Evidence that Vesicular Stomatitis Virus Produces Double-Stranded RNA That Inhibits Protein Synthesis in a Reticulocyte Lysate

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Cell-free protein synthesis by reticulocyte lysates was inhibited by a polyadenylated RNA fraction extracted from HeLa cells infected with vesicular stomatitis virus (VSV) but not by polyadenylated RNA from mock-infected HeLa cells. A similar inhibitor of cell-free protein synthesis was found in a polyadenylated fraction of RNA transcribed *in vitro* by VSV but not in untranscribed nucleocapsids. Fractionation of the VSV transcription product showed that the translation inhibitor segregated with nucleocapsids containing newly transcribed polyadenylated or non-polyadenylated RNA, as determined by oligodeoxythymidylate-cellulose chromatography. The inhibitors present in VSV-infected HeLa cells and in VSV *in vitro* transcripts both appeared to be double-stranded RNA, as judged by the characteristics for inhibition of reticulocyte cell-free protein synthesis described by Hunter et al. (*J. Biol. Chem.* **250**:409-417, 1975). The double-stranded nature of the VSV RNA inhibitor was supported by the finding that the translational inhibitory effect was inactivated by melting the inhibitor in the absence of salt and by micrococcal nuclease.

Infection with wild-type vesicular stomatitis virus (VSV) results in rapid inhibition of cellular protein synthesis, a genetically determined event that requires functional viral transcription (18). This inhibitory effect appears to be directed at the initiation of translation (23), but the underlying mechanism is not understood. It has been clearly shown, however, that double-stranded RNA (dsRNA), natural or synthetic, can serve as a potent inhibitor of protein synthesis in various *in vitro* translation systems derived from mammalian cells (10, 15, 28). Protein synthesis in reticulocyte lysates is inhibited by dsRNA, which activates a protein kinase which phosphorylates the small subunit of eucaryotic initiation factor 2 (12). In addition, dsRNA has been shown to be capable of inducing interferon (7) and activating interferon-induced enzymes which, in turn, inactivate eucaryotic initiation factor 2 and activate an endonuclease and perhaps other enzymes which inhibit protein synthesis (3, 21, 27).

Viral dsRNA may play a role in the inhibition of protein synthesis in cells infected with certain viruses. The presence of dsRNA has been detected in cross-linking studies in HeLa cells infected with encephalomyocarditis virus or the tsG114 mutant of VSV (22). mRNA prepared from reovirus transcribed *in vitro* and polyadenylated [poly(A)⁺] RNA from vaccinia virus

transcribed *in vitro* have been shown to contain dsRNA which also inhibits protein synthesis in cell-free systems (1, 19).

We report here the presence in VSV-infected HeLa cells but not in uninfected cells of an inhibitor of cell-free protein synthesis which behaves like dsRNA. We also found a similar dsRNA inhibitor of protein synthesis after *in vitro* VSV transcription of poly(A)⁺ and poly(A)⁻ RNA associated with viral nucleocapsids.

MATERIALS AND METHODS

Cell cultures and viruses. The wild-type San Juan strain of the Indiana serotype of VSV was originally obtained from the U.S. Agriculture Research Center, Beltsville, Md. (29).

BHK-21 cells were cultivated as previously described (2). VSV was grown in BHK-21 cells as outlined previously (9). HeLa S3 cells were grown in suspension cultures in Joklik modified minimum essential medium (MEM) with glutamine (2 mM) and 10% calf serum.

Chemicals and radioisotopes. Rabbit globin mRNA was purchased from Bethesda Research Laboratories, Rockville, Md., and polyinosinate-polycytidylylate [poly(I):poly(C)] was purchased from Miles Biochemicals, Elkhart, Ind. [³⁵S]methionine (1,174.9 Ci/mmol), [³H]uridine (38.8 Ci/mmol), and uniformly labeled ³H-amino acids (>75 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass.

Preparation of poly(A)⁺ RNA. HeLa cells in 2-liter

suspension cultures were grown to a density of 4×10^5 cells per ml and harvested by low-speed centrifugation. Cells were then suspended in 200 ml of MEM and divided into two equal portions. One portion was mock infected with MEM, and the other was infected with VSV suspended in MEM at a multiplicity of infection of 10. After adsorption of the inoculum for 30 min at room temperature, each portion was diluted to a total volume of 1 liter with MEM. At 2.5 h postinfection (p.i.), [^3H]uridine was added to each culture to a concentration of 5 $\mu\text{Ci/ml}$. At 4.5 h p.i., both cultures were harvested by low-speed centrifugation, washed in phosphate-buffered saline, and subjected to detergent-phenol-chloroform extraction as described by Palmiter (24). After ethanol precipitation at -20°C overnight, each RNA preparation was subjected to oligodeoxythymidylate (oligo-dT)-cellulose chromatography, a second ethanol precipitation as described above, and another pass over oligo-dT-cellulose. The poly(A) $^+$ RNA was recovered by ethanol precipitation and suspended in sterile water, and absorption readings were determined at wavelengths of 260 and 280 nm.

In vitro translation reactions. Micrococcal nuclease-treated rabbit reticulocyte lysate was purchased from Amersham/Searle Corp., Arlington Heights, Ill., and translation reactions were performed in reaction mixtures essentially as described by Pelham and Jackson (25). Unless otherwise noted, translation reactions were run with K^+ concentrations of 133 mM and Mg^{2+} concentrations of 1.83 mM.

