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NOTES

Encephalomyocarditis Viral RNA Can Be Translated Under Conditions of Poliovirus-Induced Translation Shutoff In Vivo

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Superinfection with poliovirus of HeLa cells already infected with encephalomyocarditis (EMC) virus does not inhibit translation of EMC viral mRNA, whereas residual host translation is completely inhibited. This result indicates that the cap recognition factors inactivated by poliovirus are not required for translation of EMC viral mRNA in vivo, in agreement with previous in vitro experiments. This raises the question of why EMC virus has evolved a cap-independent translation mechanism.

Selective inhibition of translation of cellular mRNA's is a common and well-documented feature of picornavirus infection (2, 14). Until recently, it has been assumed that all picornaviruses accomplish host translation "shutoff" by similar mechanisms. However, a comparison of the effects of poliovirus and encephalomyocarditis (EMC) virus infection on cellular protein synthesis in a single cell type (HeLa cells) revealed significant differences in the mechanism by which selective inhibition is induced by the two viruses (12). For example, poliovirus infection resulted in extensive shutoff of host translation early in infection, in contrast to the slow decline in cell-specific polypeptide synthesis induced by EMC virus infection. In addition, the relative translation rates of certain host proteins were affected differently by the two viruses. Finally, crude initiation factor preparations (ribosomal salt washes) from poliovirus-infected cells were defective for the translation of capped mRNA's, whereas those from EMC virus-infected cells were not (12).

Because picornaviral mRNA's are not capped (10, 16), it has been suggested that alteration of an initiation factor activity required only by capped mRNA's is the mechanism of poliovirus-induced shutoff of host translation. That this factor is inactivated after poliovirus infection has been demonstrated unequivocally (9, 18, 21, 23; J. Hansen and E. Ehrenfeld, personal communication). The hypothesis is attractive, but, given the evidence cited above, this mechanism cannot be general for all picornaviruses. These observations raise important questions, such as: Why should EMC virus have evolved a separate means of inhibiting translation of cellular messages? Is it because the cap-specific factor(s) is an integral participant in initiation on EMC viral mRNA? The available evidence suggests that this is not the case, since EMC viral RNA is translated relatively well in a fractionated system containing ribosomal salt wash from poliovirus-infected HeLa cells (12). The result of this in vitro experiment is surprising because it implies that EMC virus has evolved mechanisms of translation independent of the cap-specific factor, without concomitant evolution of the means to inactivate it.

To test directly whether EMC viral mRNA can be translated under the conditions of poliovirus-induced shutoff in vivo, it is necessary to examine proteins synthesized in HeLa cells infected with both EMC virus and poliovirus. Similar types of experiments with poliovirus and members of the cardiovirus group have been reported previously (15, 20). However, these investigations employed protocols involving either poliovirus infection of cells prior to cardiovirus infection or simultaneous infection with both viruses. Our procedure entailed preinfection with EMC virus (see below). In addition, those studies involved the use of a variant cell line restrictive for the growth of EMC virus, or drugs (e.g., actinomycin D) which themselves may have effects on host translation inhibition (12). Moreover, actual polyacrylamide gel identification of polypeptides made in doubly infected cells was not done. Thus we felt it was important to repeat these experiments in the absence of such restrictions and to examine the translation products. The results described below indicate

clearly that EMC viral translation is not inhibited by poliovirus superinfection, confirming that EMC viral mRNA does not require the function inactivated by poliovirus in vivo.

HeLa M cells (provided by the Basic Cancer Research Center of Washington University School of Medicine) were maintained in suspension culture in Joklik-modified minimal medium (MEM-J) supplemented with 5% fetal calf serum and 5% calf serum (or in MEM-J containing 10% calf serum) as described (12). HeLa monolayer cultures were prepared by plating 1.2×10^6 cells in 2 ml of culture medium in 35-mm cluster dishes (Falcon) and growing overnight to near confluency. Preparation and titration of poliovirus (type 1, Mahoney) and EMC virus stocks and virus infection of monolayer cells (multiplicity of infection = 20 PFU/cell for both viruses) were essentially as described (12) except that virus inocula were 0.3 ml and poliovirus adsorption was performed at 37°C. The commencement of each virus infection was taken as the beginning of incubation at 37°C.

