The Regulation of Translation in Reovirus-infected Cells

By ALBERTO MUÑOZ, MIGUEL ANGEL ALONSO AND LUIS CARRASCO*

Departamento de Microbiologia, Centro de Biologia Molecular, Universidad Autónoma de Madrid and CSIC, Canto Blanco, Madrid-28049, Spain

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SUMMARY

The regulation of translation in reovirus-infected cells has been investigated by double-infection experiments. Different cell lines were able to translate uncapped encephalomyocarditis (EMC) virus mRNA and capped vesicular stomatitis virus (VSV) mRNAs both early and late during reovirus infection. These results are not fully in agreement with a previously suggested model in which, in the early phase of reovirus infection, only capped mRNAs are translated, whereas in the late phase the cells are modified to translate exclusively uncapped mRNAs. The observations that EMC virus and VSV shut down host protein synthesis more efficiently than reovirus translation in the late phase in double-infections indicate that competition for a message-discriminatory factor may not be involved in the shut-off of host protein synthesis in these animal virus-infected cells.

INTRODUCTION

The regulation of translation in virus-infected animal cells has been a matter of intensive research during the last few years. In most animal systems studied, as for example picornavirus-infected cells, there is a potent inhibition of host protein synthesis after viral infection, followed by massive translation of viral mRNAs (Carrasco & Smith, 1976, 1980; Lucas-Lenard, 1979). In other instances, such as in some reovirus-infected cells, host and viral protein synthesis co-exist, with no apparent shut-down of host translation (Walden et al., 1981; Detjen et al., 1982). In encephalomyocarditis (EMC) virus-infected cells, inhibition of protein synthesis appears to be related to a modification of membrane permeability which leads to changes in the ionic conditions in the cells, with the result that translation of cellular mRNA is blocked, while viral protein synthesis is still favoured (Carrasco & Smith, 1976; Carrasco, 1977; Lacal & Carrasco, 1982). The suggestion that EMC virus mRNA is preferentially translated in infected cells because it is better able to compete for a limiting factor involved in protein synthesis has also been proposed (Golini et al., 1976). However, the possibility that competition is the cause of the shut-off phenomenon in EMC virus-infected cells has been ruled out for two main reasons: (i) shut-off still occurs even in the absence of viral mRNA production and (ii) if host mRNAs are replaced by more efficient viral mRNAs, then no inhibition of total protein synthesis should be observed (Steiner-Pryor & Cooper, 1973; Bablanian, 1972; Holland, 1964; Collins & Roberts, 1972; Carrasco & Smith, 1980; Muñoz & Carrasco, 1981).

Competition has also been considered to operate in reovirus-infected SC1 cells. Indeed, in a series of publications, Thach and co-workers have developed a kinetic model to explain the behaviour of protein synthesis in reovirus-infected cells (Walden et al., 1981; Brendler et al., 1981a, b; Godefroy-Colburn & Thach, 1981; Detjen et al., 1982). According to this model, viral and cellular mRNAs compete for a message-discriminatory component that participates in translation, before the mRNA binds to the 40S initiation complex. This component has been tentatively identified as initiation factor eIF-4A (Ray et al., 1983). Evidence was obtained showing that reovirus mRNAs are poor initiators relative to host mRNAs. Thus, the following order in competition was established: EMC virus > host > reovirus mRNA. Therefore, it was proposed that the translation strategy adopted by reovirus is to overwhelm the cellular synthetic...
apparatus with a large excess of weakly initiating messages (Walden et al., 1981). In addition, it was observed that the degree of competition between host and viral mRNA largely depends on the ionic concentration in the cell-free system (Brendler et al., 1981a, b).

Contrary to this view, Skup & Millward (1980) have suggested that the inhibition of translation after reovirus infection of L cells occurs by a mechanism similar to that already suggested for poliovirus. The model proposes that the reovirus mRNA synthesized early in infection is capped, and the infected cells translate this kind of mRNA efficiently, but not uncapped mRNAs. Late in infection, the viral mRNA produced is uncapped and it is efficiently translated because the protein-synthesizing machinery has been modified in such a way that only uncapped mRNAs are recognized (Skup & Millward, 1980; Skup et al., 1981). However, the validity of the reovirus and poliovirus models has been questioned recently (Detjen et al., 1982; Alonso & Carrasco, 1982a, b).