The in vitro translation reaction mixtures were incubated at 31°C for the times indicated in a total reaction volume of 25 μl . In addition to the reticulocyte lysate, the following preparations to be tested for their effect on translation were prepared by being lyophilized under nitrogen gas: mixtures of either ^3H -amino acids at 0.6 $\mu\text{Ci}/\mu\text{l}$ or [^{35}S]methionine at 1 $\mu\text{Ci}/\mu\text{l}$ and poly(A) $^+$ RNA from either mock- or VSV-infected cells or various fractions of RNA made in vitro during a VSV transcription reaction. Also present, where indicated, were lyophilized rabbit globin mRNA and poly(I):poly(C). Reactions were then initiated by adding 25 μl of micrococcal nuclease-treated reticulocyte lysate which had been previously combined with the reaction mix by the method described by Pelham and Jackson (25). The lysate also contained hemin, 0.02 mM; creatine phosphate, 10 mM; creatine kinase, 50 $\mu\text{g/ml}$; Ca^{2+} , 1 mM; EGTA [ethyleneglycol-bis(2-aminoethyl ether)- N,N' -tetraacetic acid], 2 mM; Mg^{2+} , 1.83 mM; and K^+ , 133 mM (except where indicated otherwise, when K^+ was added as the acetate salt). Translation reactions were terminated and assayed for acid-precipitable material by transferring either 2.0- or 5.0- μl portions into 100 μl of Laemmli buffer and then adding 1 ml of 10% trichloroacetic acid (TCA). Samples were then collected on Whatman GF/A filters, washed with 5% TCA and 95% ethanol, air dried, and counted in Beckman Ready-Solv EP scintillation fluid in a Beckman LS-230 scintillation counter.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Portions (15 μl) from the in vitro translation reactions were diluted with 50 μl of sample buffer containing 2% sodium dodecyl sulfate, 25 mM Tris (pH 6.8), 100 mM dithiothreitol, and 20% glycerol, with bromophenol blue. Samples were then kept at 100°C for 3 min and loaded on a

stacking gel consisting of 4% acrylamide, 0.2% bisacrylamide, 0.1% sodium dodecyl sulfate, and 0.125 M Tris (pH 6.8) for electrophoresis by the method of Laemmli (16) as modified by Carroll and Wagner (4). Samples were then run for 2.5 h at 200 V on a gel containing 12.5% acrylamide, 0.1% bisacrylamide, 0.1% sodium dodecyl sulfate, and 0.375 M Tris (pH 8.7). Gels were fixed, stained with Coomassie blue, destained, and dried onto Whatman 3MM filter paper. Autoradiography was done with Kodak SB-5 X-ray film.

In vitro transcription by VSV. The Indiana serotype of VSV was cultivated and purified from BHK-21 cells as previously described (9), and the protein concentration was determined by the method of Lowry et al. (17). As previously described (4), purified VSV at a protein concentration of 2.0 mg/ml in reticulocyte standard buffer, pH 8.0, with 15% glycerol was solubilized for 3 min at 4°C with an equal volume of Triton-high salt solubilizer consisting of Triton X-100, 4%; glycerol, 15%; NaCl, 1.4 M; dithiothreitol, 7.4 mM; and reticulocyte standard buffer (pH 8.0), 50%. To this mixture was then added 5 volumes of prereaction mixture consisting of 2.0 mM each of ATP, GTP, and CTP, 0.2 mM of [^3H]UTP (244 mCi/mM), and 15% reticulocyte standard buffer-30% glycerol (pH 8.0). Transcription was then initiated by the addition of 3 volumes of 0.1 mM HEPES (N -2-hydroxyethylpiperazine- N' -2-ethanesulfonic acid) buffer (pH 8.0) containing 16 mM magnesium acetate. The reaction mixtures were incubated for 3 h at 31°C , and the RNA was then extracted with phenol-chloroform and precipitated with ethanol. Where noted, the transcription reactions were centrifuged for 2 h at 4°C in a Beckman SW50.1 rotor at 48,000 rpm to remove the nucleocapsids before phenol-chloroform extraction of the RNA. The nucleocapsid pellet was recovered by suspension in 0.01 M Tris (pH 7.4), and RNA from both the supernatant and the nucleocapsid was phenol-chloroform extracted and ethanol precipitated.

RESULTS

Comparative translational inhibitory effects of poly(A) $^+$ RNA extracted from VSV-infected or uninfected HeLa cells. The initial experiments were designed to determine the degree to which viral RNA newly synthesized in infected HeLa cells would inhibit protein synthesis in the reticulocyte lysate system. To this end, poly(A) $^+$ RNA was isolated 4 h p.i. from mock- or VSV-infected suspension cultures of HeLa cells by detergent-phenol-chloroform extraction followed by oligo-dT chromatography. Various concentrations of poly(A) $^+$ RNA recovered from either VSV- or mock-infected HeLa cells were then added to micrococcal nuclease-treated rabbit reticulocyte lysates, and cell-free protein synthesis was measured by the incorporation of [^{35}S]methionine (or of ^3H -amino acids). The addition of 0.5 to 1.0 μg of exogenous globin mRNA per ml to the nuclease-treated lysates increased the synthesis of globin two- to four-fold. Three types of experiments were performed: (i) relatively low concentrations of

