Cell-Free Translation of Paramyxovirus Messenger RNA

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Polypeptides corresponding in electrophoretic mobility to virion polypeptides 1, 3, and 5 were made in a reticulocyte cell-free system to which 18S RNA from Sendai virus-infected cells was added. Immune precipitation was used to select relevant polypeptides from endogenous products. The cell-free product corresponding to virion polypeptide 3 (the nucleocapsid structure unit) was the most abundant; its tryptic peptides comigrated electrophoretically with tryptic peptides of polypeptide 3 isolated from virions. Other sedimenting classes of RNA from infected cells were tested; only the 28S fraction showed slight activity. Virion 50S RNA was inactive. These findings support the hypothesis that complementary RNA transcripts of paramyxovirion RNA are the templates for viral proteins.

There is much evidence that the single-stranded RNA in paramyxoviruses (5) is not messenger RNA (1, 15). (i) Isolated virion RNA is not infectious (13). (ii) Virions contain an RNA-directed RNA polymerase which makes complementary RNA species smaller than virion RNA in vitro and in vivo (11, 26, 27, 31). (iii) Infected cells contain large amounts of complementary RNA species (most of which sediment at about 18S) late in infection (2, 4, 14, 21); these are the only virus-specific RNAs known to be associated with polyribosomes (3, 4); they contain polyadenylic acid (23), an indication of messenger function, whereas virion RNA does not (8). But a conclusive demonstration of messenger function requires translation of an RNA in a reconstituted cell-free protein synthesizing system. This report describes the synthesis of Sendai virus structural proteins in a rabbit reticulocyte lysate directed by 18S RNA from infected cells. As predicted, virion RNA was inactive in the same system.

MATERIALS AND METHODS

Virus. Previous reports describe cultivation of the clone of Sendai virus used in this work (22, 31).

Virion RNA. Unlabeled 50S RNA was isolated from egg-grown virions by sodium dodecyl sulfate (SDS)-phenol extraction and agarose chromatography (13, 22). The RNA was precipitated with ethanol, washed with ethanol, and stored as described below.

RNA from infected cells. Chicken embryo lung (CEL) cell monolayer cultures, 100 mm in diameter, were inoculated with 1 to 10 plaque-forming units of

Sendai virus per cell and were incubated at 30 C in Eagle minimal essential medium (Earle saline base) supplemented with 3% heat-inactivated fetal calf serum, penicillin, streptomycin, and Mycostatin in 5% CO₂ in air. At 48 h, the cells were scraped into phosphate-buffered saline, collected by centrifugation (500 × g, 4 C, 5 min), and suspended in 1 ml of 0.01 M sodium acetate and 0.05 M sodium chloride (pH 5.2) per culture. SDS was added to give 0.017 M and the mixture was shaken with an equal volume of phenol at 50 C (14, 29). A second phenol extraction was done at 22 C. The aqueous phase was made 0.1 M in sodium acetate (pH 5.0); 3 vol of ethanol was added, and the mixture was placed at -20 C for 16 h or longer. Precipitated RNA was collected by centrifugation at 12,000 × g, 4 C, for 15 min, washed three times with absolute ethanol, dried under vacuum, dissolved in autoclaved water, and stored at -60 C. About 100 μg of RNA were obtained from each culture.

Cell-free protein synthesis. Rabbits were treated with phenylhydrazine for 6 days as described by Gilbert and Anderson (7) and bled by cardiac puncture on the 8th day, when reticulocytosis was greater than 90%. Reticulocyte lysate (10) was stored in a liquid-nitrogen freezer. Reaction mixtures contained the ingredients specified by Housman et al. (10), with the addition of 500 μCi of ³H-amino acid mixture (Schwarz/Mann, catalog no. 3130-08) per ml. RNA solution or water (for endogenous reactions) represented 18% of the final reaction volume. Incubation was at 22 C for 90 min.

Antisera. Rabbit serum with antibodies against Sendai virion polypeptides was prepared as follows. Purified egg-grown Sendai virions (31) were suspended in 0.01 M sodium phosphate (pH 7.2) at a concentration of 2 mg of virion protein per ml and dialyzed against 1 M KCl in 0.01 M sodium phosphate

(pH 7.2). Triton X-100 was added to a final concentration of 2% (28). After 20 min at 23 C, 10 vol of ethanol was added, and the mixture was placed at -20 C for 24 h. The precipitate was collected by centrifugation, suspended in phosphate buffer, and dialyzed against phosphate buffer containing 0.17 M NaCl. The antigen was mixed with complete Freund adjuvant and 2 mg of viral protein was inoculated into each rabbit, the dose divided equally among a foot pad and two leg muscles. A month later the intramuscular inoculations were repeated, and an intravenous injection was given. Serum, collected 7 days later, had a hemagglutination-inhibition titer of 500, and precipitating antibodies against viral envelope polypeptides (28) and nucleocapsids were detected, although this was not determined quantitatively.