To investigate these models and also to measure to what extent competition plays a part in the regulation of translation in reovirus-infected cells, we have analysed protein synthesis in reovirus-infected cells, superinfected with different animal viruses with capped or uncapped mRNAs. Contrary to Skup and co-workers (Skup & Millward, 1980; Skup et al., 1981) and in agreement with Detjen et al. (1982), the results obtained led us to consider that reovirus-infected HeLa cells are able to translate capped viral mRNAs late during virus infection. However, in reovirus-infected HeLa cells, no inhibition of cellular protein synthesis is apparent at the time when most viral proteins are synthesized, which suggests that there is an excess of translation components and that no competition between cellular and viral mRNAs is established in intact infected cells. Moreover, superinfection of these cells with EMC virus shuts down cellular protein synthesis more efficiently than reovirus translation. This is another argument against competition playing a part in the shut-off phenomenon, since, as suggested by Walden et al. (1981), host mRNAs are more efficient competitors than reovirus mRNAs.

METHODS

Cells and viruses. HeLa cells and BHK-21 cells were grown in Dulbecco's modified Eagle's medium (E4D) supplemented with 10% calf serum (E4D10 medium) and incubated at 37 °C in a 5% CO₂ atmosphere. Poliovirus type 1 was grown on HeLa cells in E4D medium supplemented with 2% newborn calf serum (E4D2). Vesicular stomatitis virus (VSV) was grown in BHK-21 cells in the same medium. EMC virus was grown on L929 cells in a mixture of Eagle's medium, phosphate-buffered saline (PBS) and E4D medium (80 : 15 : 5) supplemented with 1% newborn calf serum.

In all cases, the fraction obtained after removal of cell debris by low-speed centrifugation was used as the source of virus. Reovirus type 3 Dearing strain was purchased from the American Type Culture Collection.

Virus infection, measurement and analysis of protein synthesis. Cells grown on 24-well plates (Falcon Plastics) containing 1 ml E4D10 were infected with the indicated virus at the m.o.i. described in each experiment. After 1 h incubation at 37 °C, the medium was removed and 1 ml E4D2 was added. Time of virus addition was considered as −1 h, and 0 time was taken as the time when the virus was removed. Incubation at 37 °C was continued until the labelling period. For this purpose, 0.5 ml methionine-free E4D1 medium and 5.4 μCi [35S]methionine (1100 Ci mmol⁻¹, 5.4 mCi ml⁻¹; Amersham) were added to the cells for a 1 h pulse. At the end of the pulse period, the cells were washed with 1 ml PBS and dissolved in 200 μl 0.02 M-NaOH plus 1% SDS and 200 μl sample buffer (62.5 mM-Tris-HCl pH 6.8, 2% SDS, 0.1 M-dithiothreitol, 17% glycerol and 0.024% bromophenol blue as indicator). Each sample was sonicated to reduce viscosity and heated to 90 °C for 5 min. Ten μl was applied to a 15% polyacrylamide gel and run overnight at 30 V. Fluorography of the gel was carried out with 2,5-diphenyloxazole at 20% (w/w) in DMSO. The dried gels were exposed using XS-5 X-ray films (Kodak). Ten μl more of each sample was precipitated with 1 ml 10% TCA and filtered through GF/C glass fibre filters in a Millipore apparatus. Toluene–POPOP–PPO (2.5 ml) was added to the dried filters and the radioactivity retained was estimated in an Intertechnique scintillation spectrometer.

Measurement of 86Rb⁺ content. HeLa cells grown in E4D10 medium were placed in 280 μl of a mixture of methionine-free E4D1 medium : E4D10 medium (3 : 1), 0.2 μCi 86Rb⁺ (1 mM Ci ml⁻¹; Amersham) was added, and the cells were incubated for 17 h at 37 °C. Virus infection was then carried out while the 86Rb⁺ concentration was maintained constant. At the times indicated, the cells were pulse-labelled with 0.14 μCi [35S]methionine. After 1 h incubation, the medium was removed and the cells were washed three times with 1 ml PBS, and 0.5 ml 5% TCA was added to extract the 86Rb⁺ from the cells. The radioactivity of 0.4 ml of the TCA extract was determined by estimating the Cerenkov radiation in a liquid scintillation counter. The level of protein synthesis was estimated in parallel cultures as described above.
**RESULTS**

