Shutoff of HeLa Cell Protein Synthesis by Encephalomyocarditis Virus and Poliovirus: a Comparative Study

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Previous experimental results have suggested that poliovirus and encephalomyocarditis (EMC) virus employ very different mechanisms for shutting off host protein synthesis. However, this conclusion is suspect, inasmuch as different cell types were used for the two viruses; hence the apparent mechanistic differences might be specific for cell type and not virus type. To test this possibility we compared shutoff mechanisms in poliovirus- and EMC virus-infected HeLa cells. Striking differences were seen: poliovirus-induced shutoff was much more rapid and extensive than that induced by EMC virus; relative translation rates of certain host proteins were inhibited to different extents by the two viruses; initiation factors prepared from poliovirus-infected cells were specifically defective for translation of capped mRNA's in vitro, whereas those from EMC virus-infected cells were not. These results indicate that EMC virus and poliovirus employ different mechanisms for the shutoff of HeLa cell protein synthesis. This conclusion is consistent with much earlier work and indicates that many differences previously reported are specific to virus type.

Infection of animal cells by picornaviruses results in a switch from host to viral protein synthesis (3, 6). A common and central feature of the switching process is a selective inhibition or "shutoff" of host protein synthesis. The mechanism of this shutoff has been a subject of considerable interest, and numerous theories have been advanced to explain the phenomenon. The viruses most commonly used in these studies have been poliovirus, mengovirus, and encephalomyocarditis (EMC) virus. Recently, a highly plausible mechanism to account for the poliovirus-induced inhibition of host translation has been proposed (10, 11, 14, 21). In this model, poliovirus infection results in the inactivation of an initiation factor required for the translation of capped mRNA's. Since the viral mRNA is not capped (12, 20), whereas host mRNA's are, the loss of this cap-specific factor would necessarily effect a transition from host to viral mRNA translation.

Although the proposed mechanism accommodates well the effects seen in poliovirus infection, it remains uncertain whether the same model can account for the selective inhibitions induced by other picornaviruses. Indeed, a number of studies on EMC virus- or mengovirus-infected cells have revealed no comparable mRNA-specific changes in the host translational machinery (1, 8, 18, 23). However, these past failures to detect such changes are essentially "negative" experiments, and require "positive"

control for verification. For example, it is possible that the apparent absence of virus-induced specific alterations might be due to peculiarities in the cells employed, and not in the viruses per se. To address this issue directly we have compared in a common host system, HeLa cells, the effects of EMC virus and poliovirus infection on host protein synthesis. Our results show that the selective inhibitions induced in HeLa cells by the two viruses differ in several significant aspects. Moreover, important differences were also found between the translational components derived from poliovirus- and EMC virus-infected cells. The implications of these findings for the mechanisms of host translation shutoff in picornavirus infections are discussed.

MATERIALS AND METHODS

Cells and viruses. HeLa M cells were provided by the Basic Cancer Research Center of Washington University School of Medicine. HeLa spinner cultures were grown at a density of 3×10^5 to 6×10^5 cells per ml in Joklik's modified minimal essential medium (MEM-J) supplemented with 5% fetal calf serum and 5% horse serum. HeLa monolayer cultures were prepared by plating 10^6 cells in 2 ml of MEM-J supplemented with 10% fetal calf serum into 35-mm cluster dishes (Costar) and grown overnight to near confluency in a 5% CO₂ incubator. HeLa monolayer cells were used for all experiments except in the preparation of ribosomal salt washes (RSWs). The Mahoney strain of poliovirus type 1 was a gift from E. Wimmer; crude infected-cell lysates clarified of debris by centrifuga-

tion were used as virus stock. EMC virus was grown in LPA cells and purified as described by Kerr and Martin (15). Titers for poliovirus were determined by plaquing on HeLa cell suspensions in soft agar (5). EMC virus titers were determined by plaquing on LPA monolayers (9). Previous correlation studies with EMC virus showed no differences in efficiency of infection between LPA and HeLa cells.