FIG. 1. Comparative electrophoretic analysis of endogenous proteins synthesized by nuclease-treated reticulocyte lysates in the presence of: (A) no added poly(A)⁺ RNA (control); (B) poly(A)⁺ RNA from mock-infected HeLa cells (0.5 µg/ml); (C) poly(A)⁺ RNA from VSV-infected HeLa cells (0.5 µg/ml); (D) poly(A)⁺ RNA from mock-infected HeLa cells (1.0 µg/ml); and (E) poly(A)⁺ RNA from VSV-infected cells (1.0 µg/ml). Lane F, Proteins extracted from VSV virions to serve as molecular weight markers: G, ≈69K; N, ≈50K; NS, ≈45K; and M, ≈29K. Also shown are markers for actin (Ac) and globin (gb). Poly(A)⁺ RNA was extracted from HeLa cells, mock-infected or infected with VSV (multiplicity of infection, ≈10) at 4.5 h p.i., subjected to oligo-dT-cellulose chromatography, and added to reticulocyte lysates in a complete translation reaction mixture containing [³⁵S]methionine (1 µCi/µl) for 60 min, as described in the text. Newly synthesized proteins were extracted and electrophoresed on 12.5% polyacrylamide slab gels and visualized by autoradiography.

poly(A)⁺ RNA from mock- or VSV-infected HeLa cells were compared in the reticulocyte lysate system for their effect on protein synthesis as assayed by polyacrylamide gel electrophoresis; (ii) increasing concentrations of poly(A)⁺ RNA from mock- or VSV-infected HeLa cells were assayed for their effect on [³⁵S]methionine incorporation into TCA-precipitable proteins synthesized in reticulocyte lysates in the absence or presence of added globin mRNA; and (iii) the kinetics of [³⁵S]methionine incorporation by reticulocyte lysates was compared in the presence of poly(A)⁺ RNA extracted from VSV- and mock-infected HeLa cells.

Figure 1 shows electrophoretic autoradiographs of [³⁵S]methionine-labeled proteins synthesized for 60 min by endogenous mRNA from nuclease-treated reticulocyte lysates containing either no exogenous RNA or exogenous poly(A)⁺ RNA extracted 4 h p.i. from mock- or VSV-infected HeLa cells, at concentrations of 0.5 or 1.0 µg/ml. Exogenous poly(A)⁺ RNA from VSV-infected cells substantially reduced the synthesis of endogenous reticulocyte proteins as compared with the effect of comparable

amounts of exogenous poly(A)⁺ RNA from mock-infected cells or proteins synthesized in the absence of any exogenous RNA. Laser densitometry scanning and integration of the bands shown in Fig. 1 revealed ~50% reduction in the synthesis of radioactive proteins by poly(A)⁺ RNA from VSV-infected cells (lanes C and E) compared with that of mock-infected cells (lanes B and D) (data not shown). These results indicate that *in vitro* translation is inhibited by relatively small concentrations of exogenous poly(A)⁺ RNA extracted from VSV-infected but not from uninfected HeLa cells.

We next compared the effects of various amounts of poly(A)⁺ RNA extracted from VSV- and mock-infected HeLa cells by determining the amounts of acid-precipitable protein made after incubation of reticulocyte lysates for 60 min. Controls consisted of determining [³⁵S]methionine incorporation by reticulocyte lysates to which no poly(A)⁺ RNA was added, which provided base-line data on the residual endogenous translation activity of the reticulocyte lysates. In only one experiment, endogenous mRNA was translated by the reticulocyte lysates, but in the other, exogenous globin mRNA was added to augment the endogenous mRNA. Low levels of poly(A)⁺ RNA (0.001 to 0.005 µg/ml) from either mock- or VSV-infected cells resulted in slight inhibition of [³⁵S]methionine incorporation, averaging ~15% reduction in protein synthesis (Fig. 2). When similarly low levels of exogenous globin mRNA only were added to reticulocyte lysates, the translational activity was reduced by an equivalent 15% (data not shown). Apparently, this translational system is susceptible to inhibition by low levels of nonspecific exogenous poly(A)⁺ RNA.

A more severe reduction (up to 55%) occurred in [³⁵S]methionine incorporation by reticulocyte lysates exposed to higher concentrations (0.1 to 1.0 µg/ml) of poly(A)⁺ RNA from VSV-infected cells (Fig. 2). By comparison, equivalent amounts of exogenous poly(A)⁺ RNA from mock-infected cells caused only a limited reduction in protein synthesis. Apparently, exogenous RNA in general slightly depresses the translation of endogenous reticulocyte messengers, but VSV poly(A)⁺ RNA has a more pronounced effect on the protein synthesizing machinery of reticulocyte lysates. Larger concentrations (50 µg/ml) of poly(A)⁺ RNA from VSV-infected cells resulted in greater synthesis of specific VSV proteins (data not shown) but also reversed the inhibitory effect on endogenous protein synthesis, as was expected for a preparation containing dsRNA.

The effect of poly(A)⁺ RNA from mock- and VSV-infected HeLa cells on protein synthesis by reticulocyte lysates primed with 0.5 µg of

FIG. 2. Comparative effect of poly(A)⁺ RNA from VSV- or mock-infected HeLa cells on [³⁵S]methionine incorporation into proteins synthesized by reticulocyte lysates treated with micrococcal nuclease with or without exogenous globin mRNA. Various concentrations of poly(A)⁺ RNA were added to 25 µl of a complete reaction mixture and incubated with [³⁵S]methionine (1 µCi/µl) at 31°C. Duplicate 5-µl samples were withdrawn at 60 min, TCA precipitated, and filtered; radioactivity was counted by scintillation spectrometry and averaged. The final concentrations of added HeLa cell poly(A)⁺ RNA in each translation reaction are indicated on the abscissa. [³⁵S]methionine incorporation is expressed on the ordinate as a fraction of the amount incorporated by reticulocyte lysates to which no HeLa cell poly(A)⁺ RNA was added with the exogenous globin mRNA. Poly(A)⁺ RNA was added from (▲) mock-infected HeLa cells; (Δ) VSV-infected HeLa cells; (●) mock-infected HeLa cells in the presence of globin mRNA (0.5 µg/ml); and (○) VSV-infected HeLa cells in the presence of exogenous globin mRNA (0.5 µg/ml).

