Effect of Viral Infection on Host Protein Synthesis and mRNA Association with the Cytoplasmic Cytoskeletal Structure

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ABSTRACT We studied the association of several eucaryotic viral and cellular mRNAs with cytoskeletal fractions derived from normal and virus-infected cells. We found that all mRNAs appear to associate with the cytoskeletal structure during protein synthesis, irrespective of their 5′ and 3′ terminal structures: e.g., poliovirus that lacks a 5′ cap structure or reovirus and histone mRNAs that lack a 3′ poly A tail associated with the cytoskeletal framework to the same extent as capped, polyadenylated actin mRNA. Cellular (actin) and viral (vesicular stomatitis virus and reovirus) mRNAs were released from the cytoskeletal framework and their translation was inhibited when cells were infected with poliovirus. In contrast, actin mRNA was not released from the cytoskeleton during vesicular stomatitis virus infection although actin synthesis was inhibited. In addition, several other conditions under which protein synthesis is inhibited did not result in the release of mRNAs from the cytoskeletal framework. We conclude that the association of mRNA with the cytoskeletal framework is required but is not sufficient for protein synthesis in eucaryotes. Furthermore, the shut-off of host protein synthesis during poliovirus infection and not vesicular stomatitis virus infection occurs by a unique mechanism that leads to the release of host mRNAs from the cytoskeleton.

Initiation of translation in eucaryotes is far more complex than in procaryotes. At least eight initiation factor activities along with several auxiliary factors and ATP have been demonstrated to be involved in the initiation process in eucaryotes in vitro, as compared with just three initiation factors and an ATP-independent mechanism in procaryotes. It seems plausible that this complex complement of eucaryotic initiation factors might reflect an involvement in the more intricate patterns of regulation of translation that occur in the eucaryotic system (1).

Besides the greater complexity of the factors mentioned above, the cellular architecture might also play a significant role in eucaryotic translation. There is evidence that eucaryotic mRNAs are translated only when associated with an elaborate network of filaments which extends throughout the cell and is referred to as "the cytoskeleton" (2). This fibrous network is composed of three distinct but interconnected filament systems, namely, the microfilaments, microtubules, and intermediate filaments, and other components. Wolosewick and Porter (3) have shown by high-voltage electron microscopy of intact cells that polysomes are clustered in the vicinity of the cytoskeleton structure. Subsequently, Lenk et al. (4) developed a procedure to fractionate cells into a detergent-resistant fraction which contains the cytoskeletal elements and a soluble fraction (containing soluble proteins, tRNA, monosomes, and other components) and found that polysomes invariably were associated with the cytoskeleton fraction. In further experiments, Cervera et al. (5) have shown that vesicular stomatitis virus (VSV)[1] mRNAs are translated only when associated with the cytoskeleton. Furthermore, these authors showed that dissociation of polysomes by high salt or heat treatment did not result in the release of mRNA from the cytoskeletal fraction, indicating a direct association between mRNA and the cytoskeleton which does not require mRNA–ribosome interaction. However, Howe and Hershey (6) have recently shown that ribosomal subunits can remain

[1] *Abbreviations used in this paper:* CBP, cap binding protein; DME, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; SSC, standard saline-citrate buffer; VSV, vesicular stomatitis virus.

associated with the cytoskeleton after mild RNase treatment to disaggregate polysomes.

It has been reported that shut-off of host protein synthesis in poliovirus-infected HeLa cells is accompanied by the release of host mRNAs from the cytoskeleton (7) followed by the association of virus-specific polysomes. Similarly, it has been shown that infection of human KB cells by adenovirus resulted in the shut-off of host protein synthesis with concomitant release of host mRNA from the cytoskeleton (8). In light of these observations which demonstrate a direct correlation between virally induced dissociation of host mRNAs from the cytoskeleton and cessation of host protein synthesis, it has been argued that fractionation of mRNA with the cytoskeleton reflects an involvement of cytoskeletal components in translation.

Immediate questions concern, on the one hand, the identity of the particular cytoskeletal components involved and on the other, structural features of mRNA and/or the involvement of soluble factors in the association. Consequently, we wanted to extend previous studies by using other viral systems in an attempt to address the following pertinent questions: (a) Are viral mRNAs generally associated with the cytoskeleton, irrespetive of the nature of their terminal structures? It has been suggested that two essentially ubiquitous structural features of eucaryotic mRNA, namely the cap structure [$m^7GpppX(m)$] and the 3′ poly A tail, might be implicated in this attachment (references 9 and 10, respectively). We used cDNA probes to three different viral mRNAs to follow their subcellular fractionation: (i) poliovirus RNA does not contain a 5′ cap structure but does contain a 3′ poly A tail (11), (ii) VSV mRNAs that are capped and polyadenylated (11), or (iii) reovirus mRNAs that are not polyadenylated (12) and apparently do not contain a cap structure at late times postinfection in L cells (13). (*b*) What is the degree of correlation between shut-off of host protein synthesis after viral (poliovirus, VSV, and reovirus) infection and release of host mRNAs from the cytoskeleton?

MATERIALS AND METHODS

Cells and Virus: CV-1 (African green monkey kidney cells) were grown in monolayers with Dulbecco's modified Eagle's medium (DME) supplemented with 5% fetal bovine serum (FBS). Cells were infected with poliovirus type 1 (Mahoney strain) at a multiplicity of infection of 200 plaque forming units/cell as described by Doyle and Holland (14) or with VSV, heat-resistant strain (HR) of Indiana serotype, obtained from C. P. Stanners (McGill University, Montréal, Québec) (15) at an MOI of 10 plaque forming units/cell, or with reovirus type 3 (Dearing strain) at an MOI of 50 plaque forming units/cell.

Cytoskeleton Extraction: Extraction conditions were as described by Cervera et al. (5) except that Ca^{++} was omitted from the extraction buffer and 0.1% Triton X-100 was used throughout the experiments. Cells grown on petri dishes were treated with extraction buffer (10 mM PIPES, pH 6.8, 100 mM KCl, 2.5 mM $MgCl_2$, 0.3 M sucrose, and 0.1% Triton X-100) at 4°C for 1 min, followed by one wash with cold extraction buffer without Triton X-100. All subsequent manipulations were performed at 4°C. The material obtained from the cells under these conditions was referred to as the soluble fraction. The cell remnants were collected with a policeman and centrifuged at 2,000 rpm. The pellet was resuspended in 20 mM HEPES (pH 7.5) buffer containing 0.5 M NaCl, 30 mM $Mg(OAc)_2$, 0.5% deoxycholate, and 1% Tween 40 and left for 5 min on ice, and the suspension was passed through a low-gauge needle. The suspension was then centrifuged for 5 min in an Eppendorf microfuge; the supernatant is referred to as the cytoskeletal fraction whereas the pellet contained mostly the nuclear matrix with associated DNA and heterogeneous nuclear RNA (see Results).