Virus infection. HeLa monolayer cells in 35-mm wells were washed once with warm MEM-J. Each well was overlaid with 0.5 ml of virus in MEM-J. The multiplicity of infection used was 20 PFU/cell for poliovirus or EMC virus. Virus attachment proceeded at room temperature for 30 min, with occasional swirling of the infection solution. At the end of the attachment period, the infection solution was replaced with 1 ml of growth medium at 37°C. Virus infections were then allowed to proceed by incubation at 37°C in a 5% CO₂ incubator. The commencement of virus infection was taken as the beginning of incubation at 37°C. HeLa cells in spinner culture were infected as follows. Suspended cells were pelleted by centrifugation at 1,000 rpm for 5 min and washed three times with ice-cold MEM-J. Washed cells were suspended to 2×10^7 cells per ml and infected with an equal volume of virus in MEM-J to a multiplicity of infection of 20 PFU/cell. Virus attachment proceeded at 4°C for 30 min, with occasional swirling of the cell suspension. At the end of the attachment period, cell suspensions were diluted to 4×10^6 cells per ml with warm MEM-J and supplemented with fetal calf serum and horse serum to 5% of each. The cell suspensions were then incubated first in a 37°C shaking water bath to facilitate temperature equilibration and then in a 37°C warm room with stirring.

Radiolabeling of protein synthesis in vivo and gel analysis of proteins. Cell monolayers were washed twice with warm Earle basal salt solution. Washed monolayers were incubated with 0.5 ml of methionine-free MEM containing 50 μ Ci of [³⁵S]methionine (Amersham; highest specific activity) per ml at 37°C for 20 min. The label was chased for 10 min in the presence of 1.2 mg of nonradioactive methionine per ml. Labeled monolayers were washed with ice-cold Earle basal salt solution, and each was lysed with 1 ml of buffer saline containing 1% Nonidet P-40 and 1% sodium deoxycholate. The lysates were clarified of nuclei by centrifugation. Quantitations of radiolabel incorporated into protein were done by precipitating small aliquots of lysate with 10% trichloroacetic acid. Precipitates were collected on Whatman GF/C filters and washed with 5% trichloroacetic acid and ethanol. Radioactivity present on dried filters was determined by liquid scintillation. To analyze labeled proteins on polyacrylamide gels, cell lysates were precipitated by addition of trichloroacetic acid to a concentration of 10%. The precipitates were spun down and freed of trichloroacetic acid by three cycles of acetone washes. Dry samples were dissolved in sample buffer and analyzed on the gel system of Laemmli (16). Sodium salicylate fluorography of gels was carried out according to J. Chamberlain (personal communication), except for the fluorogram used in calculation of data in Table 2, which was done using the PPO (2,5-diphenyloxazole)-dimethyl sulfoxide method (17).

Quantitation of radioactivity in gel slices. Specific protein bands in gels were located by superposition of fluorograms and then excised and divided into slices of 0.5 cm² or less. Each slice was rehydrated with 0.3 ml of water. Labeled proteins were eluted from each slice by digesting overnight at 37°C with 1 ml of Protosol (New England Nuclear Corp.). Each digest was neutralized with 1 ml of 1 M Tris-hydrochloride, and the mix was counted using 6 ml of 3a70 (Research Products International Corp.). Sample activities were normalized using the channel ratio method.

Preparation of RSW and analysis of activity in fractionated translation systems. RSWs were prepared from HeLa suspension-grown culture according to the procedures of Golini et al. (7). The activity of RSWs to support globin and/or EMC virus translation was analyzed using the fractionated translation system described by Golini et al. (7). Reactions (25 μ l) contained 20 mM Tris-hydrochloride (pH 7.4), 3 mM MgCl₂, 100 mM KCl, 1.5 μ g of spermidine, 2 mM dithioerythritol, 1 mM ATP, 0.1 mM GTP, 0.6 mM CTP, 10 mM creatine phosphate, 4 μ g of creatine kinase, 0.09 unit of absorbancy at 260 nm of tRNA, 2.5 μ g of carrier rRNA, 0.36 μ g of globin or 0.46 μ g of EMC virus RNA (where indicated), 19 amino acids at 40 μ M each, 0.36 μ M methionine, 5 μ Ci of ³⁵S-labeled methionine, 0.03 unit of absorbancy at 260 nm of 40S ribosomes, 0.12 unit of absorbancy at 260 nm of 60S ribosomes, 10 to 25 μ g of RSW, and 25 μ g of pH 5 fraction.

