column at 45 C.

It can be concluded that at least 50% of the mRNA's responsible for the in vitro synthesis of proteins M and NP contains long poly(A) stretches. However, considerable heterogeneity exists in the size of the poly(A) chain, since we have been able to detect some viral mRNA activity in non-oligo(dT)-cellulose-bound, sucrose gradient-fractionated RNA from the same infected cell (not shown). This is probably a very general observation among eukaryotic mRNA's (25).

Can virion RNA serve as an mRNA? Virion

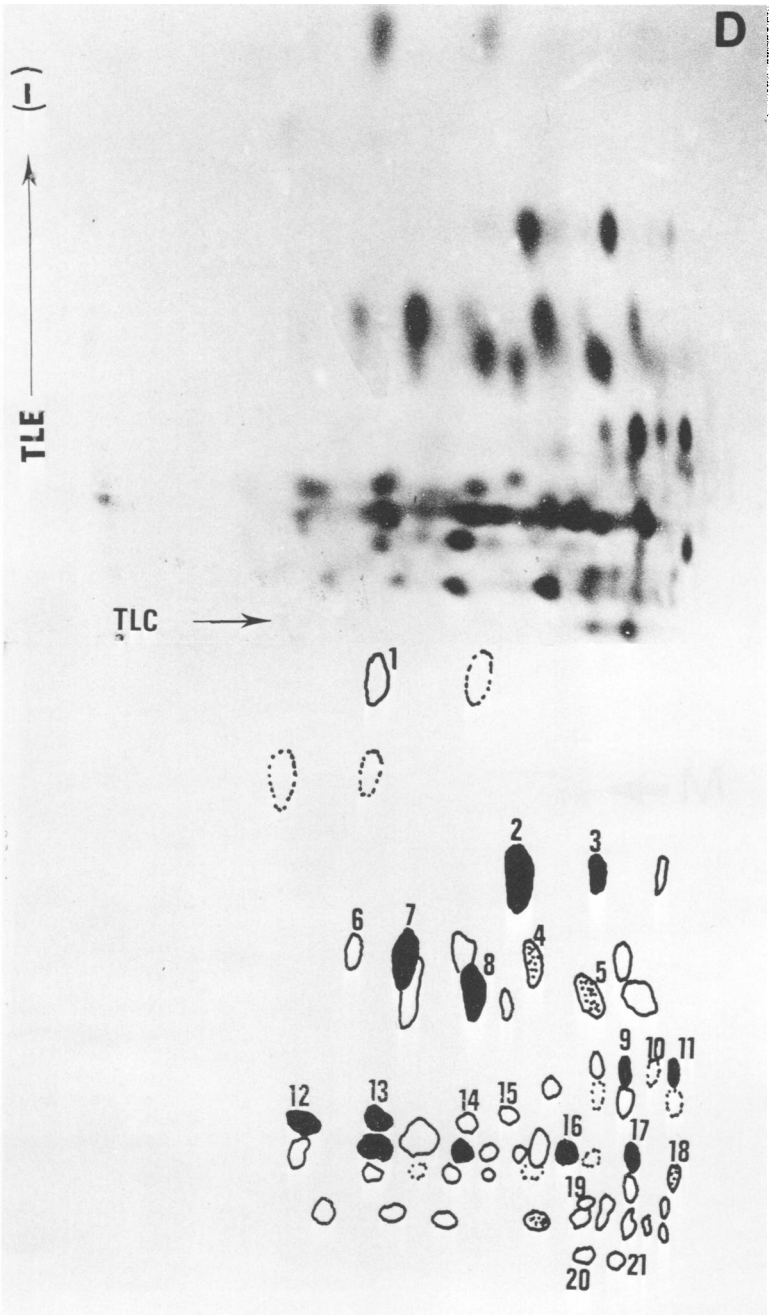


FIG. 4D

RNA was extracted from purified, egg-grown FPV. When this RNA was ionophorized on 6 M urea-agarose-acrylamide gel it showed a typical distribution of eight different segments, with molecular weight ranging between about 3.0×10^5 and 1.2×10^6. No evidence of degradation of this RNA is apparent on the gel (Fig. 6). As indicated in Fig. 1, this RNA can stimulate to some extent the incorporation of [^{35}S]methionine into protein when added to the wheat germ system, but this occurs with a much lower efficiency, on a weight basis, as compared to the poly(A)-rich RNA extracted from the cytoplasm of infected cells. This is even more