exogenous globin mRNA per ml was also determined (Fig. 2). Under these conditions, low concentrations (0.001 to 0.05 µg/ml) of poly(A)⁺ RNA from either mock- or VSV-infected cells had no effect on reticulocyte lysate translation. In fact, higher concentrations of poly(A)⁺ RNA from mock-infected HeLa cells (0.1 to 1.0 µg/ml) did not impair *in vitro* translation of globin mRNA. In sharp contrast, concentrations of poly(A)⁺ RNA from VSV-infected cells at levels of ≥0.1 µg/ml markedly reduced [³⁵S]methionine incorporation in the globin mRNA-primed reticulocyte lysates. Similar results were obtained when ³H-amino acid instead of [³⁵S]methionine incorporation was used to measure protein synthesis (data not shown). Polyacrylamide gel electrophoresis of [³⁵S]methionine-labeled proteins synthesized under these conditions revealed proportionate reductions in

globin and all other endogenous proteins (data not shown).

It was also of interest that less inhibition of protein synthesis was caused by 1.0 than by 0.1 or 0.5 µg of poly(A)⁺ RNA from VSV-infected cells per ml regardless of whether the translation reaction was primed with exogenous globin mRNA (Fig. 2). Protein synthesis inhibition occurred over a narrow concentration range (0.1 to 0.5 µg/ml) of VSV-infected cell poly(A)⁺ RNA and was partially reversed by a higher concentration (1.0 µg/ml). A similar phenomenon has been reported repeatedly with dsRNA, which inhibits cell-free protein synthesis at low or intermediate concentrations, but the inhibitory effect of which is reversed at high concentration (13).

Kinetics of protein synthesis inhibition by VSV-infected cell poly(A)⁺ RNA and by synthetic dsRNA. The time course of protein synthesis inhibition was studied in an attempt to shed light on the nature of the reaction. These studies were done by adding poly(A)⁺ RNA (0.5 µg/ml) from either mock- or VSV-infected HeLa cells or synthetic poly(I):poly(C) (0.5 µg/ml) to reticulocyte lysates and then measuring [³⁵S]methionine incorporation into acid-precipitable material at intervals after starting the reaction; no exogenous globin mRNA was added, and therefore protein synthesis represented translation predominantly from endogenous reticulocyte mRNA. Essentially parallel incorporation of [³⁵S]methionine occurred for the first 10 min in reticulocyte lysates in which poly(A)⁺ RNA (0.5 µg/ml) was present from either mock- or VSV-infected cells (Fig. 3). Thereafter, there was a marked decline in, followed by cessation of, protein synthesis in reticulocyte lysates containing VSV-infected cell RNA but not in those containing mock-infected cell RNA.

Since the kinetics of protein synthesis inhibition by poly(A)⁺ RNA from VSV-infected cells resembled that previously reported for protein synthesis inhibition by dsRNA (13), we tested the effect of poly(I):poly(C) on the reticulocyte lysate. Poly(I):poly(C) at the same concentration (0.5 µg/ml) which had been found to be optimal for inhibiting translation had an almost identical effect on protein synthesis as did poly(A)⁺ RNA from VSV-infected cells.

If inhibition of protein synthesis caused by VSV-infected cell RNA is due to the presence of dsRNA, then very high concentrations of a synthetic dsRNA such as poly(I):poly(C) should reverse the inhibitory effect, as was reported by Hunter et al. (13). High concentrations (50 µg/ml) of poly(I):poly(C) were effective in reversing the inhibition of protein synthesis produced by poly(A)⁺ RNA from VSV-infected cells (Fig. 3). Moreover, poly(I):poly(C) at a concentration of

FIG. 3. Effect of poly(A)⁺ RNA from VSV- or mock-infected HeLa cells and/or poly(I):poly(C) on the kinetics of protein synthesis by nuclease-treated reticulocyte lysates primed with globin mRNA. Complete reaction mixture samples of 25 μ l each were incubated at 31°C in the presence of [³⁵S]methionine. Samples of 2.5 μ l each removed at intervals thereafter were TCA precipitated, and their radioactivity was counted by scintillation spectrometry. Added to the reticulocyte-lysate translation mixture were (●) poly(A)⁺ RNA (0.5 μ g/ml) from mock-infected HeLa cells; (■) poly(A)⁺ RNA (0.5 μ g/ml) from VSV-infected HeLa cells; (○) poly(A)⁺ RNA (0.5 μ g/ml) from mock-infected HeLa cells and poly(I):poly(C) (50 μ g/ml); (□) poly(A)⁺ RNA from VSV-infected HeLa cells plus poly(I):poly(C) (50 μ g/ml); (▲) poly(I):poly(C) alone (0.5 μ g/ml).

50 μ g/ml had no effect on the translation reaction containing poly(A)⁺ RNA from mock-infected HeLa cells.

These studies suggest that the protein synthesis-inhibitory factor in poly(A)⁺ RNA from VSV-infected cells is dsRNA or behaves like dsRNA, unlike the poly(A)⁺ RNA from uninfected cells.