RNA Isolation and Analysis: Soluble and cytoskeletal fractions were treated with proteinase K (200 μg/ml) at 37°C for 30 min and RNA was extracted with phenol/chloroform/isoamyl alcohol (24:24:1) and ethanol precipitated. For dot-blot analysis, RNA was resuspended in 50 μl of H_2O followed by the addition of 30 μl of 20 × standard saline-citrate buffer (20 × SSC, 3 M NaCl, 0.3 M Na citrate, pH 7.0) and 20 μl of 37% (wt/wt) formaldehyde. RNA was denatured by incubation at 65°C for 15 min. RNA from equivalent amount of cells was twofold serially diluted in 15 × SSC and spotted on GeneScreen (New England Nuclear, Boston, MA [NEN]) or nitrocellulose paper (Schleicher & Schuell, Inc., Keene, NH), which had been presoaked consecutively in H_2O and 20 × SSC, using a BRL-dot blot apparatus (Bethesda Research Laboratories, Inc., Gaithersburg, MD). Hybridization to cDNA probes was done as described by Lesure et al. (16) with modifications. Blots were baked for 2 h at 80°C and prehybridized with 6 × SCP buffer (20 × SCP: 2 M NaCl, 0.01 M EDTA, 0.6 M Na_2HPO_4, pH 6.2), 1% sarcosyl, 2.5% dextran sulfate, and 100 μg/ml of carrier DNA for 2 h at 65°C. Blots were then hybridized in 6 × SCP buffer with 1 × 10^6 cpm of nick-translated cloned cDNA probes (labeled to a specific activity of 1–3 × 10^8 cpm/μg with α-^{32}P-dATP according to Rigby et al. [17] and boiled for 10 min and cooled down before hybridization). After hybridization at 65°C overnight, blots were washed twice for 10 min with 2 × SSC buffer containing 0.1% SDS followed by 0.1 × SSC buffer containing 0.1% SDS at room temperature. Alternatively, RNA from equal amount of cells was analyzed on 1% agarose gels containing 5 mM methyl mercury hydroxide (18). RNA from gels was transferred to GeneScreen sheets by electrophoresis overnight at 150 mA in 25 mM sodium phosphate buffer (pH 5.5). Hybridization to cDNA probes was performed as for the dot blot-analysis except that blots were washed with 2 × SSC buffer containing 0.1% SDS and 0.1% Na pyrophosphate twice for 15 min at 65°C and twice for 15 min at room temperature. Dried blots were exposed against XAR-5 Kodak film with Cronex Hi plus intensifying screens (DuPont Co., Wilmington, DE) for 1–2 days and dot or band intensity was quantified by soft laser densitometry (LKB Instruments, Inc., Gaithersburg, MD). Probes used for hybridization were: pBR322 containing the cloned poliovirus genome (pVR104, reference 19) obtained from V. Racaniello (Columbia University); pBR322 containing the cloned genome encoding the VSV G protein (pG1, reference 20) and M protein (pM309, reference 20) provided by J. K. Rose (Salk Institute); chicken β-actin gene cloned in pBR322 (21) from S. Farmer (Boston University); and a mouse histone 3-2 gene cloned in pLL10 (pRAH3-2, reference 22) from W. Marzluff (Florida State University at Tallahassee). A clone of reovirus S4 cDNA in pBR322 was a generous gift from Rhonda Bassel-Duby of this laboratory.

RESULTS

Previous studies characterizing the association of polysomes with the cytoskeleton were performed primarily with HeLa and KB tumor cell lines (4–8). These cell lines do not exhibit anchorage-dependent growth properties (in contrast to normal cells) and their rate of protein synthesis does not respond to changes in cell shape and surface contact signals (23). Therefore, these cells might have lost important features related to the regulation of protein synthesis. We chose to use an African green monkey kidney cell line, CV-1, which is fibroblastic in nature, exhibits anchorage-dependent growth properties, and can be infected by poliovirus type 1.

Initial experiments were performed to establish optimal conditions for cytoskeleton extraction. These conditions specify that most of the soluble components are removed from the cytoskeleton whereas polysomes are retained. We lysed cells with an extraction buffer similar to that described by Cervera et al. (5), except that the concentration of Triton X-100 was reduced from 0.5 to 0.1%, since we found that at the higher concentration, a significant percentage of polysomes was released from the cytoskeleton. In addition, we changed the protocol of Cervera et al. (5) for the separation of the cytoskeleton fraction from the nuclei. Instead of using homogenization in the presence of deoxycholate and Tween 40, we treated the cells with high concentrations of NaCl (0.5 M) in the presence of the two detergents and passed the suspension through a low-gauge needle. We found that under these conditions we obtained consistently better recovery of polysomes in the cytoskeletal fraction. However, by this procedure, the nuclei are disrupted and the fraction termed cytoskeleton in our studies, contains in addition to cytoplasmic

cytoskeletal components, nuclear proteins. The fraction termed nuclei (Table I) contains mainly DNA and RNA which are stably associated with the nuclear matrix (24–26). This explains the low percentage of proteins (2%, Table I) in the nuclear fractions as compared with other reports (e.g., Ben-Ze'ev et al., reference 27). The amount of protein appearing in the soluble fraction reached a maximum of 74% of the total protein content after 2 min of extraction, while at the same time the percentage of protein in the cytoskeletal fraction decreased to 24% of total protein (Table I). A similar kinetic pattern was also observed for RNA fractionation (Table I), whereby the RNA extracted in the soluble fraction reached a maximum of ~50% after 2 min of incubation with extraction buffer while the amount of RNA in the cytoskeletal fraction reached ~25%. The unchanged percentage of the uridine-labeled material in the nuclei indicates that although the nuclei were broken during extraction, most if not all of the heterogeneous nuclear RNA was pelleted together with the nuclear matrix. This explanation is also consistent with our finding that after a short pulse with [^{3}H]uridine, most of the labeled material is associated with the nuclear matrix (data not shown).

We wanted to examine the distribution of polysomes between the cytoskeletal and soluble fractions as a function of extraction time. Fig. 1 shows the profiles of polysome distribution after an extraction time of 1 min. The results indicate that under these conditions almost all of the polysomes are associated with the cytoskeletal fraction (Fig. 1*A*). In this experiment, cells were incubated in the presence of low concentrations of cycloheximide to recruit most of the mRNA into polysomes. This might also explain the lack of monosomes in the soluble fraction (Fig. 1*B*), since most ribosomes are associated with mRNAs to form polysomes. Breakdown of monosomes to subunits in the cytoskeletal fraction (Fig. 1*A*) is probably due to the high concentration of salt used during the extraction. Extraction times of >1 min generally resulted in the dissociation of polysomes from the cytoskeletal fraction (data not shown). Consequently, we chose an extraction time of 1 min for our experiments.