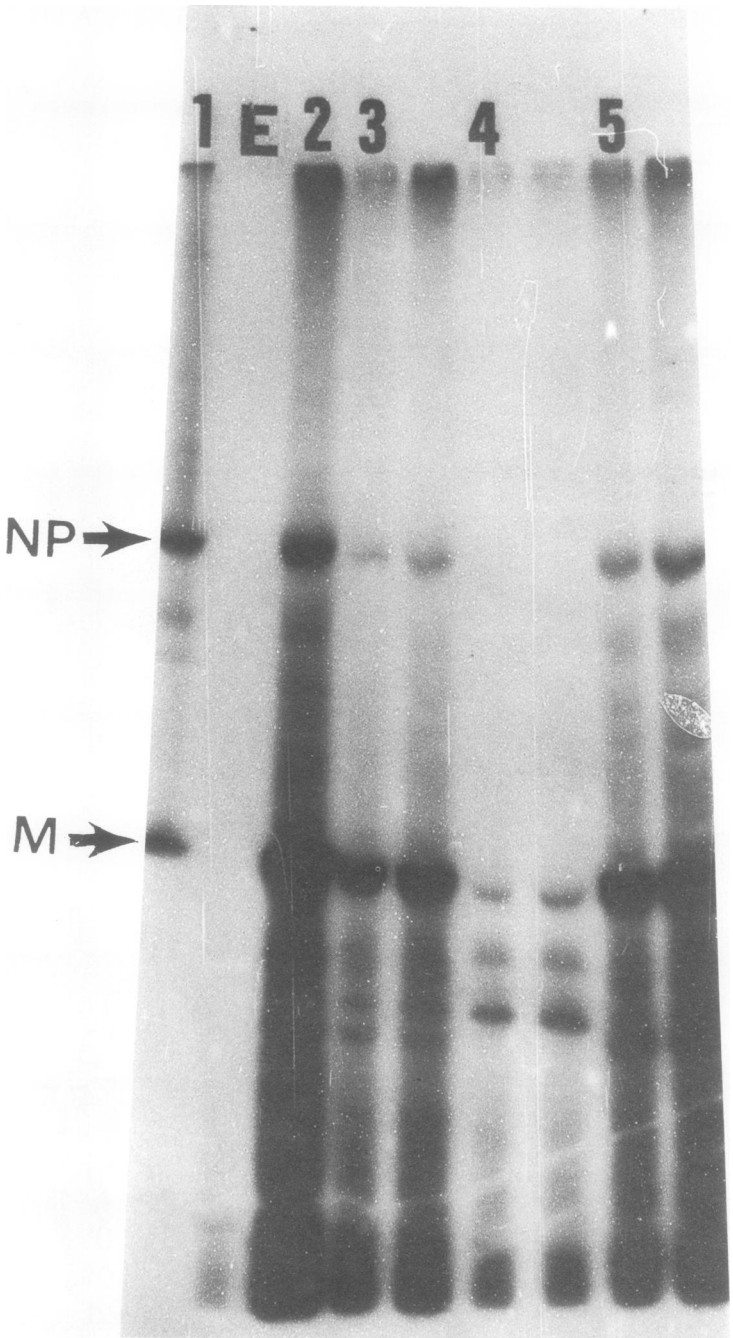
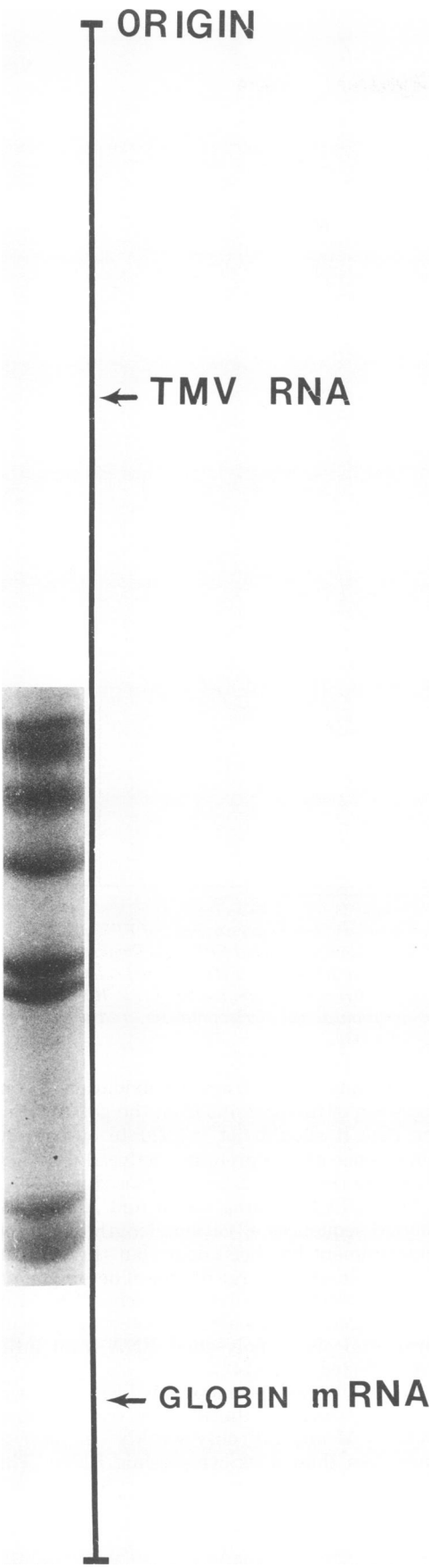