RESULTS

Comparison of effects of poliovirus infection and EMC virus infection on protein synthesis in HeLa cells. Protein synthesis during the course of viral infection was examined by pulse-labeling with [³⁵S]methionine. Synthesis rates were determined in terms of radioactivity incorporated into trichloroacetic acid-precipitable material. The patterns of proteins synthesized were analyzed by gel electrophoresis and fluorography.

The effects of poliovirus infection on the kinetics and on the pattern of protein synthesis in HeLa cells are shown in Fig. 1 and 2, respectively. Poliovirus infection resulted in the inhibition of cellular protein synthesis beginning at 2 h postinfection. This inhibition was extremely rapid and was virtually complete within 1 h after the onset of inhibition. Poliovirus protein synthesis appeared sometime during the decline of cellular protein synthesis and increased in the ensuing 2 h before declining in late infection. Viral protein synthesis appeared not to be sensitive to the process that inhibited cellular protein synthesis. This insensitivity was suggested by the observation that during the transition period viral protein synthesis was increasing rapidly even as cellular protein synthesis was sharply declining. Thus, the switch to viral protein synthesis during poliovirus infection ap-

pears to be a direct consequence of a selective inhibition that suppresses cellular protein synthesis without affecting poliovirus protein synthesis, as noted previously by others (3, 10, 11, 14, 21).

The effects of EMC virus infection on the kinetics and on the pattern of protein synthesis in HeLa cells are shown in Fig. 3 and 4, respectively. Similar to the course of poliovirus infection in HeLa cells, EMC virus infection resulted

in viral protein synthesis commencing at midinfection. In contrast to poliovirus infection, however, EMC virus protein synthesis was not preceded or accompanied by an abrupt inhibition of cellular protein synthesis. Consequently, concurrent synthesis of cellular and viral proteins was seen throughout the remainder of the infectious cycle. EMC virus infection did result in a gradual decline in host protein synthesis in HeLa cells beginning slightly before or concomitant with the appearance of viral protein synthesis. (In the experiment shown in Fig. 3, this decline started at 3 h postinfection, whereas in the Fig.

FIG. 1. Rates of protein synthesis in poliovirus-infected HeLa cells. Poliovirus-infected HeLa monolayer cells were pulse-labeled with [35 S]methionine at various times postinfection. Rates of protein synthesis, in terms of radioactivity incorporated into trichloroacetic acid-precipitable material, were normalized relative to those of the mock-infected control. Details of the procedures used are described in the text.

FIG. 3. Rates of protein synthesis in EMC virus-infected HeLa cells. The rates were determined as described for Fig. 1. (Points at 3.2, 3.7, and 4.3 h were left off the line, because our cumulative experience from many such experiments is that the decline in protein synthesis rate is not irregular.)

FIG. 2. Protein synthesis in poliovirus-infected HeLa cells. HeLa monolayer cells were infected with poliovirus in the presence or absence of 5 μ g of actinomycin D per ml. Infected cells were pulse-labeled at the indicated times postinfection with [35 S]methionine. The labeled proteins were analyzed in 7 to 15% sodium dodecyl sulfate-polyacrylamide gels. Sodium salicylate fluorography was used. Details of the procedures used are described in the text.

