Action of Nucleotide Derivatives on Translation in Encephalomyocarditis Virus-infected Mouse Cells

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SUMMARY

The infection of animal cells by encephalomyocarditis (EMC) virus lead to a drastic change in membrane permeability towards low mol. wt. compounds. Addition of the nucleotide analogue GppCH₂P to the culture medium resulted in a specific inhibition of protein synthesis in EMC virus-infected 3T6 cells. This inhibition was not observed when GTP or ATP were present nor in control mock-infected 3T6 cells. The induction of membrane leakiness after viral infection was not specific for 3T6 cells, as it was also detected in mouse L cells, hamster BHK-21 cells and monkey CV1 cells. The inhibitory action produced by GppCH₂P in virus-infected cells was fully reversed upon addition of fresh medium. Moreover, analysis of the proteins synthesized after medium replacement showed a preferential synthesis of cellular proteins. The presence of zinc ions resulted in an inhibition of the cleavage of large viral polypeptide precursors to mature viral proteins. Under these conditions, membrane leakiness as measured by GppCH₂P, was not observed. However, this seems to be an effect of zinc ions themselves on the membrane, because inhibition of mature protein formation by other means, such as the presence of amino acid analogues, did not prevent inhibition of translation by GppCH₂P in virus-infected cells.

Addition of the cap analogues 7mGppp and 2'-O'-mGppp, resulted in specific stimulation of viral protein synthesis in EMC virus-infected 3T6 cells. On the other hand, the presence of 7mGp had no effect on translation. We propose that a specific capping of viral mRNA takes place in the presence of these compounds, and leads to increased stability and greater efficiency in the translation of viral mRNA.

INTRODUCTION

The infection of mouse cells by picornaviruses leads to profound alterations in the permeability properties of the infected cells to compounds to which control uninfected cells are normally impermeable (Carrasco, 1978, 1979; Carrasco & Smith, 1976; Contreras & Carrasco, 1979; Egberts et al., 1977; Farnham & Epstein, 1963; Nair et al., 1979). The GTP analogue, GppCH₂P, which is unable to inhibit protein synthesis in normal 3T6 cells specifically blocks translation in encephalomyocarditis (EMC) virus-infected cells at the onset of viral protein synthesis, suggesting that the nucleotide analogue enters the virus-infected cells (Carrasco, 1978). A similar effect was also observed with other translation inhibitors, such as blasticidin S, edeine, gougerotin and hygromycin B (Contreras & Carrasco, 1979). Other evidence indicates that membrane leakiness after viral infection, as measured by inhibitors that do not penetrate into normal cells, is a general phenomenon observed when the

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shut-off of host protein synthesis occurs. It has been observed in cells infected with picornaviruses, togaviruses (Carrasco, 1978), papovaviruses (Carrasco, 1979; Contreras & Carrasco, 1979), rhabdoviruses, herpesviruses and paramyxoviruses (Benedetto et al., 1980).

Some characteristics of the inhibition of protein synthesis by GppCH$_2$ in EMC virus-infected 3T6 cells and the action on translation of cap analogues to which virus-infected cells are permeable, is presented in this paper.

**METHODS**

**Chemicals.** Radioactive compounds were purchased from The Radiochemical Centre, Amersham. GppCH$_2$p was from Boehringer, Mannheim. All the cap analogues used were from P-L Biochemicals, Milwaukee, Wis., U.S.A.

**Cells and viruses.** Mouse fibroblast 3T6 cells, L cells, monkey cells (CV1) and BHK-21 cells were propagated in 100 mm Petri dishes containing 10 ml Eagle's medium, modified by Dulbecco, supplemented with 10% foetal calf serum. EMC virus was grown on 3T6 cells. Cell debris was removed by centrifugation before the virus was used to infect cells.

