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Reversion by Hypotonic Medium of the Shutoff of Protein Synthesis Induced by Encephalomyocarditis Virus

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Infection of human HeLa cells by picornaviruses produces a drastic inhibition of host protein synthesis. Treatment of encephalomyocarditis virus-infected HeLa cells with hypotonic medium reversed this inhibition; no viral protein synthesis was detected. The blockade of viral translation by hypotonic conditions was observed for a wide range of multiplicities of infection. However, only with low virus-to-cell ratios did cellular protein synthesis resume. The ratio of cellular to viral mRNA translation was strongly influenced by the concentration of monovalent ions present in the culture medium: a high concentration of NaCl or KCl favored the translation of viral mRNA and strongly inhibited cellular protein synthesis, whereas the opposite was true when NaCl was omitted from the culture medium. Once viral protein synthesis had been blocked by hypotonic medium treatment, it resumed when the infected cells were placed in a normal or hypertonic medium, indicating that the viral components synthesized in the infected cells were not destroyed by this treatment. These observations reinforced the idea that ions play a role in discriminating between viral and cellular mRNA translation in virus-infected animal cells.

Infection of animal cells by a cytolytic virus induces a specific inhibition of host macromolecular synthesis. The inhibition of protein synthesis in picornavirus-infected cells, the so-called shutoff phenomenon (1, 7, 28), was first described 20 years ago (8). Since then, studies in a number of laboratories have established that the bulk of viral protein synthesis takes place when cellular mRNA translation is drastically inhibited.

Several theories have been proposed to explain the specific regulation of translation observed in animal cells infected with viruses (2, 7). These theories can be divided in two main groups: some suggest the generation of a specific inhibitor of host protein synthesis after viral infection (10, 16), whereas others explain the discrimination between host and viral mRNA translation by changes in the protein-synthesizing machinery of the cell (12, 15, 24, 29, 30).

We advanced the hypothesis that the modification of the cell membrane after viral infection could alter the ionic milieu in the cellular cytoplasm, and, in turn, this change would differentially influence cellular and viral protein syntheses. In support of this model, we know that the in vitro translation of cellular mRNA's is inhibited by monovalent ions at the same time that viral protein synthesis is stimulated (5, 6). A similar discrimination exerted by monovalent ions on translation has also been described in cells infected by viruses (for a review, see reference 7). Moreover, the permeability of virus-infected cells to ions and metabolites changes when the shutoff of host protein synthesis takes place (3, 9, 11, 20). These changes in membrane permeability probably reflect the interaction of a virion component with the cell membrane and are observed early during viral attachment, as well as late in infection when massive amounts of viral coat protein are synthesized. In any event, it is clear that changes in the intracellular cation concentration of a magnitude sufficient to selectively inhibit host translation by the same mechanism as that which occurs in vitro occur late in the course of infection (9).

In this study, we show that the removal of sodium chloride from the culture medium of encephalomyocarditis (EMC) virus-infected cells stopped viral protein synthesis; at the same time, host mRNA translation resumed.

MATERIALS AND METHODS

Cells and virus. HeLa cells were propagated in culture flasks (Falcon Plastics) containing 6 ml of Eagle medium as modified by Dulbecco (E_4D), supplemented with 10% newborn calf serum (GIBCO Laboratories; E_4D_{10}), and incubated at 37°C in a 5% CO_2 atmosphere.

EMC virus was grown on L-929 cells in a mixture of Eagle medium, phosphate-buffered saline, and E_4D (80:15:5), supplemented with 1% newborn calf serum (E_4D_1). The fraction obtained after the removal of cell

debris by low-speed centrifugation was used as a source of virus.

Conditions of EMC virus infection and protein labeling. HeLa cells were grown in 3.5-cm-diameter petri dishes (Falcon Plastics) containing 2 ml of E_4D_{10}. When the cell monolayer was confluent, the medium was removed and the cells were infected with EMC virus in phosphate-buffered saline at a multiplicity of infection of 5 PFU per cell for 1 h at 37°C. After this time, the inoculum was removed and replaced with 1 ml of E_4D_1. At the indicated times, the medium was removed and replaced with 0.5 ml of methionineless E_4D_1 (E_4D_1 − Met) or with 0.5 ml of E_4D_1 − Met with no NaCl (E_4D_1 − Met − NaCl). Labeling of newly synthesized proteins was carried out by adding 5.7 μCi of [^{35}S]methionine (Amersham Corp.; 740 Ci/mmol, 5.7 mCi/ml) per culture dish, and incubation was continued for 1 h at 37°C.