We first wished to determine mRNA distribution in the cell using cDNA probes for a cellular mRNA. In addition, we wanted to analyze the effect of poliovirus infection on cellular mRNA distribution, since it has been reported that infection causes release of cellular mRNAs from the cytoskeleton (7). We used as a probe for cellular mRNA, chicken actin cDNA cloned in pBR322 (provided by S. Farmer), which was shown to react specifically with chicken actin mRNA by Northern blotting and is not species specific since it cross-hybridizes with β- and γ-actin mRNA sequences from mouse (28). The relative amounts of actin mRNA in the cytoskeleton and soluble fractions were determined by extraction of total RNA and hybridization to ^{32}P-nick translated cDNA probe. The results of such a dot-blot experiment performed at 3 h post-infection (Fig. 2) show that ~90% of actin mRNA (based on densitometry tracings of spots obtained at the different dilutions) fractionated with the cytoskeleton in uninfected cells, whereas in poliovirus-infected cells only ~30% was in the cytoskeletal fraction. We were unable to demonstrate the release of more than ~80% of actin mRNA or other host (e.g., tubulin) or viral mRNAs (see below) even after longer periods of infection, although the inhibition of the synthesis of the corresponding proteins was >90%, as determined by [^{35}S]methionine incorporation into proteins and analysis by SDS PAGE. One possible explanation is that the fraction of actin mRNA not released from the cytoskeleton upon poliovirus infection represents nonspecific entrapment of mRNA in the cytoskeletal pellet. In any event, the results demonstrate that our system resembles previously described systems (4–8),

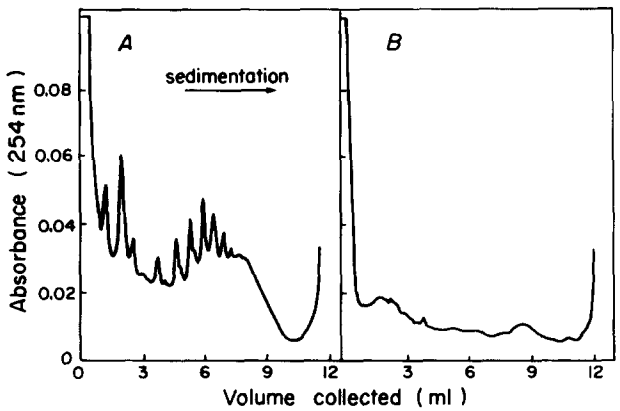

FIGURE 1 Polyribosome distribution between cytoskeleton and soluble fractions in CV-1 cells. Cells (5×10^6) grown in DME were incubated with 50 μg/ml cycloheximide for 30 min and fractionated as described in Materials and Methods. The soluble and cytoskeleton fractions were layered on 15–40% sucrose gradients containing 500 mM NaCl, 20 mM HEPES (pH 7.5), 30 mM $Mg(OAc)_2$, and 50 μg/ml cycloheximide. Gradients were centrifuged for 150 min in an SW40 rotor at 35,000 rpm at 4°C and absorbance was recorded with an ISCO spectrophotometer flow cell (ISCO Lincoln, NE) at 250 nm. (*A*) Cytoskeletal fraction; (*B*) soluble fraction.

TABLE I

*Percentage Distribution of Proteins and RNA as a Function of Extraction Time**

	Extraction time (*min*)									
	0		1		2		3		5	
Fractions	Protein	RNA	Protein	RNA	Protein	RNA	Protein	RNA	Protein	RNA
Cytoskeleton	93	83	45	46	24	26	22	22	24	29
Soluble	2	2	51	31	74	51	76	62	73	53
Nuclei	5	15	4	23	2	23	2	16	3	18

* To radiolabel proteins, CV-1 cells grown in 60-mm petri dishes (3×10^6 cells/dish) were incubated with 20 μCi/ml of [^{35}S]methionine (>1,000 Ci/mmol, NEN) overnight in MEM lacking methionine supplemented with 10% FBS. To label RNA, cells were incubated with 5 μCi/ml of [^{3}H]uridine (40 Ci/mmol, NEN) overnight in DME supplemented with 10% FBS. Cells were extracted and fractionated as described in Materials and Methods for the indicated periods of time. Aliquots from [^{3}H]uridine-labeled cells were precipitated with cold 10% trichloroacetic acid for 20 min on ice, collected on GF/C filters, rinsed with ethanol, dried, and counted in a toluene-based scintillation fluid. Aliquots from [^{35}S]methionine-labeled cells were spotted on a 3-MM paper and boiled in 10% trichloroacetic acid for 10 min, followed by successive washes in 5% trichloroacetic acid, ethanol, ethanol/ether (1:1), and ether. Filters were dried and counted in a toluene-based scintillation fluid. Time 0 values were determined by the addition to cells of extraction buffer without Triton X-100 followed immediately by centrifugation of the cells and further manipulations (see Materials and Methods).

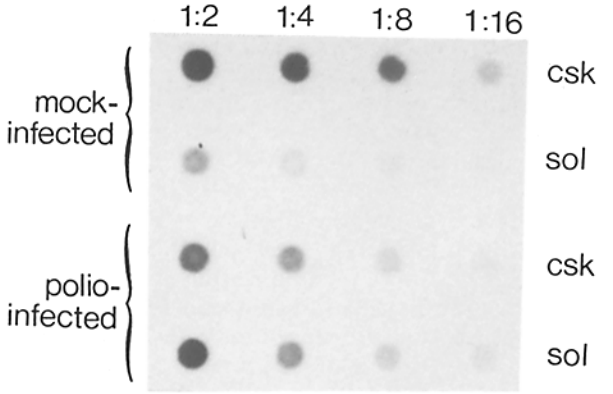

FIGURE 2 Dot-blot analysis of actin mRNA distribution in mock and poliovirus-infected CV-1 cells. Cells grown in 150-mm petri dishes (1.1×10^7 cells/dish) were infected with poliovirus or mock-infected as described in Materials and Methods. After infection for 3 h at 37°C, cells were extracted with cytoskeleton extraction buffer, RNA was purified, and dot-blot analysis was performed at twofold serial dilutions on nitrocellulose paper (Schleicher & Schuell, Inc.) by hybridization with ^{32}P-labeled nick translated actin cDNA. Dried blots were autoradiographed and quantified by soft laser densitometry as described in Materials and Methods. Fractions analyzed are indicated in the figure. *csk*, Cytoskeletal fraction; *sol*, soluble fraction.

in which the majority of host mRNAs are normally found attached to the cytoskeleton framework and are released as a consequence of poliovirus or adenovirus infection.

We chose two other viral systems to examine the correlation between the ability of viruses to shut-off host protein synthesis and the effect on host mRNA association with the cytoskeletal framework. Infection with certain strains of VSV (e.g., HR strain of Indiana serotype) results in drastic shut-off of host protein synthesis (29), and although the molecular mechanism for the inhibition has not been established, there are some indications that inactivation of eIF-2 is involved (30). When VSV-infected cells are superinfected with poliovirus, there is a precipitous inhibition of VSV-directed protein synthesis (14, 31). Thus, the mechanisms of inhibition of protein synthesis induced by VSV and poliovirus are apparently different. Consequently, it was of interest to determine the cellular distribution of host mRNAs following VSV infection and the distribution of VSV mRNAs following poliovirus superinfection.