FIG. 5. *Autoradiograms of sodium dodecyl sulfate-polyacrylamide gel electrophoretic slabs of polypeptides directed by poly(U)-Sepharose-fractionated, poly(A)-rich RNA from FPV-infected cells in the wheat germ system. Cytoplasmic RNA from FPV-infected chicken embryo fibroblasts was extracted 16 h after infection and enriched in poly(A)-containing mRNA by oligo(dT)-cellulose chromatography. This material was then adsorbed on poly(U)-Sepharose in Tris-hydrochloride (0.01 M, pH 7.5), NaCl (0.3 M), EDTA (1 mM), and sodium dodecyl sulfate (0.2%) and eluted in the same buffer without NaCl, first at 20 C and then at 45 C. The three fractions were precipitated with ethanol, and various amounts were added to the wheat germ system. Products were analyzed as described in Fig. 2. (1) [^{35}S]methionine proteins from marker FPV; (E) wheat germ system, endogenous activity: no mRNA added; (2) 1 μg of oligo(dT) retained infected-cell RNA; (3) fraction of this RNA not retained on poly(U)-Sepharose: 2 and 4 μg/50 μl; (4) fraction of this RNA eluted from the poly(U)-Sepharose in low-salt buffer at 20 C: 2 and 4 μg/50 μl; (5) fraction of this RNA eluted from the poly(U)-Sepharose in low-salt buffer at 45 C: 2 and 4 μg/50-μl reaction mixture.*



striking if one considers that virion RNA is nearly pure, whereas at least 50% of the cytoplasmic oligo(dT)-bound RNA is still rRNA (Content, unpublished data). When the products of such a reaction are analyzed by polyacrylamide gel electrophoresis, a 10 times longer exposure of the autoradiograms is needed to detect one band, which coincides with protein M, and a second very weak band with the same electrophoretic mobility as NP (Fig. 7).

In addition, although at least a large fraction of the cytoplasmic viral mRNA contains poly(A), it has been demonstrated that influenza virion RNA does not contain any poly(A) sequences. For these two reasons, and since influenza RNA can often be contaminated with detectable amounts of 28*S* and 18*S* rRNA (11), it seems justified to propose that the low level of activity detected here with virion RNA might be due to some degree of contamination of the preparation (or of the virion itself) by cytoplasmic mRNA. Since only part of the cytoplasmic mRNA contains some poly(A) sequences, oligo(dT)-cellulose chromatography would not be an efficient way to eliminate this possible contamination.

DISCUSSION

We have shown that a poly(A)-rich fraction of cytoplasmic mRNA extracted from FPV-infected cells can be translated in vitro in the wheat germ system. Two major components have been obtained in vitro, which correspond to the two main virion structural proteins and also represent more than 60% of the total protein synthesized in the infected cells 6 h after infection (Fig. 2B).