*Protein synthesis and $^{86}\text{Rb}^+$ content in reovirus-infected cells*

The reovirus genome comprises ten different pieces of dsRNA, each one coding for a single protein (Joklik, 1981). These different proteins have been classified into groups according to size. Infection of HeLa cells with reovirus type 3 led to massive synthesis of reovirus proteins starting from about 6 h post-infection and continuing at very high levels for several hours. It is striking that infection did not lead to inhibition of total protein synthesis as occurs with most animal viruses, but instead total protein synthesis was stimulated almost 100% compared to uninfected cells. Also, cellular protein synthesis continued unabated for long periods of time, indicating that cellular and viral protein synthesis co-exist (Fig. 1). However, infection of L cells by reovirus interfered with cellular protein synthesis from about 10 h post-infection, and from 15 h onwards the cell translated viral mRNAs exclusively (Fig. 1). Analysis of membrane permeability to monovalent cations in these cells indicated that a close parallel exists between variations in protein synthesis and modifications in $^{86}\text{Rb}^+$ content. For HeLa cells, there was an increase in $^{86}\text{Rb}^+$ until 11 h post-infection, followed by a gradual decline, but even at 20 h post-infection the $^{86}\text{Rb}^+$ content was similar to that in uninfected cells (Fig. 1). Analysis of $^{86}\text{Rb}^+$ uptake by very short pulses (15, 30 and 60 s) indicates a rapid increase in the uptake of this cation, which was maximal (250% of control) at 11 h post-infection (results not shown).
Theoretically, the higher uptake of this cation should be compensated by a higher release of sodium ions, since at that time there is no loss of other cellular metabolites. The behaviour of reovirus-infected L cells was similar to that of HeLa cells during the first 13 h of infection. After that time, a drastic release of $^{86}$Rb$^+$ occurred, accompanied by a cessation of synthesis of several reovirus proteins. However, the synthesis of other reovirus proteins was particularly resistant to these ionic modifications (Fig. 1).

Protein synthesis in HeLa cells infected with reovirus and superinfected with VSV, poliovirus or EMC virus

According to the model proposed by Skup and co-workers (Skup & Millward, 1980; Skup et al., 1981) for regulation of translation in reovirus-infected cells, a modification in the protein-synthesizing apparatus late in reovirus infection takes place, thus preventing the cell from translating capped mRNAs. To test this, reovirus-infected HeLa cells were superinfected late in infection with VSV, a virus that possesses typical capped mRNA, after which the synthesis of VSV proteins was analysed. Fig. 2 shows that VSV mRNAs were translated in these double-infected cells, suggesting that the protein-synthesizing machinery is in fact able to translate capped mRNAs. As regards shut-off of protein synthesis by VSV in this system, it is worth noting that cellular mRNA translation was shut down before reovirus protein synthesis. Twenty h after reovirus infection, which corresponds to 5 h after VSV superinfection, no cellular proteins were made, and there was then a significant decrease in reovirus protein synthesis (Fig. 2). Since reovirus mRNA is a weaker competitor than cellular mRNA (Walden et al., 1981), this result does not support the idea that the interference of VSV with translation is carried out by competition with preexisting mRNAs.
Fig. 3. Effect of guanidine on protein synthesis in reovirus-infected HeLa cells superinfected with poliovirus. Cells grown on 24-well Linbro dishes were infected with reovirus (20 p.f.u./cell) at −1 h, and superinfected with poliovirus (75 p.f.u./cell) at either (a) + 1 or (b) + 15 h. Cells were treated with guanidine (3 mM) where indicated from the beginning of poliovirus infection until the end of the labelling period. Bands corresponding to some poliovirus (NCPV1α, NCPV1β, NCPV2, VPO, VP3) and reovirus (λ, μ, σ) proteins are indicated. O, Untreated cells; ●, guanidine-treated cells.