CV-1 cells were infected with VSV alone or were superinfected with poliovirus after 2 h of VSV infection. Proteins were labeled with [^{35}S]methionine 2 h after poliovirus infection and resolved on SDS polyacrylamide gels. The profile of synthesized proteins shown in Fig. 3 indicates that VSV infection of CV-1 cells resulted in a dramatic inhibition of host protein synthesis (compare Fig. 3, lanes *1* and *2*). After 5 h of infection, the only proteins synthesized were VSV encoded. Poliovirus infection of CV-1 cells caused similar repression of host protein synthesis followed by exclusive synthesis of poliovirus-coded polypeptides (Fig. 3, lane *3*). When VSV-infected cells were superinfected with poliovirus, VSV-programmed protein synthesis was reduced significantly (compare lane *4* with lane *2* in Fig. 3). However, translation of the different VSV polypeptides was inhibited to different extents; while translation of the G and M mRNAs was reduced by 95%, translation of N+NS proteins was reduced by only ~60% as determined by densitometric tracing of the autoradiograph shown in Fig. 3. From Fig. 3 it is also evident that poliovirus-directed protein synthesis in VSV preinfected cells was significantly lower (~30-fold) than in cells infected by poliovirus alone (compare Fig. 3, lanes *4* and *3*). Consequently, poliovirus-superinfected cells synthesize more VSV proteins than poliovirus protein. These findings differ from those in previous studies (14, 31), which showed that poliovirus protein synthesis is normal in poliovirus-infected, VSV pre-infected cells, and therefore were repeated several times

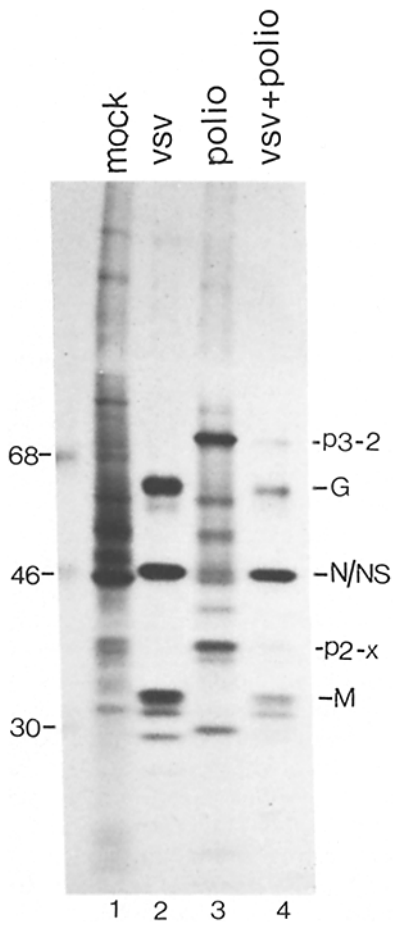

FIGURE 3 SDS polyacrylamide gel analysis of proteins synthesized in VSV-infected (lane *2*), poliovirus-infected (lane *3*), or VSV-infected, poliovirus-superinfected (lane *4*) cells. CV-1 cells grown in 60-mm petri dishes (3×10^6 cells/dish) were mock-infected (lane *1*) or infected with VSV. After adsorption for 45 min at 37°C in 0.5 ml of DME, 4 ml of DME supplemented with 2% heat-inactivated FBS was added and cells were incubated for 2 h at 37°C. 2 h after VSV infection, VSV-infected cells were washed with cold PBS and mock-infected or superinfected with poliovirus. Alternatively, mock-infected cells were infected with poliovirus. Adsorption of poliovirus for 45 min at 37°C in 0.5 ml DME was followed by the addition of 4 ml DME supplemented with 2% heat-inactivated FBS. [^{35}S]Methionine was added 2 h after poliovirus infection to a final concentration of 50 μCi/ml in 4 ml of Eagle's minimal essential medium without methionine and supplemented with 2% heat-inactivated FBS. Labeling for 30 min at 37°C was followed by cell lysis in cytoskeletal extraction buffer containing 1% Nonidet P-40 and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged at 2,000 *g* for 10 min and the supernatant was used for further analysis. Samples obtained from the same number of cells were resolved on a 7.5–15% gradient SDS polyacrylamide gel, which was dried and autoradiographed. Numbers at left represent molecular weight $\times 10^{-3}$.

with reproducible results. The most straightforward explanation of these differences might be that different strains of VSV were used in our studies and those of Doyle and Holland (14) and Ehrenfeld and Lund (31). We used the HR strain of Indiana serotype of VSV which has been shown to induce a more precipitous shut-off of host protein synthesis than the other strains of Indiana serotype used by the latter investigators (32). The lesion in initiation of protein synthesis caused by the HR strain has been traced to eucaryotic initiation factor 2 (30) which is required for ternary complex formation in the initiation step of protein synthesis and is most probably required for poliovirus protein synthesis. Consequently, it is not surprising that cells infected with the HR strain of VSV do not allow efficient translation of poliovirus mRNA. In addition, our experiments were conducted with CV-1 cells, while the previous studies were carried out with HeLa cells. This might also lead to different patterns of shut-off of host protein synthesis.

To examine the relationship between the shut-off of host protein synthesis and cellular mRNA association with the cytoskeleton after VSV infection, we examined the subcellular distribution of actin mRNA by dot-blot analysis. Poly $(A)^+$ cellular RNA from mock-infected and VSV-infected cells was spotted on nitrocellulose filter paper followed by hybridization with ^{32}P-nick translated cloned actin cDNA. The results in Fig. 4 show that actin mRNA was not released from the cytoskeleton after VSV infection. In both infected and mock-infected cells, ~80% of cellular actin mRNA was found associated with the cytoskeletal fraction. Despite the continued association of actin mRNA with the cytoskeleton following VSV infection, the mRNA was not translatable, indicating that the mere association of mRNA with the cytoskeleton is not sufficient for translation. Furthermore, these results show that inhibition of host protein synthesis during viral infection does not necessarily lead to release of host mRNAs from the cytoskeleton as shown in the case of poliovirus infection.

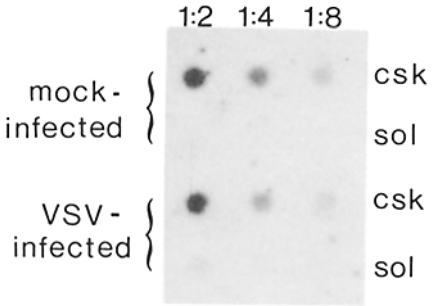

FIGURE 4 Actin mRNA distribution between cytoskeletal and soluble fractions of mock and VSV-infected CV-1 cells. CV-1 cells grown in 150-mm petri dishes (1.1×10^7 cells/dish) were infected with VSV or mock-infected as described in Fig. 3. After adsorption for 45 min at 37°C in 4 ml of DME, 15 ml of DME supplemented with 2% heat-inactivated FBS was added and cells were incubated at 37°C. 6 h after VSV infection, cytoskeleton and soluble fractions were extracted and RNA was prepared as described in Materials and Methods. Poly(A)$^+$ RNA was purified by one cycle of oligo (dT)-cellulose chromatography (33). Dot-blot analysis of actin mRNA performed at twofold serial dilutions, on nitrocellulose filters (Schleicher & Schuell, Inc.) by hybridization with ^{32}P-labeled nick translated actin cDNA was followed by autoradiography as described in Materials and Methods. The autoradiograph is shown. Fractions analyzed are indicated in the figure. *csk*, cytoskeletal fraction; *sol*, soluble fraction.