**Conditions of infection and labelling with $^3$S-methionine.** 3T6 cells were grown in 30 mm Petri dishes containing 2 ml Eagle's medium, modified by Dulbecco, supplemented with 10% foetal calf serum. Once the cell monolayer was confluent, the medium was removed and the cells were mock-infected or infected with EMC virus (2 p.f.u./cell). After incubation for 1 h at 37 °C the medium was removed, 1 ml medium containing 2% calf serum was added and the dishes were incubated for a further 4 h at 37 °C. The medium was then replaced by 0.5 ml medium without methionine supplemented with 1% calf serum. The indicated nucleotide was then added and incubation was continued for 30 min at 37 °C. After this time, 2 μCi $^3$S-methionine (1000 Ci/mmol; 2 mCi/ml) were added and the dishes were further incubated for 1 h at 37 °C. The medium was then removed and the cell monolayer was washed with 2 ml phosphate buffer. The cells were dissolved in 0.15 ml 0.1 M-NaOH containing 0.1% sodium dodecyl sulphate and 0.15 ml sample buffer. Each sample was sonicated to reduce the viscosity and heated to 90 °C for 10 min. Samples of 10 μl were analysed by polyacrylamide gel electrophoresis using 15% acrylamide gels. The gels were run overnight at 25 V. When the bromophenol blue tracking dye reached the bottom of the gels, electrophoresis was stopped and the gels were stained and destained. Fluorography with 20% (w/w) dimethyl sulfoxide–2,5-diphenyloxazole was then carried out and the gels were dried. The gels were subjected to autoradiography (Kodak XH1 film) and the developed film was analysed on a densitometer.

**RESULTS**

**Characteristics of the inhibition of translation by GppCH$_2$p in EMC virus-infected cells**

Addition of some nucleotides to the medium of cells growing in culture produces changes in the activity of some membrane-bound enzymes and in the permeability of those cells to ions (Giotta et al., 1978; Rozengurt & Heppel, 1975; Spiegel et al., 1977). We considered it of interest to determine whether the inhibition of protein synthesis by GppCH$_2$p in EMC virus-infected 3T6 cells was specific for that compound. Fig. 1 shows that specific inhibition of translation in virus-infected cells did not occur in the presence of ATP or GTP and that only the protein synthesis inhibitor GppCH$_2$p blocked translation in those cells. This result indicated that GppCH$_2$p does not inhibit protein synthesis by a non-specific mechanism involving, for instance, magnesium or pH changes in the culture medium.

The concentration of serum in the medium has a number of effects on the transport to the cytoplasm of several compounds (Plagemann & Rickey, 1974). Moreover, it has recently been observed that some nucleotides do cross the cell membrane (Cohen & Plunkett, 1975)
Control

Action of nucleotides in viral translation

Fig. 1. Effect of nucleotides on protein synthesis in EMC virus-infected 3T6 cells. 3T6 cells grown in 30 mm Petri dishes were infected with EMC virus (3 p.f.u./cell). After incubation for 1 h at 37 °C the medium was removed, 1 ml Eagle's medium supplemented with 2% calf serum was added and the cells were incubated for a further 4 h at 37 °C. The medium was then replaced by 0.5 ml medium without methionine and supplemented with 1% calf serum. Half of the cultures were made 1 ml with respect to GppCH₂p and GTP or ATP (1 mM) or no further additions were made to these and the control cultures and incubation continued for 30 min at 37 °C. After this time 2 µl [³⁵S]-methionine (1000 Ci/mmol; 2 mCi/ml) were added and the dishes were further incubated for 1 h at 37 °C. Finally they were processed as described in Methods. The densitometric scan of the autoradiograph is shown. The translation of the EMC virus RNA gives rise to three primary translation products: A, F and C. A is further cleaved through a series of steps to α, γ and ε. During assembly ε is cleaved to β and δ. C is cleaved to D and finally the cleavage of D gives rise to the replicase E (Carrasco & Smith, 1980). Ac, Actin; Or, origin.

by a transport mechanism which is dependent on the presence of serum in the medium. The effect of calf serum on the permeability of EMC virus-infected 3T6 cells to GppCH₂p was measured by its ability to inhibit translation in those cells. The concentrations of calf serum used ranged from 0 to 10% and under all conditions GppCH₂p passed into virus-infected cells (results not shown). Membrane leakiness in EMC virus-infected 3T6 cells, as measured by the inhibition of translation by GppCH₂p, is not, therefore, a serum-dependent process.

The influence of the host cell on the induction of membrane leakiness by EMC virus infection was also tested. The infection of mouse 3T6 cells, L cells, BHK-21 cells and monkey CV1 cells with EMC virus resulted, in all cases, in the appearance of membrane permeability changes to GppCH₂p at the time when viral proteins were being synthesized (Fig. 2). The extent of the inhibition varied depending on the cell type. This could be due to differences in the plasma membrane response of those cells to induction of membrane leakiness or to some variation in the multiplication cycle of EMC virus in those cells. The possibility that different cells are affected to different extents by the translation inhibitor used is not excluded.

The inhibition of protein synthesis by GppCH₂p in vitro involves the interaction of this compound with several components of the protein-synthesizing apparatus, giving rise to stable complexes inactive in translation (Vázquez, 1979). Such inhibition is reversed upon removal of GppCH₂p. Similar behaviour in intact cells is shown in Fig. 3, implying that its mechanism of action in EMC virus-infected cells does not involve the irreversible modification of any of the components that participate in mRNA translation. Interestingly, after reversal, there is preferential translation of cellular mRNAs as compared to infected cells which had been maintained as controls.