In experiments where HeLa cells were to be infected with both EMC virus and poliovirus, EMC virus adsorption was first carried out at room temperature for 0.5 h in serum-free medium, as in the single infection. At the end of the attachment period, the infection solution was replaced with 1 ml of culture medium at 37°C. The infection was placed in a 5% CO_2 incubator at 37°C for 1.5 h. At this point the growth medium was removed and replaced with poliovirus inoculum in 0.3 ml of MEM-J. The virus was given 0.5 h at 37°C for attachment, and then the inoculum was replaced with 1 ml of serum-containing medium and the infected cells were returned to the incubator. (This attachment period was considered as part of the total infection time.)

This infection protocol, featuring superinfection with poliovirus 1.5 h after EMC virus infection, was chosen to allow sufficient synthesis of EMC viral RNA before induction of host translation shutoff by poliovirus to insure that EMC virus-specific translation could be measured. At the time when poliovirus-induced shutoff first becomes apparent (3.5 h after EMC virus infection), EMC viral RNA synthesis should be well into the exponential phase (3, 4).

At various times after infection with one or both viruses, viral and cellular protein synthesis was examined by labeling in vivo with [^{35}S]methionine and analysis on polyacrylamide gels, according to the following protocol. Cell monolayers were washed twice with Earle balanced salt solution. Washed monolayers were incubated with 0.5 ml of methionine-free Eagle minimal essential medium (or minimal essential medium supplemented with 10 μM unlabeled methionine) containing 20 to 50 μCi of [^{35}S]methionine (Amersham; highest specific activity) per ml at 37°C for 10 min. The label was chased for 20 min in the presence of 1.2 mg of nonradioactive methionine per ml. Labeled monolayers were either washed three times with phosphate-buffered saline (without $CaCl_2$ or $MgCl_2$) and lysed directly into 0.5 ml of sample buffer (20 mM Tris-hydrochloride, pH 6.8, 2% sodium dodecyl sulfate, 20% glycerol, 5% 2-mercaptoethanol, and bromophenol blue), or lysed and processed as described (12). Samples of the lysates were analyzed on 6 to 15% sodium dodecyl sulfate-polyacrylamide gels and autoradiographed on Kodak XR-5 film. Densitometry of the autoradiograms was performed using a Joyce-Loebl microdensitometer (13).

The results of polyacrylamide gel analysis of proteins pulse-labeled during the course of single infection of HeLa cells with poliovirus or EMC virus are shown in Fig. 1. Shutoff of host protein synthesis in poliovirus-infected cells began between 2.1 and 2.7 h postinfection, well before the onset of viral protein synthesis under these conditions. The peak rate of poliovirus-specific translation occurred later, at approximately 4 h after infection. In contrast, the first EMC virus-specific products could be observed in addition to the full spectrum of cellular polypeptides at about 4 h after infection. Decline in synthesis of host-specific proteins did not begin until nearly 5 h in these cells, at the same time as the peak rate of viral protein synthesis was achieved.

Figure 2 shows kinetics of protein synthesis during superinfection of EMC virus-infected HeLa cells with poliovirus. In this infection, inhibition of synthesis of host-specific polypeptides took place at 3.5 to 4.5 h (2.0 to 3.0 h after superinfection), and poliovirus-specific products first appeared at 4.5 h (3.0 h after superinfection). The fact that the timing of these events is the same as in the infection with poliovirus alone suggests that poliovirus replication proceeds normally in the EMC virus-infected cell. Host translation shutoff must be due to a poliovirus function, because it occurs sooner than would be expected for EMC virus-induced shutoff, and because it is characterized by a very rapid and complete inhibition of cellular translation, as opposed to the gradual decline typical of EMC virus-infected HeLa cells.

In the absence of translation of cellular messages, formation of EMC viral products was clearly evident. Moreover, poliovirus superinfection of EMC virus-infected cells allowed synthesis of EMC virus-specific polypeptides on schedule (compare Fig. 1, lanes 8 to 13, with Fig. 2, lanes 2 to 5), and the replication of poliovirus in