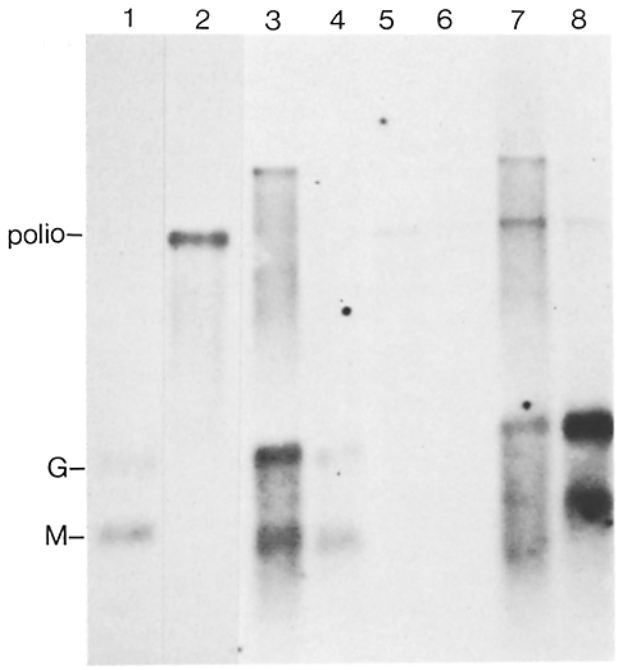

FIGURE 5 Viral mRNA distribution between cytoskeletal and soluble fractions of VSV-infected, poliovirus-infected, and VSV-infected, poliovirus-superinfected CV-1 cells. CV-1 cells grown in 75-cm^2 flasks (8×10^6 cells/flask) were infected with VSV or poliovirus or VSV-infected and poliovirus-superinfected as described in Fig. 3. 3 h after poliovirus infection, cells were extracted with cytoskeleton extraction buffer and RNA was prepared, run on an 1% agarose gel containing 5 mM methyl mercury hydroxide, transferred to GeneScreen paper by electrophoresis, and hybridized with a mixture of ^{32}P-nick translated G and M VSV cDNAs and ^{32}P-nick translated poliovirus cDNA, as described in Materials and Methods. Blots were exposed against x-ray film and the autoradiograph is shown: (lane *1*) total VSV mRNAs; (lane *2*) poliovirus genomic RNA; (lane *3*) cytoskeletal fraction from VSV-infected cells; (lane *4*) soluble fraction from VSV-infected cells; (lane *5*) cytoskeletal fraction from poliovirus-infected cells; (lane *6*) soluble fraction from poliovirus-infected cells; (lane *7*) cytoskeletal fraction from VSV-infected, poliovirus-superinfected cells; (lane *8*) soluble fraction from VSV-infected, poliovirus-superinfected cells.

The fate of VSV mRNAs after poliovirus infection was determined by Northern blotting after resolution of total RNA from the cytoskeletal and soluble fractions on methyl mercury hydroxide gels and probing with cDNA clones of poliovirus RNA and VSV G and M mRNAs. The results (Fig. 5) show that in VSV-infected cells, ~70% of G and M mRNAs were found associated with the cytoskeletal fraction (Fig. 5, lanes *3* and *4*) and 75% of poliovirus RNA in poliovirus-infected cells was associated (compare Fig. 5, and lanes *5* and *6*). Moreover, almost all of the VSV virion 42S RNA was associated with the cytoskeleton (Fig. 5, lane *3*) as previously observed (5). When VSV-infected cells were superinfected with poliovirus, the great majority of G and M VSV mRNAs were released from cytoskeleton, as these mRNAs were found in the soluble fraction (Fig. 5 lanes *7* and *8*; 97 and 88% of G and M mRNAs, respectively, were in the soluble fraction). Poliovirus RNA, under these conditions, remained associated with the cytoskeleton (75%; lanes *7* and *8*) as was the case after poliovirus infection alone (Fig. 5, lanes *5* and *6*). It is noteworthy that the 42S VSV minus strand RNA association with the cytoskeleton was not affected by superinfection with poliovirus (Fig. 5, lanes *7* and *8*), indicating that there is

preferential release of mRNAs and that the release of VSV mRNAs from the cytoskeleton following poliovirus infection is not due to nonspecific interference with RNA–cytoskeleton association. This experiment was performed with total cellular RNA and therefore poliovirus RNA migrating at 35S included both plus and minus strands, which can hybridize to ^{32}P-nick translated cDNA. However, further experiments with oligo (dT)-selected RNAs gave similar results in which most poliovirus poly $(A)^+$ RNA was associated with the cytoskeleton under the different conditions described above (data not shown). One finding in Fig. 5 deserves further consideration. Although poliovirus protein synthesis was significantly lower in poliovirus-superinfected, VSV-infected cells as compared with cells infected by poliovirus alone, the fraction of poliovirus RNA bound to the cytoskeleton in both cases was similar (~75%; note the reduced level in the amount of poliovirus RNA in Fig. 5 lane *5* compared with lane *7*. This reduction is particular for this experiment and might be due to loss of material). This result is further evidence that the association of mRNA with the cytoskeleton by itself is not a sufficient prerequisite for protein synthesis, consistent with the results shown in Fig. 4.

Similar experiments were performed to examine the association of reovirus S4 mRNA with the cytoskeleton and to analyze the effects of reovirus infection on host mRNA subcellular distribution and host protein synthesis. None of the 10 reovirus mRNAs contain a poly A tail (12) and it has been reported that at late times after infection of L cells, reovirus mRNAs do not possess a cap structure, either (13). The latter findings were not corroborated in another study using a different mouse cell line, SC-1 grown in monolayers, in which it was suggested that reovirus mRNAs are capped at late times postinfection (34). We first determined the kinetics of reovirus protein synthesis in CV-1 cells and found that synthesis of $\sigma 3$ reovirus protein (which is the major viral protein synthesized) begins ~6 h after infection and then levels off ~12 h postinfection (Fig. 6). It is also clear from Fig. 6 that host protein synthesis is not inhibited even at 12 h postinfection when reovirus-directed protein synthesis is maximal. This pattern of protein synthesis is similar to the one previously observed in reovirus-infected mouse SC-1 monolayer cells, where host protein synthesis is not shut-off after reovirus infection (35). This is in contrast to the significant inhibition of host protein synthesis observed in reovirus-infected L cells (13). Superinfection of reovirus-infected CV-1 cells with poliovirus results in the reduction of reovirus and host protein synthesis. [^{35}S]-Methionine-labeled proteins at 3 h after poliovirus infection were resolved on a 10% SDS polyacrylamide gel followed by autoradiography. The results (Fig. 7) show that when cells were superinfected by poliovirus from 6 h up to 15 h after reovirus infection, synthesis of reovirus and host proteins was inhibited. These results indicate that poliovirus shuts off reovirus and host protein synthesis by the same mechanism.