Goat serum with antibodies against rabbit gamma globulin was a gift of Luis Borella.

Immune precipitation. Triton X-100 (final concentration 0.5%) and 2 μ liters of rabbit anti-Sendai virion serum were added to 70 to 150 μ liters of reaction mixture. After incubation at 22 C for 30 min, 40 μ liters of goat anti-rabbit serum was added, and incubation was extended for 2 h more at 22 C. Immune precipitates were collected by centrifugation and washed three times with 0.01 M sodium phosphate, 0.15 M NaCl, 0.01 M EDTA, and 1% Triton X-100 (pH 7.2). Precipitates were dissolved in 0.01 M sodium phosphate and 0.034 M SDS (pH 7.2) at 100 C for 2 min. Incubation of reaction mixtures with antibodies at 37 C (9) or in the presence of 0.5% sodium deoxycholate (20, 24) were found to increase nonspecific precipitation, and were therefore avoided.

Polyacrylamide gel electrophoresis. Immune precipitates containing 50 to 100 μ g of protein were electrophoresed in 6-mm diameter 10% polyacrylamide gels, and the gels were sliced and processed for counting as described (30).

Tryptic peptide analysis. 14 C-amino acid-labeled Sendai virion polypeptides were separated in 6-mm polyacrylamide gels (30). 3 H-amino acid-labeled immune-precipitated products of a 1-ml reaction programmed by 18S RNA from infected cells were separated in a 25-mm diameter gel. In both cases, radioactive polypeptides were eluted by incubating gel slices in 0.01 M sodium phosphate, 0.003 M SDS, and 0.001 M NaN₃ (pH 7.2) at 37 C for 24 h. Gel fragments were removed by passing the eluates through type HA membrane filters (Millipore Corp.). Polypeptides were reduced and alkylated (12) in the presence of 100 μ g of ovalbumin (Worthington) and concentrated by precipitation with 15% trichloroacetic acid. Three washes with 5% trichloroacetic acid and three washes with ethanol were followed by drying under vacuum. Each sample was suspended in 1 ml of 0.1 M NH₄ HCO₃ (pH 8.0) and digested with 10 μ g of TPCK-treated trypsin (Worthington) for 3 h at 37 C. An additional 5 μ g of trypsin was then added, and incubation continued for 12 h. Samples were dried at 60 C in a stream of dry nitrogen, dissolved in pyridine-acetic acid-water (100:4:896, pH 6.4), and separated in the same buffer on Whatman 3MM paper at 2,500 V, 10 C, for 2 h. The dried paper was cut into 1-cm segments which were placed in liquid

scintillation counting vials containing 1 ml of 0.1 M NaOH. After 30 min at 22 C, 10 ml of PCS (Amersham/Searle) were added, the mixtures were shaken, and they were counted after 24 h, by which time chemiluminescence had decayed to an undetectable level.

RESULTS

Messenger activity in total RNA from infected cells. Schimke and co-workers (20, 24) have shown that a messenger RNA need not be purified for cell-free translation if there is a way, such as immune precipitation, to isolate the relevant product. Apparently, ribosomal RNA in large amounts does not interfere. The simplicity of this approach is particularly advantageous in work with paramyxoviruses, because they grow poorly in suspension cultures, which is a handicap to producing large amounts of polyribosomes. In addition, large amounts of candidate paramyxovirus message are not polyribosome associated (4, 15).

Accordingly, as infected CEL cell monolayer cultures became available, they were extracted as described in Materials and Methods, and the RNA was kept as a precipitate in ethanol at -20 C until several milligrams were accumulated. When this material was added to the reticulocyte protein-synthesizing system, several peaks of radioactivity were seen after gel electrophoresis of the immune precipitate (Fig. 1). These correspond in mobility to virion polypeptides 1, 3, and 5, with a suggestion of material in the region of polypeptide 6. Polypeptide 1 is implicated in viral transcriptase function (30); polypeptide 3 is the nucleocapsid structure unit (18); there are several polypeptides in region number 5, at least one of which is glycosylated (19); and polypeptide 6 probably resides on the inside of the viral envelope (19).