4 experiment it began somewhat later, at about 4.3 h.) This decline resulted in the eventual total inhibition of protein synthesis late in infection. Analysis of the ratio of viral to host protein synthesis reveals that the viral portion of the total synthesis steadily increased with the course of infection (data not shown). The outcome of this trend is the switch to predominantly viral protein synthesis seen late in infection (Fig. 4).

It seemed possible that the EMC virus-induced inhibition of host protein synthesis may be an attenuated form of that induced in poliovirus infection. We examined for this possibility by comparing the rates of decrease of synthesis of various cellular proteins in response to infection by the two viruses. The rationale for this approach was that if EMC virus-induced inhibition were merely a slower form of that induced by poliovirus, then the relative rates of decrease for various cellular proteins should be the same. However, markedly different sensitivities to the two viruses were exhibited in the synthesis of certain HeLa cellular proteins. This effect is evident in Table 1, where the rates of synthesis of actin and a high-molecular-weight protein (designated H₂ and H₁, respectively, in Fig. 2 and 4) have been quantitated by scanning autoradiograms with a densitometer. It is clear from these data that the two viruses have quantitatively very different effects on the two host proteins, H₁ being relatively more sensitive to EMC virus than to poliovirus infection. The same kind of differential effects between the two viruses in their inhibition of cellular protein

synthesis has also been observed (data not shown). These differences suggest that the mechanism of inhibition of cellular protein synthesis may differ between poliovirus and EMC virus infections, although they provide no insights as to molecular details. To obtain the latter, *in vitro* studies were conducted.

Comparison of translational activities of RSWs from poliovirus-infected and EMC

TABLE 1. *Relative translation rates of specific host proteins in EMC virus- and poliovirus-infected cells^a*

Virus	Time (h) postinfection	H ₁ synthesis ^b	H ₂ synthesis ^b	H ₁ /H ₂ ^c
EMC virus	4.8	3.3	16.9	0.19
	5.0	2.3	15.3	0.15
	5.4	1.1	10.3	0.10
	5.8	~0	4.6	~0
Poliovirus	2.3	10.3	17.8	0.58
	2.8	5.6	13.3	0.42
	3.3	3.6	8.6	0.42
	4.3	3.0	6.2	0.48

^a Fluorograms similar to those shown in Fig. 2 and 4, but prepared using a PPO-dimethyl sulfoxide fluor to provide higher resolution, were scanned in a Joyce-Loebl densitometer. Care was taken to insure that exposure of fluorograms was within their linear range. Peak height was used as a measure of the relative radioactivity in a given polypeptide band, and the data presented are in centimeters above the no-sample base line. This method gives results very similar to those obtained when peak areas are measured (unpublished data).

^b Refer to Fig. 2 and 4 for identification of these polypeptides.

^c This value for uninfected or mock-infected cells was 0.56.

FIG. 4. Protein synthesis in EMC virus-infected HeLa cells. Procedures described for Fig. 2 were used.

virus-infected HeLa cells. Others have reported that the poliovirus-induced selective inhibition is due to the inactivation of a translational initiation factor exclusively required for the translation of capped messages (11, 21). To examine whether similar changes occurred during the EMC virus infection of HeLa cells, we prepared RSWs from EMC virus- and poliovirus-infected HeLa cells and compared their activities to support translation of capped and uncapped messages in a fractionated translation system.

RSWs were prepared from HeLa suspension cultures. RSWs from poliovirus-infected cells were prepared at 2.25 and 4.0 h postinfection, and those from EMC virus-infected cells were prepared at 2.5 and 4.0 h postinfection. The infectious cycles of poliovirus and EMC virus were observed to be faster in suspension-grown HeLa cells than in HeLa monolayer cells. Consequently, the virus-induced inhibitions were shifted to earlier times. Gel analysis showed that poliovirus infection of suspension-grown cells by 2.25 and 4.0 h postinfection had inhibited host protein synthesis by approximately 75 and 95%, respectively, relative to the uninfected control. Similarly, EMC virus infection of suspension-grown cells by 2.5 and 4.0 h postinfection had inhibited total protein synthesis by 50 and 80%, respectively, relative to the control. Gel analysis showed that EMC virus infection of suspension-grown cells by 4.0 h postinfection resulted in a switch to predominantly EMC virus protein synthesis, so that host translation inhibition was roughly 95%.