To analyze the subcellular distribution of reovirus and host mRNAs after reovirus infection, we probed with a cDNA clone to S4 mRNA (prepared by R. Bassel-Duby, unpublished data), and mRNA levels were determined by dot-blot hybridization analysis (Fig. 8). More than 90% of reovirus S4 mRNA was bound to the cytoskeleton in reovirus-infected cells at 12 and 18 h postinfection. (Dot-blot analysis was also performed at 6 h postinfection but the signal was too weak for accurate analysis). Following poliovirus infection for 3 h, ~50% of reovirus S4 mRNA present at 12 h after reovirus infection

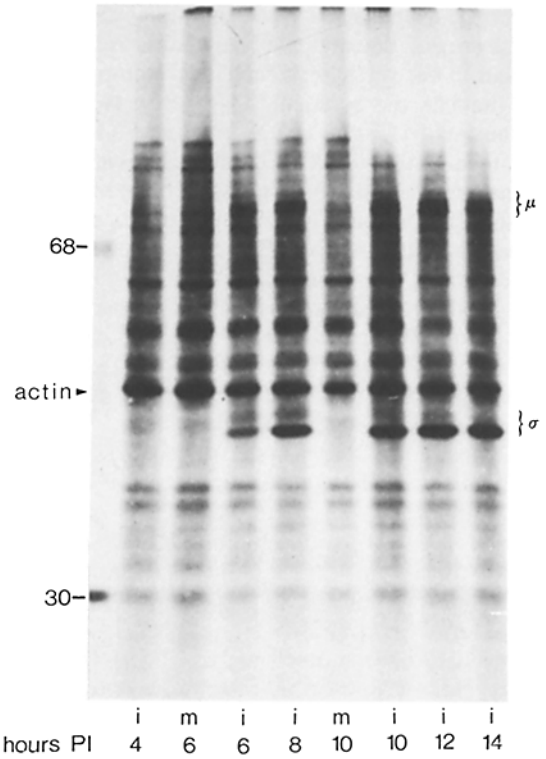

FIGURE 6 SDS polyacrylamide gel analysis of proteins synthesized in mock-infected or reovirus-infected cells. CV-1 cells grown in 60-mm petri dishes (2×10^6 cells/dish) were mock-infected or infected with reovirus. After adsorption for 1 h at 37°C in 0.5 ml PBS, 4 ml of DME supplemented with 2% heat-inactivated FBS was added and cells were labeled after various periods of incubation, as indicated in the figure, with [^{35}S]methionine for 30 min and extracted as detailed in Fig. 3. Samples obtained from the same number of cells were resolved on a 10% SDS polyacrylamide gel which was dried and autoradiographed. *i*, Infected; *m*, mock-infected; *PI*, postincubation.

was released from the cytoskeleton, while 70% of the cytoskeleton-associated S4 mRNA from 18 h postinfected cells was released. Actin cDNA clone was used as a probe for cellular mRNA distribution between the cytoskeletal and soluble fractions in reovirus-infected and poliovirus-superinfected cells (Fig. 8). As one would predict from the pattern of actin protein synthesis in reovirus-infected cells (which is not changed during infection [Fig. 7]), actin mRNA was associated with the cytoskeleton in reovirus-infected cells, but was released from the cytoskeleton after poliovirus infection in agreement with the results in Fig. 2. These results demonstrate that infection of cells with a virus that does not shut off host protein synthesis also does not cause the release of host mRNA from the cytoskeleton. In addition, the association of reovirus S4 mRNA with the cytoskeleton indicates that the poly A tail is not implicated in this interaction. Nevertheless, it is possible that reovirus has evolved an alternative mechanism for attachment of mRNA to the cytoskeleton. Consequently, we wanted to examine the cytoskeleton association of a cellular mRNA that does not contain a poly A tail.

For a cellular mRNA that does not contain a 3′ poly A tail, we chose histone mRNA (22) and probed for mRNA distribution between the cytoskeletal and soluble fractions with a mouse H3-2 histone cDNA clone which we assumed would behave in a manner similar to the rest of the histone mRNAs. To obtain higher levels of histone mRNAs to facil-

itate their quantitation by hybridization, we treated CV-1 cells with the drug aphidicolin, which synchronizes cells by arresting them at the G_1/S boundary (36). Removal of the drug allows the cells to proceed into the S phase. It has been shown that the level of histone mRNAs at the S phase is 100-fold higher than at the G_1 phase of the cell cycle (37). The dot-blot analysis of RNA from the cytoskeletal and soluble fractions from the S phase (Fig. 9) gave results similar to those obtained with actin mRNA (Fig. 2), showing that most of the histone mRNA (~80%) is found associated with the cytoskeleton. Moreover, this association is characteristic of that of actin mRNA in that it is sensitive to poliovirus infection, resulting in the release of the mRNA (~50% released) from the cytoskeleton (compare Figs. 2 and 9). Thus, the presence of a poly A tail on either cellular or viral eucaryotic mRNAs does not correlate with their ability to associate with the cytoskeleton structure.

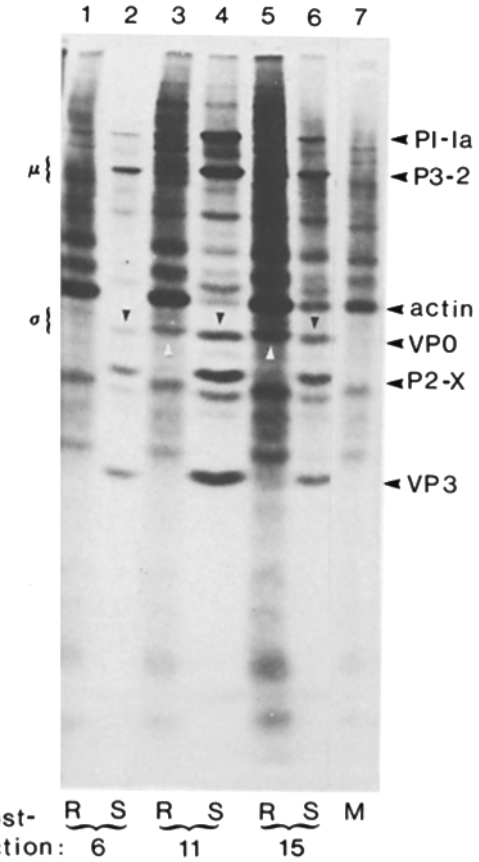

FIGURE 7 SDS polyacrylamide gel analysis of proteins synthesized in reovirus-infected and reovirus-infected poliovirus-superinfected cells. CV-1 cells grown in 60-mm petri dishes (2×10^6 cells/dish) were infected with reovirus or mock-infected as described in the legend to Fig. 6. After infection for different periods, as indicated in the figure, cells were either mock-infected or infected with poliovirus as described in Materials and Methods. After adsorption for 1 h at 37°C in 0.5 ml of PBS, 4 ml of DME (supplemented with 2% heat-inactivated FBS) was added and cells were incubated for 3 h at 37°C. Cells were then incubated with [^{35}S]methionine and lysed as described in the legend to Fig. 3. Samples obtained from the same number of cells were resolved on a 10% SDS polyacrylamide gel, which was dried and autoradiographed. *R*, Lanes contained material from reovirus-infected cells. *S*, Lanes contained material from reovirus-infected, poliovirus-superinfected cells. Lane *7* (indicated by *M*) contained material from mock-infected cells. Black arrowheads (in lanes *2*, *4*, and *6*) point to poliovirus-coded VPO polypeptide, and white arrowheads (in lanes *3* and *5*) point to reovirus-coded σ3 polypeptide.