Evidence that the protein synthesis inhibitor in VSV-infected cells is dsRNA. To test the hypothesis that the protein synthesis inhibitor is dsRNA, we tested the susceptibility of the inhibitory poly(A)⁺ RNA from VSV-infected HeLa cells to melting, micrococcal nuclease, and the K⁺ optima. Heating the RNA to 100°C and cooling it quickly in HEPES-KOH buffer abolished the capacity of infected-cell poly(A)⁺ RNA to inhibit protein synthesis in a reticulocyte lysate, whereas heating and cooling it under reannealing conditions in 0.3 M NaCl had no effect on its inhibitory properties (Table 1). Similar treatment did not affect the inactive poly(A)⁺ RNA from mock-infected cells, but the capacity of poly(I):poly(C) to inhibit protein synthesis was similarly abolished by melting it at 100°C and cooling it quickly in HEPES buffer. It seems likely, therefore, that double-strandedness or significant secondary structure are as essential for the inhibitory effect of VSV-infected cell poly(A)⁺ RNA on in vitro protein synthesis as they are for the effect of poly(I):poly(C).

Micrococcal nuclease is capable of digesting double-stranded nucleic acids (10). Therefore, we tested this enzyme by the procedure of Pelham and Jackson (25) for nuclease treatment

TABLE 1. Effect of melting and micrococcal nuclease on the residual fractional capacity of poly(A)⁺ RNA from VSV-infected HeLa cells and poly(I):poly(C) to inhibit protein synthesis by reticulocyte lysates^a

RNA (source)	Fractional [³⁵ S]methionine incorporation after treatment in indicated medium				
	Melting (100°C for 1 min) ^b			Micrococcal nuclease ^c	
	Control	HEPES	0.3 M NaCl	Control	Treated
Poly(A) ⁺ (mock infected)	1.01	1.04	1.06	0.84	0.96
Poly(A) ⁺ (VSV infected)	0.65	1.02	0.58	0.57	1.03
Poly(I):poly(C)	0.68	1.09	0.57	0.76	1.08

^a Parallel translation reactions of micrococcal nuclease-treated reticulocyte lysates containing exogenous globin mRNA were carried out in the presence or absence of RNA inhibitors. Reaction mixtures were incubated at 31°C in the presence of [³⁵S]methionine for 60 min, and then duplicate 5- μ l samples were TCA precipitated and filtered, and the radioactivity was counted by scintillation spectrometry. The data shown represent [³⁵S]methionine incorporation expressed as a fraction of that incorporated in an equivalent reaction mixture containing globin mRNA (0.5 μ g/ml) but no other exogenous RNA.

^b Poly(A)⁺ RNA (0.1 μ g/ml) isolated from either mock- or VSV-infected HeLa cells or poly(I):poly(C) (0.5 μ g/ml) in HEPES-KOH (pH 7.2) was incubated at 100°C for 1 min and then immediately quenched on ice. Duplicate poly(A)⁺ RNA or poly(I):poly(C) samples were similarly treated in the presence of 0.3 M NaCl or carried through the same procedure without heating (controls).

^c Poly(A)⁺ RNA (0.1 μ g/ml) isolated from mock- or VSV-infected HeLa cells or poly(I):poly(C) (0.5 μ g/ml) in 20 mM Tris (pH 8.2) and 0.01 M CaCl₂ were treated with 7.5 U of micrococcal nuclease per ml by the method of Pelham and Jackson (25). After treatment for 30 min at 20°C, the nuclease was inactivated by adding EGTA to the reaction mixture to a concentration of 0.02 M. Each control was treated identically but the micrococcal nuclease was omitted.

Fig. 4. K^+ optima for in vitro translation by mRNA-dependent reticulocyte lysates in the presence of (Δ) poly(A)⁺ RNA from mock-infected HeLa cells (0.5 μ g/ml); ($\bullet$) rabbit globin mRNA (0.5 μ g/ml); ($\circ$) poly(A)⁺ RNA from VSV-infected HeLa cells (0.5 μ g/ml); ($\square$) poly(A)⁺ RNA from VSV-infected HeLa cells (0.5 μ g/ml) plus poly(I):poly(C) (50 μ g/ml); ($\blacksquare$) poly(I):poly(C) alone (0.5 μ g/ml). All reactions in complete translation mixtures were incubated at 31°C for 60 min in the presence of [35 S]methionine (1 μ Ci/ μ l); duplicate samples of 5 μ l each were then withdrawn, TCA precipitated, filtered, and counted by liquid scintillation spectrometry.

of reticulocyte lysates to determine its effect on the capacity of poly(A)⁺ RNA from uninfected and VSV-infected cells and of poly(I):poly(C) to inhibit protein synthesis. Micrococcal nuclease treatment of 0.5 μ g of poly(A)⁺ RNA from VSV-infected cells per ml or of 0.5 μ g of poly(I):poly(C) per ml abolished their ability to inhibit protein synthesis by reticulocyte lysates reconstituted with exogenous globin mRNA. Micrococcal nuclease had little or no effect on poly(A)⁺ RNA controls from mock-infected HeLa cells (Table 1).

The effects of melting and micrococcal nuclease treatment indicate that double-strandedness may be the critical property of infected-cell poly(A)⁺ RNA that enables it to inhibit protein synthesis. Another property of dsRNA is its ability to shift the optimum concentrations of potassium required for translation of vaccinia virus transcripts in the reticulocyte lysate translation system (1). Baglioni et al. (1) reported that the optimum concentration of salt for the translation of vaccinia virus mRNA is influenced by the presence of a dsRNA inhibitor which copurifies with vaccinia virus poly(A)⁺ RNA. Therefore, we chose to examine the K^+ concentration required for optimal reticulocyte lysate translation under conditions of dsRNA inhibition or no inhibition. Poly(I):poly(C) or VSV-infected cell poly(A)⁺ RNA (0.5 μ g/ml) shifted the K^+ con-