Polyacrylamide gel electrophoresis. After incubation of cells in the presence of [^{35}S]methionine, the medium was removed and the cell monolayer was washed with 2 ml of phosphate buffer; the cells were dissolved in 0.100 ml of 0.02 N NaOH containing 0.1% sodium dodecyl sulfate plus 0.200 ml of sample buffer (62.5 mM Tris [pH 6.8], 2% sodium dodecyl sulfate, 0.1 M dithiothreitol, 17% glycerol). Each sample was sonicated to reduce viscosity and heated at 90°C for 2 min. Samples (0.005 ml each) were analyzed by polyacrylamide gel electrophoresis, using 15% acrylamide gels. The gels were run overnight at 30 V, stained, and destained, and fluorography was carried out with 2,5-diphenyloxazole–dimethyl sulfoxide (20%, wt/wt). The dried gels were exposed by using XS-5 X-ray films (Kodak). Densitometric profiles of the gel were done in an Optronics P1700 microdensitometer.

Measurement of protein synthesis. A 0.010-ml portion of each sonicated sample was precipitated with 1 ml of 10% trichloroacetic acid, heated for 10 min at 90°C, and filtered in GF/C glass fiber filters (Whatman, Inc.). The radioactivity retained on the filters was determined in an Intertechnique scintillation spectrometer.

RESULTS

Hypertonic medium treatment inhibits the initiation of protein synthesis in normal cells (31) and has a differential effect on viral and cellular protein syntheses in virus-infected cells (4, 22, 23, 27). The interpretation currently given to these findings is that hypertonicity induces an unspecific inhibition on the initiation of translation of both viral and cellular mRNA's (21). Nevertheless, a differential effect could be observed if viral mRNA's have a higher initiation rate for translation as compared with the α- and β-globin mRNA's (19). According to this interpretation, any unspecific inhibition of the initiation of protein synthesis in virus-infected cells will result in a similar effect. In contrast, our early observations on the action of monovalent ions on translation strongly suggested that ions cannot be regarded as unspecific inhibitors of the initiation of translation, but rather that they behave as specific effectors, because they inhibit one kind of mRNA at the same time that they stimulate the translation of other mRNA's (5, 6). The prediction derived from these observations was that a decrease in the concentration of ions would preferentially inhibit viral as opposed to cellular protein synthesis. To test this hypothesis, human HeLa cells were infected with EMC virus, and protein synthesis was measured over the time course. At the moment when the shutoff of host protein synthesis was apparent and viral proteins started to be synthesized, the culture medium was replaced by a medium without NaCl, and the proteins synthesized in the virus-infected cells under those conditions were analyzed by polyacrylamide gel electrophoresis. Figure 1 shows that whereas viral protein syn-

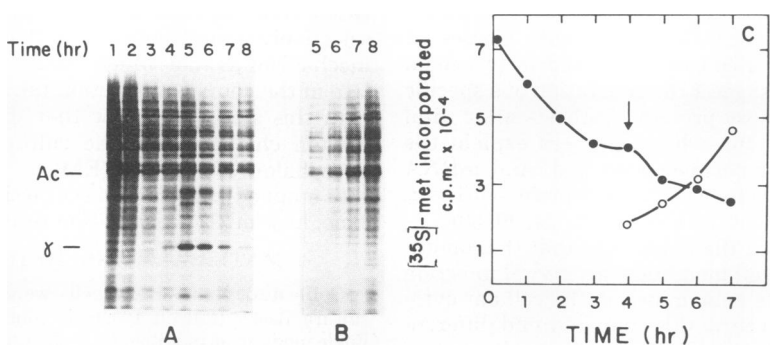

FIG. 1. *Effect of hypotonic medium on protein synthesis in EMC virus-infected HeLa cells. Conditions of infection and labeling were as described in the text. At 4 h postinfection, the medium was removed and replaced by normal (E_4D_1) or hypotonic (E_4D_1 − NaCl) medium; protein synthesis continued until 10 h postinfection. (A and B) Autoradiography of the polyacrylamide gel of the proteins synthesized in normal and hypotonic media, respectively. The numbers above the lanes indicate the time interval of protein labeling with [^{35}S]methionine. Cellular protein actin (Ac) and viral protein are indicated. (C) Time course of protein synthesis in EMC virus-infected cells in the presence of normal (●) or hypotonic (○) medium. The arrow indicates the time when hypotonic medium was added.*