Much less radioactivity was present in a gel separation of the immune precipitate of an endogenous reaction. The only discernible peak was in the region of virion polypeptide 1 (Fig. 1); this probably represents the "70,000" molecular weight reticulocyte polypeptide described by others (17, 20).

Messenger activity of virion RNA and cell RNA fractions. To learn which sedimenting class of RNA contained messenger activity, total cell RNA was centrifuged in sucrose gradients, and fractions were taken as shown in Fig. 2. The 18S pool was active in stimulating amino acid incorporation into polypeptides which migrated electrophoretically like virion polypeptides 1, 3, and 5 (Fig. 3); material like polypeptide 6 was not clearly resolved.

As with reactions directed by total cell RNA,

FIG. 1. Polyacrylamide gel electrophoresis of polypeptides in immune precipitates of cell-free reactions. (O) Endogenous reaction, no added RNA; (●) 1,020 μ g of total RNA from Sendai virus-infected cells added per milliliter. The numbers in this and later figures refer to the positions of virion polypeptides as assigned by Mountcastle et al. (18). In Fig. 1, 3, and 4 these positions were determined in stained gels run in parallel.

FIG. 2. Separation of RNA from Sendai virus-infected cells in a sucrose gradient. About 2 mg of RNA in 2 ml of 0.005 Tris-hydrochloride, 0.001 M EDTA, 0.1 M NaCl, and 0.017 M SDS (pH 7.4) were layered on a 34-ml linear 15 to 30% sucrose (wt/vol) gradient in the same buffer and centrifuged at 18,000 rpm, 20 C, for 16 h. Collection was from the top, with continuous automatic monitoring of ultraviolet absorbance. The indicated volumes were collected separately, precipitated with ethanol, and prepared for cell-free protein synthesis as described in Materials and Methods.

there was little or no radioactivity in the position of virion polypeptide 2, the major virion glycopolypeptide (19).

Virion 50S RNA was inactive. The acrylamide gel pattern of a reaction receiving virion RNA could not be distinguished from the en-

dogenous pattern (Fig. 3).

Also inactive, giving results identical to endogenous reactions, were the 4S and "50S" peaks from infected cells (data not shown).

The 28S pool of cell RNA directed the synthesis of small amounts of polypeptides which

FIG. 3. Electrophoresis of immune precipitates of reactions programmed by 33 μ g of 50S RNA from Sendai virions per milliliter (O); 490 μ g of 18S RNA from infected cells per ml (●).

FIG. 4. Electrophoresis of the immune precipitate of a reaction programmed by 480 μ g of 28S RNA from infected cells per milliliter.

migrated like virion polypeptides 1 and 3 (Fig. 4), suggesting that some of the more rapidly sedimenting viral messenger RNAs spilled over into this fraction.

Table 1 summarizes the total incorporation in the various reactions and the yields in the

TABLE 1. *Effects of RNA fractions on synthesis of protein and anti-Sendai virion-precipitable radioactivity in the reticulocyte lysate system*

RNA added	$\mu\text{g/ml}$	Total counts/ min $\times 10^{-3}/$ 0.1 ml ^a	% Precip- itated ^b
None		8.4	0.70
Total cell	1,020	6.6	2.0
4S cell	560	5.2	1.3
18S cell	490	7.4	2.4
28S cell	480	6.6	1.1
"50S" cell	52	7.7	1.0
50S virion	33	7.1	0.85

^a Hot trichloroacetic acid-precipitable radioactivity.

^b Precipitated by anti-Sendai virion serum as described in Materials and Methods.

immune precipitates. It can be seen that all added RNA species depressed overall incorporation moderately and that differences between immune precipitates of active and inactive reactions (with respect to virion polypeptide synthesis) were not as marked as in the acrylamide gel analyses. Presumably, contaminating globin, which migrated out of the gels, accounts for the differences between total radioactivity precipitated and radioactivity recovered in the gels.

Authenticity of the cell-free products. Co-electrophoresis of ¹⁴C-virion polypeptides with the immune-precipitated ³H-products of a reaction directed by 18S RNA from infected cells revealed close correspondence of peaks 1, 3, and 5 (Fig. 5).