The translation of uncapped mRNAs in reovirus-infected cells was analysed by superinfection with two different animal viruses, poliovirus and EMC viruses. Both are thought to block protein synthesis by different mechanisms (Jen et al., 1980; Detjen et al., 1981). According to the model suggested for reovirus-infected L cells, the translation of reovirus uncapped mRNAs is blocked early during infection, and thus is only possible in the late phase (Skup & Millward, 1980; Skup et al., 1981). Fig. 3 and 4 indicate that this does not hold for picornavirus mRNA translation in reovirus-infected HeLa cells, because poliovirus and EMC virus mRNAs were translated both early and late during infection. Poliovirus protein synthesis is blocked in interferon-treated cells (Muñoz & Carrasco, 1983, 1984) and also by the presence of guanidine (Fig. 3) (Lacal & Carrasco, 1983). However, even though viral protein and RNA synthesis is profoundly inhibited under those conditions, the shut-off of protein synthesis still occurs (Muñoz & Carrasco, 1983; Muñoz et al., 1983). Analysis of the blockade by poliovirus of host and reovirus protein synthesis late in reovirus infection of HeLa cells and in cells treated with guanidine leads to a number of conclusions. First, both host and reovirus translation are blocked by poliovirus infection in control cells, but host protein synthesis is more sensitive. This raises the question that if reovirus and poliovirus block protein synthesis by a mechanism similar to that already suggested (Skup & Millward, 1980; Skup et al., 1981) why is reovirus protein...
Fig. 4. Protein synthesis in reovirus-infected HeLa cells superinfected with EMC virus. Cells grown on 24-well Linbro dishes were infected with reovirus (10 p.f.u./cell) at -1 h, and superinfected with EMC virus (100 p.f.u./cell) at either +1 (O) or +11 (▲) h. Cells infected with EMC virus only were taken as control (●). Bands corresponding to some EMC virus (A, D, E, ε, α, γ, H, I) and reovirus (λ, μ, σ) proteins are indicated.

synthesis inhibited? In addition, we know that EMC virus and poliovirus translation are compatible in double-infected cells (Alonso & Carrasco, 1982a). On the other hand, inhibition of poliovirus replication by means of guanidine still allows the shut-off of protein synthesis to take place. However, it is particularly clear in guanidine-treated cells that the inhibition of host protein synthesis occurs before the inhibition of reovirus protein synthesis. Moreover, not all reovirus proteins behave in the same way, because some of them are more resistant to inhibition than others. This result is difficult to explain if we assume that poliovirus destroys a factor involved in cap recognition, necessary for the translation of capped reovirus mRNAs (Rose et al., 1978). The pattern of translation after EMC virus superinfection was similar, i.e. EMC virus protein synthesis occurred early and late during reovirus infection of HeLa cells, and shut-off of host protein synthesis occurred before that of reovirus protein synthesis (Fig. 4). Three h after EMC virus superinfection of reovirus-infected HeLa cells (14 h post-infection) no host
EMC virus superinfection at +1 h

**Fig. 5.** Effect of EMC virus superinfection on protein synthesis in different reovirus-infected mammalian cells. Cells grown on 24-well Linbro dishes were infected with reovirus (20 p.f.u./cell) at -1 h, and superinfected with EMC virus (100 p.f.u./cell) at either +1 or +11 h. Bands corresponding to EMC virus (A, D, E, ε, F, α, γ, H, I) and reovirus (λ, μ, σ) proteins are indicated.

translation was evident, whereas reovirus protein synthesis continued unabated. This indicates once again that EMC virus does not block protein synthesis by competition for a message-discriminatory factor.

**Comparison of different cell lines infected with reovirus and superinfected with EMC virus or VSV**

The inhibition of host translation after reovirus infection differed according to the cell type analysed. For some cells like HeLa (Fig. 1) or SC1 (Walden et al., 1981) there was hardly any inhibition of cellular protein synthesis, whereas for others like L cells or BHK cells (Fig. 1) the blockade of host translation was noticeable. The model suggested by Skup and Millward was based on experiments in L cells (Skup & Millward, 1980; Skup et al., 1981). It was therefore of interest to compare the behaviour of different cell lines infected with reovirus, as regards the translation of capped or uncapped mRNAs both early and late during infection. Since poliovirus only infects cells of simian origin, we chose EMC virus as representative of a virus with an uncapped mRNA and VSV to analyse capped mRNA translation in cells superinfected with these viruses. Fig. 5 shows that, on superinfection with EMC virus of a variety of cell lines...
previously infected with reovirus, expression of EMC virus proteins occurred early during infection. This leads to the conclusion that in none of the cell lines tested, including L cells, was there any restriction of translation of uncapped EMC virus mRNA. On the other hand, in some of the cell lines tested, such as L, HEp-2 and BHK-21 cells, no EMC virus proteins were detected late in infection. This might be either because this uncapped mRNA is not translated in these cells or, more likely, because viral replication cannot occur in these cells in the late phase of reovirus infection.