thesis did not take place in hypotonic medium, cellular protein synthesis did. Hypotonic medium per se caused an inhibition of total protein synthesis of around 50% in both normal and virus-infected cells; similar results were obtained in the presence of actinomycin D. This is in agreement with the findings indicating that cellular mRNA's are not degraded after picornavirus infection (18). To our knowledge, this is the first experiment indicating (i) that the shutoff of host protein synthesis by picornavirus infection can be reversed and (ii) that viral protein synthesis in virus-infected cells may be specifically suppressed. Our unpublished data also indicate that viral RNA synthesis was not affected by the change to hypotonic conditions. On the other hand, an analysis of the proteins synthesized in 10-min pulses from the time that the cells were shifted to hypotonic medium showed that viral protein synthesis was still apparent during the first 10 min and was virtually absent in cells placed in hypotonic medium for longer periods of time (unpublished data).

The shutoff of host protein synthesis is influenced by the multiplicity of infection (1, 7, 28). Figure 2 shows that under all multiplicities of infection tested the shift to hypotonic medium resulted in an inhibition of viral protein synthesis; however, cellular protein synthesis was restored during the first 90 min of treatment only when moderate multiplicities of infection were used.

Our view that monovalent ions act as effectors of protein synthesis and do not unspecifically

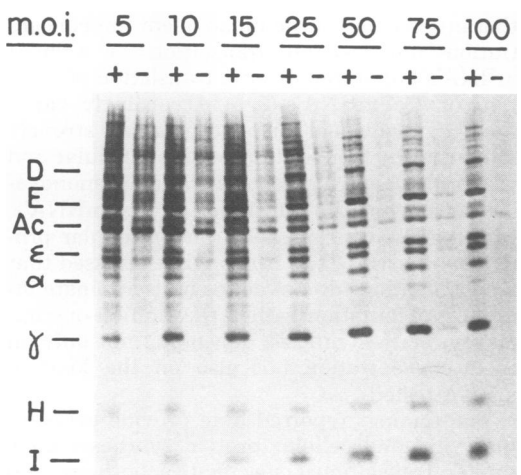

FIG. 2. *Dependence of the shutoff reversion by hypotonic medium on the multiplicity of infection (m.o.i.). At 4 h postinfection, the medium was removed and replaced by 0.5 ml of E_4D_1 (+) or E_4D_1 − NaCl(−). Labeling of newly synthesized proteins was from 4.5 to 5.5 h postinfection.*

inhibit protein synthesis on all kinds of mRNA indicates that an increase in ions above a certain concentration inhibits protein synthesis of cellular mRNA but stimulates the translation of viral mRNA, whereas a decrease in the concentration of monovalent ions will produce the opposite effects. To test this view, EMC virus-infected cells were placed in media with different concentrations of either NaCl or KCl, and the proteins synthesized were analyzed by polyacrylamide gel electrophoresis. The syntheses of cellular and viral proteins and, hence, the shutoff of host protein synthesis at a given moment after infection were strongly influenced by the tonicity of the medium (Fig. 3). In the absence of NaCl, no viral proteins were detected and only cellular mRNA's were translated, whereas the opposite was true under hypertonic conditions. Furthermore, Fig. 3 also shows that potassium ions could substitute for sodium ions for this effect to occur, in agreement with previous observations in cell-free systems (28).

The regulatory role of divalent cations in animal cells is well documented (25, 26). However, variations in magnesium or calcium either in the culture medium or in cell-free systems have no discriminatory effects on cellular and viral protein syntheses (unpublished data).

The above observations raise several questions. Does the cell survive if viral protein synthesis stops after a hypotonic medium shift? Moreover, can we cure the infected cells by placing them in a hypotonic medium after infection? Is viral mRNA stable in cells under hypotonic conditions? To answer some of these questions, we subjected EMC virus-infected HeLa cells to various rounds of hypotonic medium treatments in the presence of 10 μg of actinomycin D per ml. Viral and cellular protein syntheses could be switched on and off by manipulating the ionic conditions in the medium (Fig. 4). The shift to hypotonic conditions stopped the synthesis of viral proteins, and the cells continued to synthesize exclusively cellular proteins even 5 h after the medium change. Moreover, when the cells that had been exposed to hypotonic medium for 2 h were placed in a hypertonic medium, viral protein synthesis immediately resumed. In addition, the replacement of NaCl by sucrose did not inhibit viral translation, indicating that a low concentration of sodium ions was not the sole cause of the inhibition of viral translation. As the concentration of monovalent ions in the cytoplasm depended on the medium tonicity, the decrease in monovalent ions in the cells was probably only obtained at lower medium tonicities. One conclusion from the above results is that viral RNA, although not translated in infected cells under hypotonic