There is some evidence that a viral gene product is involved in the induction of membrane leakiness in cells infected with picornaviruses (Carrasco, 1978). Taking advantage of the fact that the virion proteins are generated by a series of proteolytic cleavages (Carrasco & Smith,
Fig. 2. Inhibition of protein synthesis by GppCH2p in a variety of cells mock-infected or infected with EMC virus. Conditions were as described for Fig. 1. The upper panels represent the percentage of the control with no GppCH2p added. O, Protein synthesis in mock-infected cells: 100% of control was 261671 ct/min for 3T6 cells; 100629 ct/min for L cells; 347094 ct/min for BHK-21 cells; and 72957 ct/min for CV1 cells. O, Protein synthesis in EMC virus-infected cells: 100% of control was 47169 ct/min for 3T6 cells; 12221 ct/min for L cells; 11770 ct/min for BHK-21 cells; and 19164 ct/min for CV1 cells. The lower panels show autoradiographs of the proteins synthesized in EMC virus-infected cells after separation by polyacrylamide gel electrophoresis.

In 1980, we asked whether the continual synthesis of virion proteins was necessary for the maintenance of membrane leakiness. For this purpose, inhibitors of the cleavage process, such as zinc ions and amino acid analogues, were added to the culture medium. Fig. 4 shows that the presence of 1 or 2 mM-zinc resulted in almost total inhibition of the production of virion proteins. Under these conditions, the inhibition of protein synthesis by GppCH2p was also significantly decreased. However, the presence of the amino acid analogue canavanine and the protease inhibitor TPCK [L-(1-tosylamide-2-phenyl)ethyl chloromethyl ketone] that also interfere with the production of virion proteins had no effect on membrane leakiness. We interpret these results to mean that the changes induced in the membrane are stable once virion proteins have been synthesized and membrane leakiness appears.
Action of nucleotides in viral translation

Fig. 3. Reversion of GppCH₂p inhibition in EMC virus-infected 3T6 cells by fresh medium. One mM-GppCH₂p was added at 4 h after infection and the cells were labelled for the period of time indicated in the figure. In (a), fresh medium was added from 4.5 to 5 h; in (b), the addition of fresh medium was from 5 to 5.5 h and the cells were labelled for the same period of time. In (c), fresh medium was added at 4.5 h and the cells labelled from 4.5 to 5 h. The figure shows the densitometric scans of the autoradiograph. The numbers represent percentages of control values as measured by TCA-precipitable material. The values of protein synthesis in the same period of time in EMC virus-infected cells not treated with GppCH₂p were taken as controls.

Effect of cap analogues on protein synthesis in EMC virus-infected 3T6 cells

Unlike most eukaryotic mRNAs, picornavirus polysomal RNA does not possess a cap structure at its 5' end (Revel & Groner, 1978; Shatkin, 1976). As we have already shown, unlike normal cells, cells infected with picornaviruses are very permeable to nucleotide analogues. Hence, we tested the action of cap analogues on EMC virus-infected 3T6 cells at the time when viral proteins are being synthesized maximally and membrane leakiness has already been induced. Surprisingly, it was found that the addition to the culture medium of 7mGppp and 2'-O'-mGppp significantly and specifically increased viral protein synthesis in those cells. On the other hand, the addition of Gppp or 7mGp had no effect on translation in uninfected and infected cells (Fig. 5). It seems unlikely that 7mGppp exerts this stimulatory effect by a non-specific increase in the overall mRNA translation capacity of the cell, as it has no effect on cellular protein synthesis in virus-infected cells; this serves as an internal control.

To study this effect further, we examined the time course of protein synthesis in virus-infected cells, both in the presence and in the absence of 7mGppp. Fig. 6 shows that under normal conditions viral protein synthesis started at 4 h post-infection (p.i.) and had completely stopped by 6 h. The presence of 7mGppp prolonged viral protein synthesis to as long as 8 h p.i. Moreover, there was a net increase in viral protein synthesis and, from 6 to 8 h p.i., only viral proteins were synthesized in the infected cells if 7mGppp was present. Taking into account that the presence of all the nucleotide analogues tested drastically reduced the production of infectious virus (results not shown), it was of interest to determine whether, under conditions in which viral proteins were synthesized but no mature virions were formed, membrane leakiness still occurred. Fig. 7 shows that this was indeed the case and the addition of GppCH₂p to infected cells treated with the cap analogue 7mGppp, resulted in an almost total inhibition of translation.