centration for optimal [35 S]methionine incorporation to about 205 mM, as compared with the 100 to 150 mM K^+ concentration which was optimal for translation in the presence of equivalent concentrations of exogenous globin mRNA or poly(A)⁺ RNA from mock-infected HeLa cells (Fig. 4). Moreover, whereas inhibition by VSV-infected cell poly(A)⁺ RNA was abolished by the presence of 50 μ g of poly(I):poly(C) per ml, the K^+ concentration for optimal translation reverted to ~133 mM (Fig. 4). These results are in close agreement with the K^+ optima obtained by Baglioni et al. (1) for reticulocyte lysate translation under conditions of no inhibition or inhibition by dsRNA. Electrophoretic analysis of the protein products synthesized in the presence of VSV-infected cell poly(A)⁺ RNA inhibitors indicated that both cell- and virus-specific proteins were inhibited at the lower (100 to 150 mM) concentration of K^+ (Fig. 5). By comparing mock-infected with VSV-infected cells, it can be seen that poly(A)⁺ RNA from VSV-infected cells (0.5 μ g/ml) substantially inhibited globin synthesis (~50% reduction as determined by densitometry scanning). This inhibition of globin synthesis was reversed by increasing the K^+ concentration (compare lanes C and D [mock] with lanes C and D [VSV]). When inhibition of protein synthesis was reversed by higher

FIG. 5. Comparative electrophoretic analysis of endogenous proteins synthesized by nuclease-treated reticulocyte lysates in the presence of various K^+ concentrations and poly(A)⁺ RNA (0.5 μ g/ml) from mock-infected (lanes A-E, Mock) or VSV-infected (lanes A-E, VSV) HeLa cells. In vitro translation reactions were run in the presence of increasing K^+ concentrations (mM) of (A) 133; (B) 157; (C) 205; (D) 229; (E) 267. Reactions were incubated at 31°C for 60 min in the presence of [35 S]methionine and then subjected to electrophoresis on 12.5% polyacrylamide slab gels and visualized by autoradiography as described in the text and in the legend to Fig. 1. Migration of actin (Ac) and globin (gb) and of VSV virion protein NS and M are shown; VSV protein N was obscured by actin, and proteins G and L were not detected.

K⁺ concentrations, specific VSV proteins NS and M became more visible (Fig. 5). VSV protein N was obscured by the actin band, and VSV proteins G and L could not be detected. These data also demonstrate the presence in poly(A)⁺ RNA from infected cells of intact viral mRNA not greatly degraded in these preparations.

These data on the K⁺ optima of translation are consistent with the hypothesis that the translation inhibitor in VSV-infected cell poly(A)⁺ RNA is dsRNA.

Translation inhibitors produced by in vitro transcription of VS virions. The foregoing evidence for a dsRNA inhibitor of protein synthesis present in VSV-infected but not uninfected HeLa cells implicates either a viral product or a virus-induced cell product as the translation inhibitor. Direct evidence for a viral product was sought by testing RNA synthesized by cell-free transcription of VS virions for its ability to

FIG. 6. Comparative effect of RNA fractions extracted from in vitro transcripts of VS virions on protein synthesis by micrococcal nuclease-treated reticulocyte lysates primed with globin mRNA (1.0 µg/ml). Transcription reactions of VS virions for 60 min and extraction of RNA transcripts from a complete reaction mixture or from pelleted nucleocapsids and supernatant after centrifugation are described in the text. The conditions used for translation of reticulocyte lysates and 60-min incorporation of [³⁵S]methionine in duplicate 5-µl samples (shown on the ordinate) at each concentration of exogenous RNA (shown on the abscissa) are similar to those described in the legend to Fig. 2. The type of RNA added to each translation reaction is as follows: △, poly(A)⁺ RNA isolated from complete reaction mixtures (not centrifuged) after in vitro VSV transcription; □, poly(A)⁺ RNA isolated from the supernatant of a 60-min VSV transcription reaction after nucleocapsids were removed by centrifugation; ○, RNA isolated from pelleted nucleocapsid after in vitro VSV transcription; and ●, RNA isolated from control VSV nucleocapsid after mock transcription in the absence of nucleoside triphosphates.

inhibit protein synthesis by reticulocyte lysates. Standard procedures for in vitro transcription by purified VS virions were used to produce RNA transcripts (4), which were then separated by oligo-dT-cellulose chromatography into poly(A)⁺ and poly(A)⁻ RNA as described above. These in vitro-produced VSV transcripts contained viral mRNA that could be translated in reticulocyte lysates; as expected, the major VSV proteins synthesized were N, NS, and M (data not shown).

Figure 6 shows the effects of increasing concentrations of various RNA fractions isolated from the VS virion transcription reaction on protein synthesis by micrococcal nuclease-treated reticulocyte lysates supplemented with exogenous globin mRNA. As noted, [³⁵S]methionine incorporation was inhibited 40 to 45% by poly(A)⁺ RNA (0.5 µg/ml or greater concentrations) isolated by phenol-chloroform extraction of complete VS virion transcription reactions from which nucleocapsids had not been removed. In contrast, when transcribed nucleocapsids were removed by centrifugation, equivalent concentrations of supernatant poly(A)⁺ RNA had no effect on reticulocyte translation. The RNA pelleting with virion nucleocapsids after a 60-min transcription, or mock transcription in the absence of nucleoside triphosphates, was extracted with phenol-chloroform and tested for its effect on reticulocyte lysate translation. Protein synthesis was inhibited by RNA extracted from newly transcribed nucleocapsids at concentrations of 0.05 µg/ml or greater but not by untranscribed template RNA at any concentration (Fig. 6). These data indicate that RNA newly transcribed from virion nucleocapsids inhibits protein synthesis only in association with virion template RNA, which itself is not inhibitory.