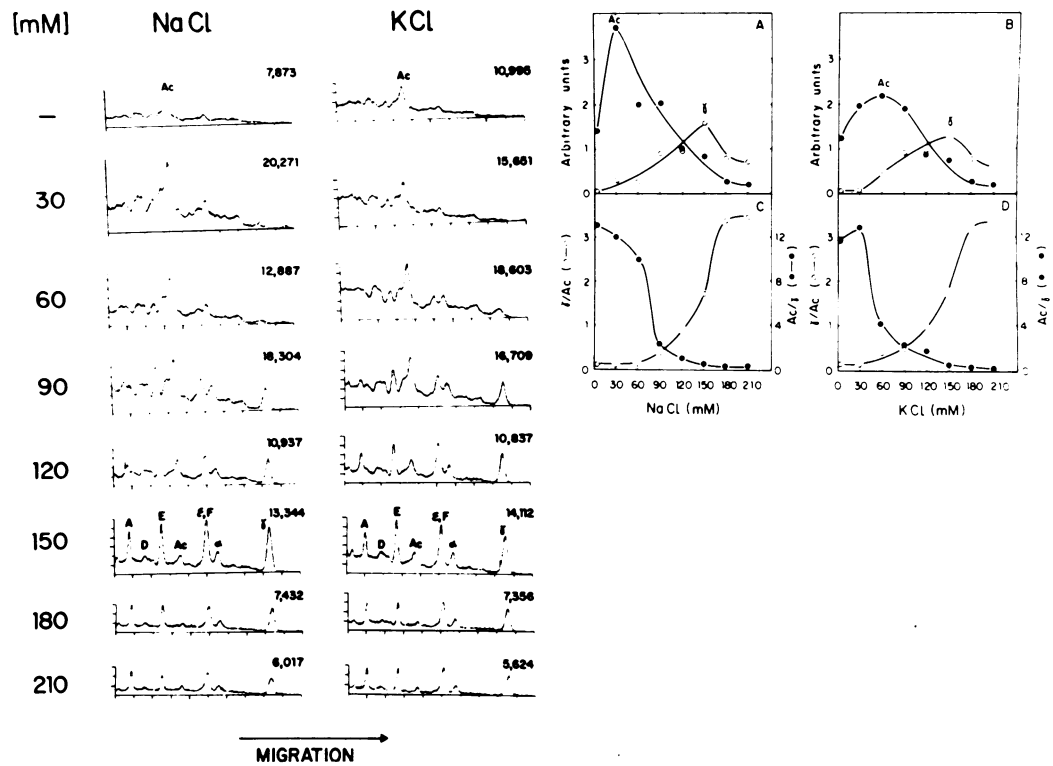

FIG. 3. *Effect of different media containing various concentrations of monovalent ions on protein synthesis. Conditions of infection were as described in the text. At 4 h postinfection, culture medium with the indicated concentrations of NaCl or KCl was added. Protein labeling with [^{35}S]methionine was from 4.5 to 5.5 h postinfection, as described in the text and in the legend to Fig. 2. The number in each densitometric scan represents the total area calculated in the microdensitometer. (A to D) Amounts of cellular protein actin (●) and viral protein γ (○) calculated from the area of each densitometric scan.*

conditions, is readily used as a messenger when the concentration of NaCl is increased; at the same time, the translation of cellular mRNA is inhibited by this increase in the NaCl concentration. The cytopathic effect could also be prevented in EMC virus-infected HeLa cells when the cells were shifted down to hypotonic medium during the first 4 h of viral infection. Under hypotonic conditions, the cells maintained a normal appearance even 2 days after infection. The addition of normal medium after this period of time did not bring about the cytopathic effect.

DISCUSSION

Two different aspects must be distinguished in the shutoff of protein synthesis induced by viral infection. One is the decrease in total protein synthesis throughout infection; the other is the specific regulation of translation, i.e., the switch from cellular to viral mRNA translation. This second phenomenon may be influenced by the changes in the monovalent ion concentration. Evidence from in vitro systems indicates that an increase in the monovalent ion concentration inhibits the translation of cellular mRNA's but stimulates the translation of a variety of viral mRNA's (5, 6). Accordingly, variations in monovalent ion concentrations strongly influence the competition between cellular and viral mRNA's: a high concentration of monovalent ions favors the translation of viral mRNA's, whereas low concentrations favor cellular protein synthesis (5, 17). It should be stressed that monovalentions do not act as indiscriminate inhibitors of initiation; rather, they inhibit or stimulate protein synthesis, depending not only on their concentration but also on the kind of mRNA tested.