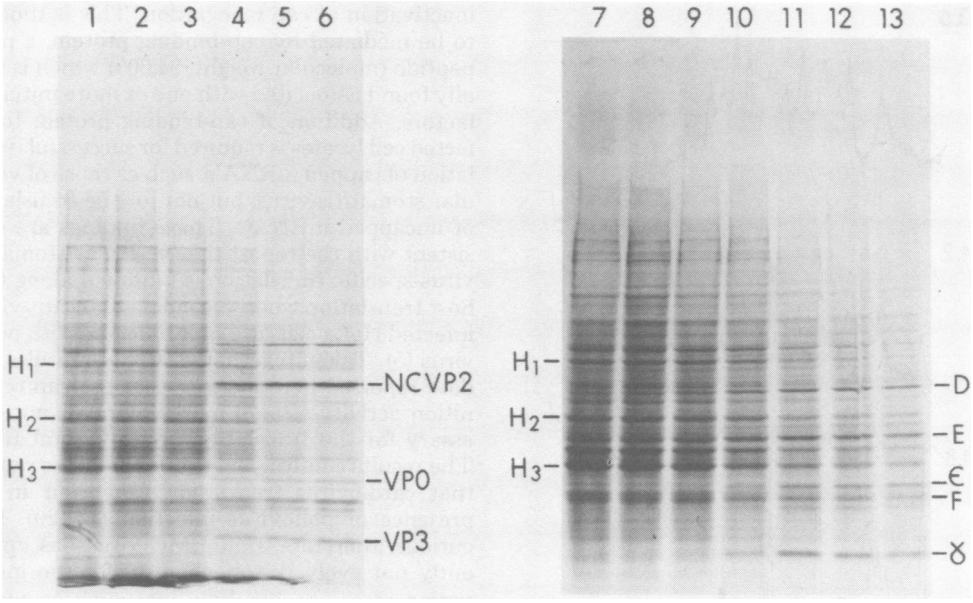

FIG. 1. *Protein synthesis in poliovirus- and EMC virus-infected HeLa cells. HeLa monolayer cells were infected with poliovirus or EMC virus at 0 h. Infected cells were pulse-labeled at various times with [^{35}S]-methionine. The labeled proteins were analyzed in sodium dodecyl sulfate-polyacrylamide gels. Lanes 1 to 6: Poliovirus-infected cells pulse-labeled at 1.5, 2.1, 2.7, 3.2, 3.5, and 3.8 h, respectively; lane 7: mock-infected cells pulse-labeled at 3.0 h postinfection; lanes 8 to 13: EMC virus-infected cells pulse-labeled at 3.0, 3.6, 4.2, 4.8, 5.0, and 5.3 h, respectively. Some cellular proteins (H_1, H_2, H_3), polioviral proteins (NCVP2, VP0, VP3), and EMC viral proteins (D, E, ϵ, F, γ) are identified (8, 17, 19).*

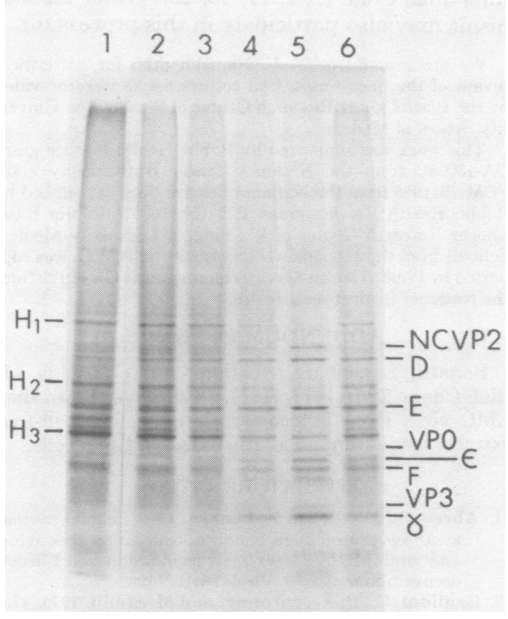

FIG. 2. *Poliovirus superinfection of EMC virus-infected HeLa cells. HeLa cells were infected with EMC virus at 0 h and superinfected with poliovirus at 1.5 h. Superinfected cells were pulse-labeled at various times with [^{35}S]methionine. Labeled polypeptides were analyzed as in Fig. 1. Lane 1: Cells mock-infected at 0 h and 1.5 h and pulse-labeled at 2.2 h; lanes 2 to 5: superinfected cells pulse-labeled at 3.5, 4.0, 4.5, and 5.0 h, respectively; lane 6: cells infected with EMC virus at 0 h, mock-infected at 1.5 h, and pulse-labeled at 5.0 h. Polypeptides are identified as in Fig. 1.*

the same milieu actually appeared to stimulate EMC virus-directed translation (compare lanes 5 and 6 in Fig. 2). Thus it is clear that EMC viral translation in vivo is not affected by the poliovirus-induced host shutoff mechanism.