The RSWs were assayed at 10 and 25 μ g of protein per 25 μ l of translation reaction. Preliminary assays determined the lower concentration to be at about the 40% saturation level in a concentration versus activity plot. We used as test messages globin mRNA for the capped message and EMC viral RNA for the uncapped message. Message-specific translation was analyzed by gel electrophoresis of the translation products, followed by excision and liquid scintillation counting of product bands. This approach has been found to be more reliable in the quantitation of message-specific translation reactions than trichloroacetic acid precipitation of translation products.

The translational activities of the RSWs assayed as described are shown in Table 2, experiment 1. Poliovirus infection altered the activity of RSWs with regard to translation of both globin and EMC virus mRNA's. However, the ability to support the translation of globin message was reduced much more rapidly, and to a lower level, than that for EMC virus message. Thus, the fractionated translation system using

TABLE 2. *Translation of globin and EMC virus mRNA in vitro using RSW preparations from infected and control cells^a*

Expt	Source of RSW	cpm incorporated with:		Globin/EMC
		Globin mRNA	EMC virus RNA	
1	Uninfected	10,100	37,600	0.27
	Mock infected, 4.0 h	13,900	33,400	0.41
	Poliovirus infected, 2.25 h	3,330	39,500	0.08
	Poliovirus infected, 4.0 h	790	19,700	0.04
	EMC virus infected, 2.5 h	8,840	26,700	0.34
	EMC virus infected, 4.0 h	8,610	24,800	0.35
2	Mock infected, 4.0 h	4,999	10,968	0.46
	Mock infected, 4.0 h, + actinomycin D	4,842	11,328	0.43
	EMC virus infected, 4.0 h	5,520	11,279	0.49
	EMC virus infected, 4.0 h, + actinomycin D	5,126	14,091	0.36
3	Poliovirus infected, 2.25 h	3,320	39,235	0.09
	Poliovirus infected, 4.0 h	795	17,307	0.05
	EMC virus infected, 4.0 h	4,730	18,995	0.25
	EMC virus infected, 4.0 h, + actinomycin D	5,280	14,725	0.36

^a Incubations were conducted and analyzed as described in the text. Absolute values of incorporations are directly comparable only within a given experiment, due to variation in the activity of components used. We used 10 μ g of RSW protein in experiments 1 and 2 and 25 μ g in experiment 3. The counts per minute (cpm) in experiment 2 represent averages of numbers obtained from duplicate incubations.

RSWs from poliovirus-infected cells reproduced in vitro the selective inhibition of cellular protein synthesis seen early in infection in vivo, as well as the decline in viral protein synthesis seen in late infection.

These changes in RSW activities are in agreement with the conclusion reached by Rose et al. (21) and Helentjaris et al. (11) in regards to the mechanism of poliovirus-induced selective inhibition, that is, the inactivation of a factor which is required for the translation of capped but not uncapped messages.

The activities of RSWs from EMC virus-infected cells to support the translation of globin and EMC virus messages were also reduced relative to those of the control. However, in contrast to the changes seen in poliovirus infection, the RSWs from EMC virus-infected cells retained most of their ability to support the translation of globin mRNA. Thus, in contrast to the case demonstrated for poliovirus infection, the fractionated translation system using RSWs

from EMC virus-infected cells did not reproduce the changes in protein synthesis seen *in vivo*.

Comparison of the ratios of globin to EMC virus translation for the various RSWs showed that changes induced by the two viruses are qualitatively different (Table 2). The ratios for RSWs from poliovirus-infected cells were much lower than the control, indicating that the virus-induced changes reduced the translation of globin much more than that of EMC virus. In contrast, the ratios for EMC virus-infected cells were not significantly different from those of the controls, indicating that the virus-induced changes were not message specific.