The findings reported here provide evidence that viral and cellular protein syntheses have similar translational specificities both in vivo and in vitro and lend support to the idea that the choice between viral and cellular mRNA's by the protein-synthesizing apparatus in cells is also dictated by modifications in the concentration of monovalent ions in the cytoplasm after viral infection.

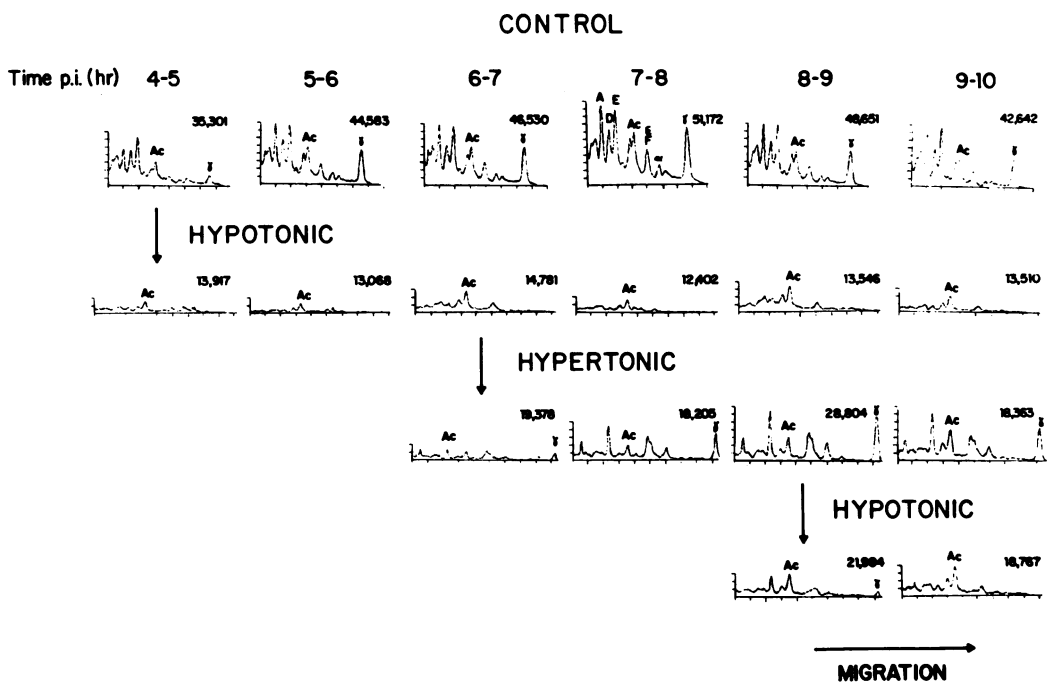

FIG. 4. *Reversibility of the hypotonic medium effect on protein synthesis in EMC virus-infected HeLa cells. Actinomycin D (Ac; 10 μg/ml) was added 1 h before virus infection and left throughout this experiment. Conditions of infection and protein labeling were as described in the text. The shifts to hypotonic (E_4D_1 − NaCl) and hypertonic (E_4D_1 plus 70 mM NaCl) media were done as indicated by the arrows. The ranges of numbers indicate the time intervals of labeling with [^{35}S]methionine. The numbers in each densitometric scan represent the counts of [^{35}S]methionine incorporated per minute in protein.*

According to this theory, EMC virus infection need not generate any stable and specific inhibitor of cellular protein synthesis, such as double-stranded RNA or viral proteins. However, the possibility that a hypotonic medium destroys an unstable inhibitor of cellular protein synthesis remains open.

As discussed previously (4), the competition between viral and cellular mRNA's must be conceived as a more dynamic process. Lebleu et al. (17) showed that the competition for the translation between viral and cellular mRNA's in vitro is strongly influenced by the concentration of monovalent ions. Our observations suggest that the competition between different types of mRNA in infected cells may also be dictated by the concentration of ions present in the cytoplasm.

An initial report indicated that S-30 systems obtained from poliovirus-infected cells are unable to support the translation of capped mRNA's; the activity of these cell-free systems was restored by the addition of eLF4B (24). It was subsequently observed that a cap binding factor that contaminated the initiation factor preparation was responsible for such restoration (23, 30). From these in vitro experiments, it was suggested that the inhibition of protein synthesis in poliovirus-infected cells might be a consequence of the inactivation of such a cap binding factor. The results presented here do not add support to the view that a similar mechanism is operative in EMC virus-infected cells. It has recently been suggested that the shutoffs induced by EMC virus and poliovirus are different (14).

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