This effect was documented further when doubly infected cells were pulse-labeled at later times, as shown in Fig. 3. In this particular experiment, polioviral translation products were detectable slightly sooner than in the previous experiment, but the data confirm the trend shown in Fig. 2: translation of cellular mRNA's was suppressed according to the schedule of the poliovirus replication cycle, and the remaining protein synthetic activity was mainly devoted to production of both EMC virus- and poliovirus-specific polypeptides. The marked reduction in incorporation into these products late in the superinfected cells is probably due to EMC virus-induced cytopathic effect (12; compare Fig. 1, lane 13).

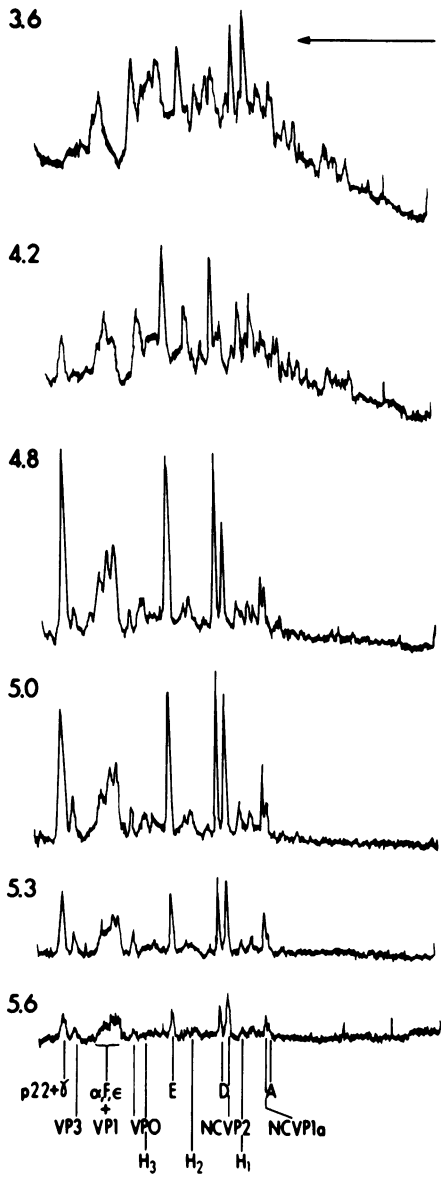

FIG. 3. *Time course of protein synthesis during poliovirus superinfection of EMC virus-infected HeLa cells. The protocol was identical to that described in Fig. 2. Densitometric scans of the autoradiogram are shown; full-scale absorbance was 1.6. The number on the left of each profile refers to the time (hours) after EMC virus infection at which the cells were pulse-labeled. Polioviral (NCVP1a, NCVP2, VP0, VP1, VP3) and EMC viral (A, D, E, ε, F, α, γ, p22) products were identified by comparison with scans of parallel lanes containing lysates of poliovirus- or EMC virus-infected cells.*

Trachsel and co-workers (23) have shown that the host translation shutoff observed in poliovirus-infected HeLa cells is correlated with the inactivation of cap recognition. This is thought to be mediated by cap-binding protein, a polypeptide (molecular weight, 24,000) which is usually found associated with one or more initiation factors. Addition of cap-binding protein to infected cell lysates is required for successful translation of capped mRNA's, such as those of vesicular stomatitis virus, but not for the translation of uncapped mRNA's. These findings are consistent with the report that vesicular stomatitis virus-specific translation is inhibited along with host translation when vesicular stomatitis virus-infected HeLa cells are superinfected with poliovirus (5). Taken together with these results, the data reported here indicate that the cap recognition activity altered by poliovirus is not necessary for the translation of EMC viral RNA. The results confirm and extend previous reports that cardiovirus translation can occur in the presence of poliovirus infection (15, 20). It is curious, therefore, that EMC virus has apparently not evolved a mechanism for the inactivation of cap recognition, inasmuch as such a mechanism would allow the virus to shut off host translation more effectively. Previous work has suggested that EMC virus selectively inhibits host translation in murine cells by an entirely different set of mechanisms, which may include a competition between viral and cellular mRNA's for host initiation factors late in the infectious cycle (1, 7, 11, 13, 22). Other mechanisms may also participate in this process (6).

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ADDENDUM IN PROOF

Bonatti, Sonenberg, Shatkin, and Cancedda (J. Biol. Chem. **255**:11473–11477, 1980) have shown that EMC virus RNA is also translated well in cell-free extracts made from poliovirus-infected HeLa cells.

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