Controlled release of mRNAs from the cytoskeleton as a means of decreasing protein synthesis is a very attractive idea and might be operative under different physiological conditions (e.g., mitosis). However, such a mechanism appears at the present time to be unique to certain viral infections (either poliovirus or adenovirus) because several treatments of cells that lead to inhibition of protein synthesis did not cause the release of cellular mRNAs from the cytoskeleton. For example, several inhibitors of initiation of protein synthesis do not induce the release of mRNAs from the cytoskeleton (5). We have confirmed these results in our system with specific cDNA probes. As shown in Fig. 10, VSV M and G mRNAs are not released from the cytoskeleton when cells are incubated in the presence of high salt (NaCl) concentrations which inhibit specifically the initiation step of protein synthesis (38), or in the presence of emetine which inhibits the elongation step of protein synthesis (39).

DISCUSSION

The results presented here are in agreement with earlier observations suggesting that eucaryotic mRNAs are associated with the cytoskeletal framework of the cell (4–8) and that this association has functional significance with respect to selection of particular mRNAs for translation, under certain conditions (5–8). The data suggest an obligatory association of mRNAs with the cytoskeleton in order for protein synthesis to take place. However, although it seems that attachment of mRNA to the cytoskeleton is required for translation (5–8), this attachment by itself is not sufficient for translation. This can be concluded from several findings in this study. (*a*) Actin mRNA was not released from the cytoskeleton after VSV infection, yet it was apparently not translated (Figs. 3 and 4). (*b*) Although the amount of poliovirus mRNA made in VSV preinfected cells was not reduced compared with yields from cells not infected with VSV, and most of the poliovirus mRNA made in VSV-infected cells was cytoskeleton associated, the translatability of poliovirus mRNA was clearly greatly reduced by VSV preinfection (Fig. 3). (*c*) Several different treatments of cells reduced protein synthesis without releasing mRNA from the cytoskeleton (Fig. 10). Furthermore, we have recently found that upon suspension of anchorage-dependent cells, mRNAs are not released from the cytoskeleton, although protein synthesis is greatly reduced (Darveau, A., unpublished data).

On the basis of the aforementioned findings, it is reasonable to assume that the release of viral (VSV and reovirus) and cellular (actin) mRNAs from the cytoskeleton upon poliovirus infection cannot be explained as resulting from inhibition of protein synthesis but, rather, as resulting from a direct effect on the process by which mRNAs are attached to the cytoskeleton. One attractive possibility is that poliovirus infection affects a cellular component that is involved in anchoring mRNAs to the cytoskeleton.

It is conceivable that the poliovirus-induced inactivation of a cap-binding protein (CBP) complex (which interacts with the 5′ cap structure of eucaryotic mRNAs) is directly or indirectly responsible for the dissociation of host mRNAs from the cytoskeleton. There is considerable evidence that a CBP complex is involved in the binding of capped mRNAs to ribosomes subsequently facilitating their translation. The

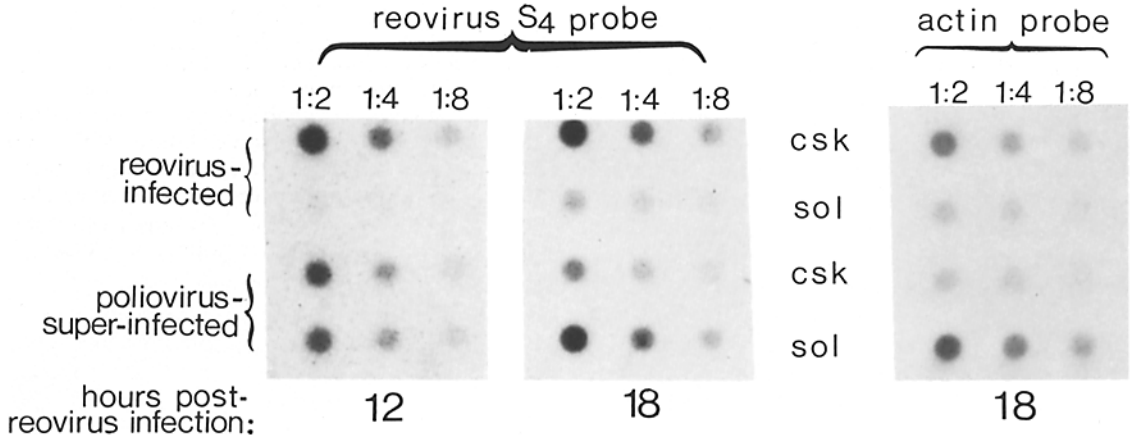

FIGURE 8 Cellular distribution of reovirus and actin mRNA in reovirus-infected cells or reovirus-infected, poliovirus-superinfected cells. CV-1 cells grown in 150-mm petri dishes (1.1×10^7 cells/dish) were mock-infected or infected with reovirus. After adsorption for 1 h at 37°C in 3 ml of PBS, 15 ml of DME supplemented with 2% heat-inactivated FBS was added and cells were incubated for 12 or 18 h at 37°C. Cells were then washed with PBS and mock-infected or super-infected with poliovirus as described in Materials and Methods. After adsorption for 45 min at 37°C in 3 ml of DME, 15 ml of DME (supplemented with 2% heat-inactivated FBS) was added and cells were incubated at 37°C. Cytoskeletal and soluble fractions were prepared 3 h postinfection with poliovirus, RNA was purified, and dot-blot analysis was performed at twofold serial dilutions on nitrocellulose paper (S&S) by hybridization with ^{32}P-labeled nick translated reovirus S4 cDNA or actin cDNA as described in Materials and Methods. Blots were exposed against x-ray film and the autoradiograph is shown. *csk*, Cytoskeletal fraction; *sol*, soluble fraction.