The two proteins synthesized in vitro have been identified with their two authentic viral protein counterparts by paper ionophoresis and also by two-dimensional separation of their tryptic peptides. Few additional peptides were found in each case among the in vitro products. This could be due to incomplete separation from some adjacent labeled protein (during the preparative gel electrophoresis); in particular, protein M might be associated with a nonstruc-

FIG. 6. *Polyacrylamide gel electrophoresis of FPV RNA. A 5-μg amount of RNA extracted from purified FPV was applied on a cylindrical composite gel (12 by 0.6 cm) containing 2% polyacrylamide, 0.6% agarose, 6 M urea (16). Electrophoresis was for 8 h at 80 V, +4 C. For comparison purified globin mRNA (mol wt, 225,000) and tobacco mosaic virus RNA (mol wt, 2.0 × 10⁶) were run in a parallel gel. The gels were stained with "Stains all" (Serva, Feinbiochemica, Heidelberg) as described previously (13). Only the central part of the gel was photographed.*

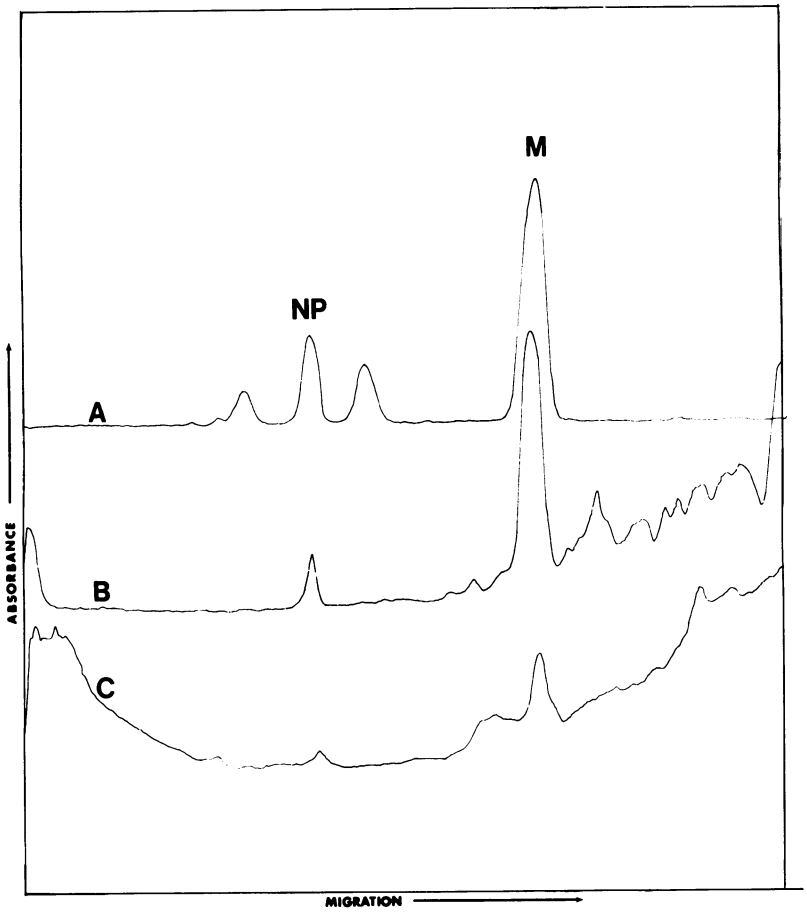


FIG. 7. *Microdensitometer tracing of autoradiograms of polyacrylamide gel analysis of FPV proteins. (A) Authentic FPV marker proteins obtained from purified [^{35}S]methionine-labeled FPV. (B) Proteins made in a cell-free extract of wheat germ directed by poly(A)-rich cytoplasmic RNA from FPV-infected cells (2 μg/50-μl reaction). (C) Proteins made in a cell-free extract of wheat germ directed by purified FP virion RNA (4 μg/50-μl reaction). Experimental conditions for incubation, polyacrylamide gel electrophoresis, and densitometry were as described. Exposure time was 7 h for (B) and 72 h for (C).*

tural protein (NS_1) not resolved on our gels and migrating slightly ahead of protein M (26, 40). This will have to be investigated in more detail. Alternatively, the products synthesized, in vitro, might be slightly larger than the "final" viral protein because some processing mechanism may be lacking in vitro. There is, however, no good evidence for cleavage of such a precursor in vivo (8, 18, 39) except for the virion hemagglutinin (HA) component, which is processed by cleavage into the two hemagglutinin subunits HA1 and HA2. The total number of peptides observed in the case of protein M also seems larger than expected for a protein of this size (a maximum of 14 oligopeptides should be obtained from a protein of 28,000 daltons containing 5% methionine). Some of the spots observed may result from air oxidation during digestion of the proteins from the gel. If this is the case it should not invalidate our conclusions, since all the proteins analyzed were submitted to the same digestion procedure.