More evidence was obtained by analysis of tryptic peptides of polypeptide 3, the most abundant product. As shown in Fig. 6, the polypeptide made in vitro closely resembled the nucleocapsid structure unit from virions. Differences in relative peak heights can be ascribed to differences in specific activities of individual

FIG. 5. ³H-polypeptides synthesized in a cell-free reaction containing 18S RNA from infected cells (●) electrophoresed in the same gel as ¹⁴C-polypeptides from Sendai virions (○).

amino acids in the two labeled amino acid mixtures used and to effects of cell pools on ^{14}C -amino acid labeling of virions.

DISCUSSION

50S Sendai virion RNA is probably a mixture of two kinds of molecules of opposite polarity, the major species being complementary to the virus-specified 18S RNA of infected cells (21, 25). It seems clear that neither kind of 50S RNA is operative as messenger for the virion polypeptides precipitated by the antiserum used in this study. This does not rule out the possibility that other viral polypeptides might be templated by 50S RNA.

Although 50S RNA from virions or infected cells was used at about 3 to 6% of the concentration of the other RNA preparations, this should have been enough for a fair test, at least of virion RNA, which is exclusively virus-specific, whereas 18S virus-specific RNA certainly represents less than 3% of the total RNA in infected cells. I have determined the amount of RNA from infected cells which binds to cellulose (22; D. W. Kingsbury, unpublished data), presumably because of its poly A content (16, 23). Only

about 0.5% of the total cell RNA bound to cellulose, and this probably includes cellular adenylylate-rich RNA as well as the majority of the viral 18S RNA (22). This material has not yet been tested in the reticulocyte system.

The 18S RNA fraction from infected cells contained the most active template. This shows that viral messenger RNA is *smaller* than virion RNA. There is already ample evidence that the virus-specific RNA which sediments at 18S is exclusively *complementary* to the major species of virion RNA (2, 4).

Double-stranded and partially double-stranded RNA species, representing template-product complexes involved in replication and transcription of viral RNA, sediment well ahead of 18S RNA; they should be present mainly in the 28S pool (22). This pool was relatively inactive, either because there is little messenger RNA that sediments that rapidly or because of inhibitory effects of double-stranded RNA on protein synthesis (6). The latter explanation seems less likely, because overall incorporation was not preferentially depressed by the 28S pool.

What was not identified among the cell-free products is as interesting as the things that

FIG. 6. High-voltage electrophoresis of ^3H -tryptic peptides (●) from polypeptide 3 made *in vitro* and ^{14}C -tryptic peptides (○) from polypeptide 3 isolated from virions. The origin is fraction 25, and the anode is at the left.

were. Nonstructural viral proteins unrelated antigenically to virion polypeptides would not have been recognized by the antiserum, and will have to be looked for by other means. Polypeptide 6 was not clearly seen; it will be necessary to learn if the antiserum contains enough antibody to it. Polypeptide 2, the major glycopolypeptide of virions, appeared to be absent. It may have been made, but not glycosylated, and therefore may lack the requisite antigenic sites. Or it may have been precipitated by antibody, but migrated anomalously under another component; more detailed peptide analysis may resolve this possibility. The most interesting possibility is that it was not made, suggesting that glycosylation and translation are coupled. Support for this idea comes from recent work on the cell-free translation of vesicular stomatitis virus complementary RNA, where little, if any, of the glycosylated virion polypeptide was produced, despite efficient synthesis of all the other major viral polypeptides (T. Morrison, et al., 1973, in press). In the Sendai virus system, the synthesis of polypeptide migrating like the minor virion glycopolypeptide number 5 argues against this idea, but all of the virion polypeptides which appear in this region of a gel are not necessarily glycosylated (19).

B. S. Collins and M. A. Bratt (Proc. Nat. Acad. Sci., in press) have recently separated Newcastle disease virus (NDV) complementary RNA into several species which differed in abundance, and there were correlations between the size and abundance of each RNA species and the size and abundance of NDV polypeptides. This suggests that paramyxovirus messenger RNAs are monocistronic. The cell-free system provides a way to test this and to identify the message for each polypeptide. It is noteworthy that no polypeptides larger than known virion components were seen in the Sendai virus cell-free system, arguing against a cleavage mechanism in the production of polypeptides 1, 3, and 5.

Assuming that each product of the cell-free system is identical to the virion polypeptide with the same electrophoretic mobility, it appears that the proportions of the cell-free products are not the same as the proportions of polypeptides in virions (Fig. 5). This is most marked with respect to polypeptide 1, which was more abundant in the cell-free product. But infected cells contain relatively more polypeptide 1 than virions do (30). Thus, the reconstituted protein-synthesizing system may indeed reflect messenger abundance or other determinants of messenger efficiency that prevail in intact cells.

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