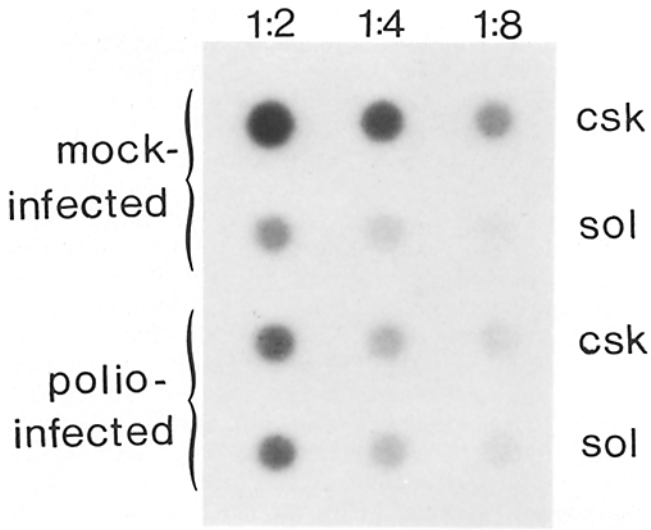

FIGURE 9 Histone mRNA distribution between cytoskeletal and soluble fractions of synchronized mock- or poliovirus-infected CV-1 cells. Subconfluent CV-1 cells, grown in 150-mm petri dishes, were incubated overnight at 37°C in DME without FBS. DME supplemented with 24% FBS containing 5 μg/ml of aphidicolin was added and cells were incubated for 20 h at 37°C. After removal of aphidicolin medium, cells were washed twice with PBS and poliovirus-infected or mock-infected as described in Fig. 3. 4.5 h after poliovirus infection, cells were extracted, the RNA of cytoskeletal and soluble fractions was purified, dot-blot analysis was performed at twofold serial dilutions with ^{32}P-labeled mouse histone probe, and dried blots were autoradiographed and quantified as described in Materials and Methods.

CBP complex is comprised of three major subunits of 220,000, 50,000, and 24,000 mol wt (40, 41), and it has been established that in extracts from poliovirus-infected cells, the CBP complex is somehow inactivated as a consequence of the proteolysis of the 220,000 mol wt subunit (42). Furthermore, a monoclonal antibody with anti-CBP activity has been shown to stain the cytoskeleton in immunofluorescence experiments, perhaps implicating some form of CBP in anchoring mRNAs to the cytoskeleton (9). Whether CBP is indeed involved in such a phenomenon and whether the activity of CBP is regulated in other cases (e.g., during normal physiological functioning of the cell) remains to be investigated.

We tried to address the question of which structural determinants of mRNA might be involved in mRNA cytoskeleton association. The best candidates for such structures are the conserved termini, i.e., the 5′ cap structure and the 3′ poly A tail. However, the results presented here demonstrate that mRNA association with the cytoskeleton can occur in the absence of these structures. Viral mRNA (reovirus) that does not contain a 3′ poly A tail is found associated with the cytoskeleton under conditions in which poly $(A)^+$ mRNAs (e.g., actin) are also associated (Figs. 2 and 8). In addition, we found that an histone mRNA (also lacking a 3′ poly A tail) associates with the cytoskeleton (Fig. 9). Furthermore, Ben-Ze'ev et al. (27) have reported that poly $(A)^-$ subsets of SV40 mRNAs are associated with the cytoskeleton. Because cellular mRNA lacking a cap structure has not been found to date, we used the naturally uncapped poliovirus RNA to probe involvement of the cap structure in cytoskeletal attachment. Our results corroborate the findings of Lenk and Penman (7) who showed by pulse labeling that poliovirus RNA associates with the cytoskeleton during translation. Although our results do not support the notion that either the poly A tail or the cap structure are involved in the attachment of mRNA to the cytoskeleton, the possibility that the relatively small number of mRNAs without a 5′ cap structure or a 3′ poly A tail have evolved an alternative structure to serve in attachment to the cytoskeleton is not precluded.

It might be argued that the release of host mRNAs from the cytoskeleton upon poliovirus-infection reflects a change in the cytoskeleton fractionation properties because of viral-induced cell injury. However, this possibility appears unlikely because polyribosomes that translate poliovirus RNA fractionate with the detergent-resistant material defined as the

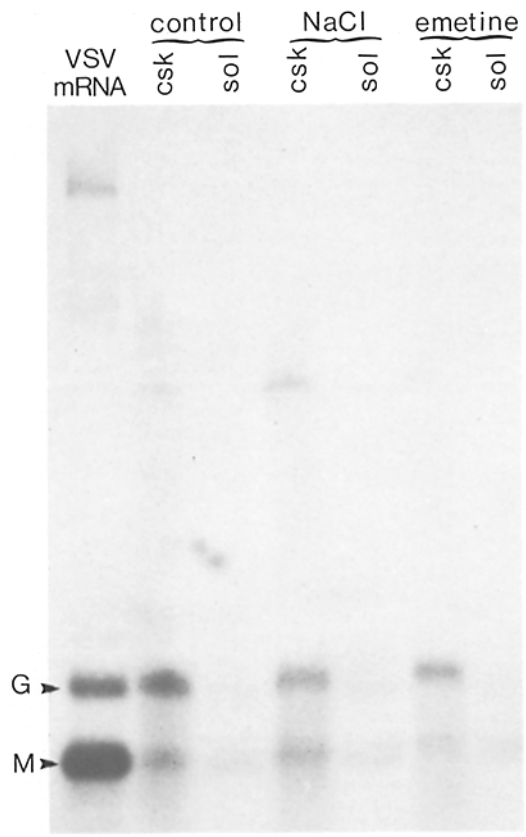

FIGURE 10 Effect of NaCl and emetine on VSV mRNA association with the cytoskeleton. CV-1 cells grown in a 75-cm^2 flask (8×10^6 cells) were infected with VSV. After 45 min of adsorption at 37°C in 2 ml of DME, 15 ml of DME supplemented with 2% heat-inactivated FBS was added and cells were incubated at 37°C. 2 h postinfection, cells were treated with emetine at 2.5 μg/ml for 20 min or with 200 mM NaCl for 15 min. Cellular cytoskeleton was extracted, and RNA was purified and analyzed on a methyl mercury hydroxide agarose gel. RNA from equal amount of cells was transferred to a GeneScreen paper and hybridized with ^{32}P-nick translated G and M VSV cDNAs, and the blot was exposed against an x-ray film, as described in Materials and Methods. The autoradiograph of the exposed blot is shown. The cellular fractions and the inhibitors of protein synthesis used are indicated in the figure. *csk*, Cytoskeletal fraction; *sol*, soluble fraction.

cytoskeleton. Moreover, we have analyzed the cytoskeleton and soluble fractions from poliovirus or VSV-infected cells on SDS polyacrylamide gels and found no gross changes in the staining pattern of the polypeptides in both fractions as compared with the corresponding fractions from mock-infected cells. These results indicate that the cytoskeletal components fractionate in a way similar to that of either uninfected or virus-infected cells.

A major area for future investigation concerns the identification of the particular cytoskeletal elements and putative soluble factors that are involved in mRNA–cytoskeleton association. One approach that might prove useful is the development of an in vitro assay in which more precisely defined components are used to reconstitute mRNA binding to the cytoskeleton.

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Note added in proof: After this manuscript was accepted for publication, we came across a report by W. R. Jeffrey (1984, *Dev. Biol.*, 103:482–492) in which the author concludes that actin and histone mRNAs are associated with the cytoskeletal framework in ascidian eggs during development.

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