Similarly, VSV protein synthesis was observed in all cell lines analysed early in infection at levels comparable to control uninfected cells, but once again no VSV proteins were detected late during reovirus infection of L cells, whereas some inhibition was observed in HEp-2 and BHK-21 cells (Fig. 6). These findings that capped mRNAs are translated late in infection in some cell lines and that uncapped EMC virus mRNA is translated early in reovirus infection in all cell lines tested do not support the idea of changes in the protein-synthesizing apparatus in every type of reovirus-infected cell, although it might operate in L cells if it is proven that the restriction of VSV replication is at the translational level.

Fig. 6. Effect of VSV superinfection on protein synthesis in different reovirus-infected mammalian cells. Cells grown on 24-well Linbro dishes were infected with reovirus (20 p.f.u./cell) and superinfected with VSV (100 p.f.u./cell) at either +1 or +11 h. Bands corresponding to VSV (L, G, N, NS, M) and reovirus (L, G, N, NS, M) proteins are indicated.
DISCUSSION

Two different models have been put forward to account for the regulation of translation in reovirus-infected cells. One suggests that early during reovirus infection the infected cells preferentially translate capped mRNAs, whereas in the late phase only uncapped mRNAs are translated. The reason for this specificity could be that the protein-synthesizing machinery is modified in a way similar to that suggested for poliovirus-infected cells (Skup et al., 1981). Indeed, cell-free systems obtained late in infection from reovirus-infected cells translate uncapped mRNAs exclusively, a result very similar to that obtained for poliovirus-infected cells (Skup et al., 1981). However, the translation of capped viral mRNAs in intact poliovirus or reovirus-infected cells occurs during the course of superinfection (Alonso & Carrasco, 1982b). This suggests that the results in vitro cannot be directly equated with the situation in vivo. The other model suggested for the regulation of translation after reovirus infection indicates that viral and cellular mRNAs compete for a message-discriminatory factor which is present in the cell in limiting amounts (Walden et al., 1981; Brendler et al., 1981a, b; Ray et al., 1983). Although host mRNAs are better competitors than reovirus mRNAs, viral protein synthesis occurs because the viral mRNAs are made in larger quantities (Walden et al., 1981). If this were so, then superinfection with EMC virus or VSV should shut down reovirus protein synthesis more effectively than host translation. Clearly, the results obtained in doubly infected cells show that the contrary is true, i.e. host translation is more sensitive to inhibition than reovirus protein synthesis. In fact, after EMC virus or VSV superinfection of reovirus-infected cells, there was a drastic decline of total protein synthesis which is hardly explicable by weak mRNAs replacing stronger mRNAs, as the competition model suggests.

As regards the possibility that reovirus-infected cells exclusively translate capped mRNAs early during infection and switch to a cap-independent mechanism at late times, the experiments reported in the present work suggest that this is not so for all cell lines analysed. Thus, mouse cells such as L and 3T6 cells efficiently translate uncapped EMC virus RNA early during reovirus infection. It might still be argued that the lack of ability of L cell extracts to translate uncapped mRNA only holds for reovirus mRNAs and not for picornavirus RNA. Besides, HEp-2, BHK-21 and L cells did not efficiently support the replication of EMC virus or VSV when they were superinfected late in the reovirus cycle. The argument that in these cell lines there is less VSV protein synthesis because these cells only translate uncapped reovirus mRNAs needs to be substantiated by experiments designed to measure the step of VSV replication inhibited late during reovirus infection of those cells.

An interesting finding was that the $^{86}\text{Rb}^+$ content paralleled the changes observed in protein synthesis both in HeLa and L cells infected with reoviruses. For L cells there was a marked decrease in total protein synthesis from 13 h post-infection, at the same time as a decrease in $^{86}\text{Rb}^+$ ions occurred. This indicates a loss of potassium ions from the cellular cytoplasm and most probably entry of sodium ions. Curiously enough, the synthesis of some reovirus proteins was more resistant than others to these ionic modifications.

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