The mRNA's coding for M and NP contain poly(A) sequences of various length. No direct measurement has been done, but the data are compatible with a large degree of heterogeneity in the size of these poly(A) stretches. This kind of heterogeneity was already detected by structural analysis in polysomal RNA from influenza-infected cells (14).

RNA extracted from a purified FP virion was found to stimulate the in vitro translation of proteins M and NP only weakly (at least 24 times less than does cytoplasmic RNA). This

very low activity is probably due to some contamination of virion RNA with cytoplasmic mRNA, which is picked up here because of the high sensitivity of the wheat germ system. Alternatively, it might be considered that virion RNA really corresponds to the mRNA strand but has to be processed in the cell to become fully active. The virion RNA tested in these experiments might have been partially processed, thus accounting for its low level of activity. There is, however, no good evidence to favor the latter hypothesis. Siegert et al. (38) showed some mRNA activity in a cell-free system of *Escherichia coli* when using influenza virion RNA. One of their translation products had the same electrophoretic mobility and antigenicity as the authentic virion NP. No comparative assay was shown in their study concerning the translation of infected-cell cytoplasmic RNA. Their observation is confirmed here in a different cell-free system, but we suggest the above-mentioned alternative explanation. Kinsbury and Webster (21) compared, in a cell-free rabbit reticulocyte system, the in vitro translation of total influenza-infected cell RNA and virion RNA. Although their system seems less efficient than ours and thus less sensitive, they observed the synthesis of protein M, as detected by its electrophoretic mobility and antigenicity. No stimulation was observed when they used virion RNA in their in vitro system.

Since only two of eight viral-coded peptides were translated in vitro in our system, we have not established whether all the monocistronic RNA segments from the virion are negative stranded (i.e., complementary to their respective mRNA's; 3). It is likely that our failure to detect in vitro P1 and P2 and also to some extent the two expected glycoproteins HA (precursor of HA1 and HA2) and NA reflects a lower concentration of the corresponding mRNA's since, first, it is obvious, in Fig. 2B, where in vivo labeled cytoplasmic products are examined at different times after infection, that some of the proteins (M and NP) are labeled more intensively than any others. As noticed by Skehel (39), some regulation exists in the infected cells, which coordinates the level of the different proteins to maintain them in a proportion similar to that found in the virus itself. Moreover, Skehel (40) has shown that a temporal regulation exists in the synthesis of the different viral proteins and has presented evidence indicating that some of the control is at the transcription level. Second, Morrison and Lodish (in press) observed in vesicular stomatitis virus-infected cells that the mRNA responsible for the synthesis of viral glycoprotein G is found exclusively on the membrane-bound polysomes.

It may thus be worthwhile to attempt the purification of some high-molecular-weight viral mRNA's according to their size to obtain an enrichment of a specific class of mRNA. Indeed it is conceivable that some competition exists as well in vitro among different mRNA's, increasing even more the preferential translation of M and NP. These proteins are translated better in vitro than any of the host cell proteins. (Table 1; Fig. 2B).

In a preliminary attempt to fractionate our active mRNA preparation in an aqueous sucrose gradient we have already been able to enrich considerably the preparation for its NP versus M translating capacity. Another approach would be to extract poly(A)-rich RNA from purified membrane-bound polysomes found in infected cells, as described by Morrison and Lodish (in press) in the case of vesicular stomatitis virus.

Only after all eight viral-coded peptides have been synthesized in vitro, and possibly on isolated purified mRNA's, will it be possible to ascribe biochemically a discrete coding function to each of the monocistronic segments of influenza virus genome.

After submission of this work, a paper was published by Etkind and Krug (15), whose conclusions are in agreement with ours.

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