RNAs extracted from pelleted nucleocapsids that had been transcribed in vitro were also fractionated by oligo-dT chromatography into poly(A)⁺ and poly(A)⁻ RNA and tested individually for their capacity to inhibit protein synthesis by reticulocyte lysates. Poly(A)⁺ and poly(A)⁻ RNA extracted from newly transcribed nucleocapsid both inhibited reticulocyte translation, whereas the RNA extracted from untranscribed VS virion nucleocapsids had no effect (Fig. 7). In fact, it appears that the poly(A)⁻ RNA from newly transcribed nucleocapsids was more inhibitory at lower concentrations than was the poly(A)⁺ RNA.

These data support the hypothesis that the translation-inhibitory RNA is of viral origin and, like the VSV-infected cell inhibitor, is dsRNA, probably formed by newly transcribed virion RNA complexed with the nucleocapsid template.

Evidence that the protein synthesis inhibitor generated by in vitro VS virion transcription behaves like dsRNA. If the inhibitor of protein synthesis made during VS virion transcription is truly dsRNA, it should behave in a manner similar to that of the poly(A)⁺ RNA inhibitor found in VSV-infected cells. To further examine this relationship, we tested the protein synthesis inhibitor in VS virion transcripts for its susceptibility to poly(I):poly(C), melting, micrococcal nuclease, and shifts in K⁺ concentration in experiments similar to those reported above for the VSV-infected cell inhibitor.

The kinetics of protein synthesis inhibition in the reticulocyte lysate system were examined in the presence of poly(A)⁺ or poly(A)⁻ RNA extracted from VS virion nucleocapsids that had undergone transcription at 31°C for 60 min. Controls contained only exogenous globin mRNA and, as described in the legend to Fig. 3, identical duplicate translation reactions were run in the presence of 50 µg of poly(I):poly(C) per ml. The results were essentially the same as those reported in Fig. 3; incorporation of

[³⁵S]methionine was not significantly different for the first 10 min in reactions containing poly(A)⁺ or poly(A)⁻ RNA extracted from transcribed nucleocapsid or controls without inhibitor. Thereafter, the rates of protein synthesis diminished greatly in reactions containing either poly(A)⁺ (0.5 µg/ml) or poly(A)⁻ (0.05 µg/ml) RNA extracted from transcribed nucleocapsids, to levels of ~40 to 50% inhibition of translation by 40 min. In each case, including the control, poly(I):poly(C) reversed the inhibitory effect of the poly(A)⁺ and poly(A)⁻ RNA extracted from transcribed nucleocapsids (data not shown). The kinetics of the translation-inhibitory effects of poly(A)⁺ and poly(A)⁻ RNA from VS virion nucleocapsid transcripts and the reversal by poly(I):poly(C) were similar to the data for VSV-infected cell RNA (Fig. 3) and provide further evidence for the dsRNA nature of these translation inhibitors present in association with transcribed nucleocapsids.

In experiments similar to those for VSV-infected cell RNA (Table 1), we found that the translation inhibitory effects of RNA from whole VS virion transcripts and nucleocapsids or poly(A)⁺ or poly(A)⁻ RNA from transcribed nucleocapsids could be inactivated by melting or by micrococcal nuclease treatment. Inhibition of protein synthesis by poly(A)⁺ and poly(A)⁻ RNA extracted from pelleted nucleocapsids after in vitro VS virion transcription reactions was reversed by exposing the RNA to 100°C for 1 min and quick cooling in 20 mM HEPES-KOH buffer (pH 8.2) before adding it to micrococcal nuclease-treated reticulocyte lysates supplemented with exogenous globin mRNA. Melting either poly(A)⁺ or poly(A)⁻ RNA in the presence of 0.3 M NaCl preserved their ability to inhibit protein synthesis. Similarly, treatment with micrococcal nuclease also reversed the protein synthesis-inhibitory activity of RNA extracted from whole VSV transcription reactions, unfractionated transcribed nucleocapsids, or poly(A)⁺ and poly(A)⁻ RNA extracted from transcribed nucleocapsids (data not shown). Micrococcal nuclease treatment and melting markedly reduced the protein synthesis-inhibitory activity of all of the nucleocapsid-RNA transcripts, as was the case for poly(A)⁺ RNA from VSV-infected cells (Table 1), thus strongly suggesting that this inhibitory effect is due to dsRNA.

Finally, we tested the K⁺ optima for cell-free protein synthesis under conditions of inhibition by poly(A)⁺ and poly(A)⁻ RNA extracted from VSV nucleocapsids after transcription for 60 min. The results were similar to those shown in Fig. 4 for K⁺ optima for translation in the presence of poly(A)⁺ RNA from VSV-infected cells as the inhibitor. The presence of poly(A)⁺

FIG. 7. Effect on protein synthesis of increasing concentrations of poly(A)⁺ and poly(A)⁻ RNA isolated from VSV nucleocapsids after in vitro transcription. As described in the legend to Fig. 5 and in the text, after 60-min transcription of purified VS virions, nucleocapsids were separated by centrifugation, and their RNA was extracted with phenol-chloroform and separated into poly(A)⁺ and poly(A)⁻ fractions by oligo-dT chromatography. Increasing concentrations of these RNA extracts and a control RNA extracted from untranscribed nucleocapsids were tested for their effect on [³⁵S]methionine incorporation by micrococcal nuclease-treated reticulocyte lysates supplemented with exogenous globin mRNA (1.0 µg/ml). The data compare the translation-inhibitory effects of (●) RNA isolated from mock-transcribed VSV nucleocapsids; (▲) poly(A)⁺ RNA isolated from VSV nucleocapsids after a 60-min transcription; and (■) poly(A)⁻ RNA isolated from VSV nucleocapsids after a 60-min transcription.