Use of actinomycin D to enhance EMC viral inhibition of host protein synthesis. It has long been known that actinomycin D enhances the shutoff of host protein synthesis induced by picornavirus infection (19). Moreover, we have observed that actinomycin D can stimulate viral translation as well as subsequent virion production. Evidence of these effects may be seen in Fig. 2 and 4. Of particular interest is the striking enhancement of the EMC virus-induced shutoff of host translation (Fig. 4), which suggests that actinomycin D might alter or supplement the EMC virus infection to render it more similar to that of poliovirus. To test this hypothesis, we looked to see whether the translation specificities reported in Table 2 were altered by the addition of actinomycin D at the time of infection. RSWs from EMC virus-, poliovirus-, and mock-infected cells, in the presence or absence of actinomycin D, were prepared and tested as described in Materials and Methods (Table 2, experiments 2 and 3). Clearly, actinomycin D did not significantly affect the specificity of the translational machinery, in spite of its ability to enhance both EMC viral translation and host shutoff severalfold (Fig. 4). This result supports the earlier conclusion that the mechanisms by which EMC virus and poliovirus inhibit host translation are qualitatively as well as quantitatively different.

The mechanism by which actinomycin D exerts its effects on virus functions is not known, but is currently being investigated.

DISCUSSION

The effects of EMC virus and poliovirus infection on HeLa cellular translation patterns have been compared *in vivo* and *in vitro*. Distinct differences in the effects of the two viruses have been noted. The polio-induced shutoff of host translation is much more rapid and extensive than that caused by EMC virus infection; the relative translation rates of certain host proteins are affected differently by the two viruses; RSWs prepared from polio-infected cells are defective

for the translation of capped mRNA's, whereas those from EMC virus-infected cells are not.

These observations suggest that EMC virus and poliovirus employ distinctly different mechanisms for the shutoff of host protein synthesis, a surprising result in view of the similarities between the two members of the picorna group. Nevertheless, our results are in close agreement with many earlier studies (1, 8, 10, 11, 14, 18, 21, 23) and provide a rational explanation for differences which had previously been thought to be irreconcilable. For example, it now seems likely that failure to detect alterations in translational specificity by EMC virus and perhaps mengovirus infection (1, 8, 18, 23) must be ascribed to the distinctive properties of these viruses, rather than to trivial anomalies in experimental protocol.

These results and conclusions raise once again the important question of how EMC virus inhibits the translation of host mRNA's. In the absence of any more plausible explanation, we are still of the opinion that mRNA competition (7, 18) may play an important role in this process. However, in the present study mRNA competition may be less important than other factors, such as cation influx or efflux (4), since the active EMC viral mRNA in infected HeLa cells is apparently present at a relatively low concentration. In particular, some additional virus-induced mechanism is required to account for the overall decline in translation rates that begins midway in the lytic cycle (at 3 h postinfection in Fig. 3).

The reason for the differences between poliovirus and EMC virus regarding their host shutoff mechanisms is obscure and an interesting subject for speculation. Suffice it to say here that the extremely rapid initiation rate enjoyed by EMC virus RNA *in vivo* (13), which has been ascribed to its unusually high affinity for one or more initiation components (2, 7), renders unnecessary any modification of the host translational machinery. Conversely, the relatively poor translatability of poliovirus RNA *in vitro* (22) suggests the necessity of eliminating competition by host mRNA's to achieve maximum efficiency of translation *in vivo*. However, much work must be done before speculations of this kind can be taken seriously.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Science Foundation (PCM 76-03079) and by Public Health Service grant CA-13008 from the National Cancer Institute.

ADDENDUM IN PROOF

Trachsel, Sonnenberg, Shatkin, Rose, Leong, Bergmann, Gordon, and Baltimore (Proc. Natl. Acad. Sci. U.S.A. 77:770-774, 1980) have shown that the cap-

specific changes induced by poliovirus infection involve the activity of the 24,000-dalton cap binding protein.

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