DISCUSSION

Very little is known about the changes in permeability that take place after infection of mammalian cells with animal viruses. Although some viral proteins are located in the plasma membrane during viral infection (Burns & Allison, 1977; Gschwender & Traub, 1979; Rifkin...
Fig. 4. Effect of proteolytic inhibitors on GppCH₂p-induced inhibition of protein synthesis in EMC virus-infected cells. At 3.5 h after infection the cleavage inhibitors were added and the cells incubated for 30 min at 37 °C. After this time 1 mM-GppCH₂p was added to half the samples as indicated, and the cells were then labelled with ³⁵S-methionine from 4.5 to 5.5 h. Finally, the samples were processed as indicated in Fig. 1 and the autoradiograph of the gel shown. 1, Control; 2, 1 mM-ZnCl₂; 3, 2 mM-ZnCl₂; 4, 2.5 mM-fluorophenylalanine plus 3.3 mM-canavanine plus 5.4 mM-azetidine-2-carboxylic acid; 5, 20 μg/ml TPCK.

& Quiglay, 1974), the actual effects that those proteins could have on the membrane and their indirect effect on cellular metabolism are still very poorly understood. We have suggested that some of these viral proteins induce profound alterations in the membrane of the infected cells, leading to a drastic modification of host metabolism (Carrasco, 1977). Indeed, in picornavirus-infected cells, the barrier to ions and metabolites that the membrane maintains gradually disappears after infection (Carrasco & Smith, 1976; Egberts et al., 1977). Analysis of the picornavirus proteins which are localized in the membrane shows that they are redistributed into patches and caps (Gschwender & Traub, 1979) which is in agreement with the membrane-leakage model (Carrasco, 1977). Similarly, cells infected with paramyxoviruses (Dubois-Dalcq & Reese, 1975; Levanon et al., 1977), papovaviruses (Norkin, 1977) and herpesviruses (Thompson et al., 1978; Wagner & Roizman, 1969; Benedetto et al., 1980)
Action of nucleotides in viral translation

Fig. 5. Effect of cap analogues on translation in mock-infected and EMC virus-infected 3T3 cells. The conditions were as described in Fig. 1. The final concentration of the nucleotides was 2 mM. The densitometric scans of the autoradiographs are shown; the numbers on the axes represent arbitrary units.
Fig. 6. Effect of 7mGppp on protein synthesis in EMC virus-infected 3T6 cells at different times after infection. Conditions were as above except that 2 mM 7mGppp was present from the onset of infection. The densitometric scans of the autoradiograph after gel electrophoresis are shown; the numbers on the axes represent arbitrary units.
Action of nucleotides in viral translation

Fig. 7. Membrane leakiness in 7mGppp-treated cells. The nucleotide analogues were added at 4 h after infection at the indicated final concentration. The EMC virus-infected 3T6 cells were labelled from 4.5 to 5.5 h as indicated in Fig. 1 and the autoradiograph shown.

also show permeability changes after infection. These include increased leakiness to ions and low mol. wt. compounds, and a drop in membrane potential (Fritz & Nahmias, 1972).

The present results lend more support to the idea that picornavirus-infected cells have a leaky membrane when viral protein synthesis takes place, because the inhibition of translation by GppCH₂p seems to be both specific for virus-infected cells and reversible. On the other hand, the cell species does not seem to influence the induction of membrane leakiness in a permanent way, as long as viral proteins are produced.

Finally, we believe that the findings on the effect of cap analogues in picornavirus-infected cells deserve further investigation. Although still unproven, the model that we favour at present to explain all the experimental results is the following: the addition of the cap analogues to the medium of picornavirus-infected cells results in some entry of this compound to the cytoplasm. Once there, the viral polysomal mRNA is capped by an enzyme that specifically uses as substrate the methylated trinucleotide. This reaction leads to the formation of viral RNA that terminates in the 5' end in mG(5')Up... This is not unlikely as we now know that naturally uncapped RNAs can be artificially capped in vitro to produce more efficient and stable mRNAs (Revel & Groner, 1978). This would explain why we found a specific increase in viral protein synthesis. On the other hand, the presence of that structure in the 5' end of viral RNA would not allow the attachment of the VPg protein (Rothberg et al., 1978) to this RNA, provided that there is an equilibrium between the attachment and the cleavage of VPg, which would result in decreased production of mature virions. Work is in progress at the moment to test this hypothesis.
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REFERENCES


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