(0.5 $\mu\text{g/ml}$) or poly(A)⁻ (0.05 $\mu\text{g/ml}$) RNA extracted from transcribed nucleocapsids shifted the K⁺ optima for protein synthesis to ~200 mM, as compared with 140 to 150 mM for uninhibited translation. Moreover, when poly(I):poly(C) was also present at concentrations of 50 $\mu\text{g/ml}$ with the poly(A)⁺ and poly(A)⁻ RNA inhibitors, the K⁺ optima for reticulocyte lysate translation reverted to 140 to 150 mM. Once again, these data are consistent with previously reported studies (1) on K⁺ optima for globin mRNA translation and reinforce the postulate that the inhibitory factor(s) behaves like dsRNA probably derived from VSV transcripts base-paired with nucleocapsid RNA.

DISCUSSION

In vitro translation by rabbit reticulocyte lysates is sensitive to inhibition by dsRNA (10, 13). Typically, inhibition by dsRNA occurs abruptly but only after a lag period of 10 to 15 min, before which time protein synthesis occurs at control rates. This inhibition of protein synthesis is associated with the disappearance of detectable met-tRNA_f-40S ribosomal subunit complexes (8). The addition of eucaryotic initiation factor 2, which under control conditions promotes the binding of met-tRNA_f to the 40S ribosomal subunit, can reverse dsRNA inhibition of protein synthesis (6, 14, 15). An inhibitor activated by dsRNA has been shown to be present in reticulocyte lysates and to have protein kinase activity that is highly selective for the small subunit of eucaryotic initiation factor 2 (11). In addition to its biphasic kinetics, inhibition of protein synthesis in these systems occurs over a limited dsRNA concentration range and can readily be reversed by the addition of high concentrations (>10 $\mu\text{g/ml}$) of either naturally occurring or synthetic dsRNA (13). Other cell-free protein synthesizing systems have been shown to be susceptible to inhibition by dsRNA, but these systems do not appear to be as sensitive to dsRNA as the rabbit reticulocyte system is (12, 28).

Several groups have also reported inhibition of cell-free translation by viral dsRNA. The replicative intermediate of poliovirus has been shown to inhibit translation in cell-free systems derived from HeLa cells (5) and rabbit reticulocytes (10). In addition, there are reports of dsRNA inhibitory activity associated with mRNA preparations. dsRNA inhibitory activity has been reported to be present in preparations of poly(A)⁺ RNA transcribed in vitro by vaccinia virus and purified by oligo-dT-cellulose chromatography (1). Such activity has also been observed in reovirus mRNA preparations transcribed in vitro (1, 19). It should be noted that poly(A)⁺ RNA isolated from the cytoplasm of

normal uninfected BSC-1 cells also induces a dsRNA-activated protein kinase in a cell-free translation system derived from interferon-treated BSC-1 cells, but with an efficiency about 1% that of pure dsRNA such as poly(I):poly(C) (26). Whether the inhibitory activity present in these mRNA preparations is due to the presence of contaminating non-mRNA dsRNA or the presence of mRNAs with sufficient dsRNA character to activate the protein kinase is not clear.

We report here that VSV-infected HeLa cells, when subjected to detergent-phenol-chloroform extraction, yield an inhibitor of cell-free protein synthesis that copurifies with poly(A)⁺ RNA. A similar RNA inhibitor was found after in vitro transcription of purified virions. The VSV RNA inhibitors described here display the following characteristics previously described by Hunter et al. (13) for dsRNA inhibitors of protein synthesis: (i) the inhibitor is effective only over a limited concentration range; (ii) inhibition occurs only after a lag period of 10 to 15 min; (iii) the inhibition can be reversed by high concentrations of a synthetic dsRNA such as poly(I):poly(C).

In addition, we have demonstrated that the VSV inhibitors of protein synthesis are inactivated by melting at 100°C and by the addition of micrococcal nuclease. Baglioni et al. (1) were able to show that the K⁺ optimum for protein synthesis in reticulocyte lysates is shifted upward by the presence of a dsRNA inhibitor. We also observed similar K⁺ optima with the inhibitors isolated from VSV-infected HeLa cells and VSV transcription reactions. Moreover, we noted that the shift to a higher K⁺ optimum in the presence of either inhibitor could be reversed by high concentrations of poly(I):poly(C).

Moyer and Banerjee (20) previously reported the formation of double-stranded transcriptive intermediates after in vitro VSV transcription reactions. They estimated that 41% of the product transcribed in vitro remained in the form of such transcriptive intermediates after transcription in vitro for 2 h. We estimate that as much as 25 to 30% of the transcribed product pellets with the nucleocapsid template after VS virion transcription; analysis of this pelleted RNA before phenol extraction by electrophoresis under non-denaturing conditions in 0.7% agarose indicates that a majority of the ³²P-labeled product runs with the unlabeled template. These results are further evidence for the presence of dsRNA transcriptive intermediates in VSV transcription reactions. Based on these observations, we propose that the inhibitors that copurify with poly(A)⁺ RNA from VSV-infected HeLa cells and VSV transcription reactions are dsRNAs.

The presence of dsRNA inhibitors in poly(A)⁺ RNA preparations has been reported for other

systems (1). At present we are unable to state unequivocally that the *in vivo*-produced inhibitor reported here is a viral product rather than a cellular product induced by viral infection. However, the presence of a similar inhibitor after transcription *in vitro* of VS virions free of cellular material supports the evidence that viral RNA is the inhibitor of protein synthesis.

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