CHLORIDE CONCENTRATION DISCRIMINATES BETWEEN FOOT-AND-MOUTH DISEASE VIRUS IRES-DEPENDENT TRANSLATION AND CLASSICAL SCANNING TRANSLATION: NEW ASPECTS OF THE PICORNAVIRUS SHUTOFF MECHANISM

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Summary. – Some picornaviruses might use the general increase of ionic strength in the host cell that occurs successively after infection to induce shutoff of host protein synthesis and to stimulate viral protein synthesis. In order to investigate this discrimination mode on a molecular level, *in vitro* experiments under different salt conditions comparing the Foot-and-mouth disease virus (FMDV) internal ribosome entry site (IRES)-dependent translation with the translation via the classical scanning mechanism were performed. For classical mRNA optimum concentrations of all investigated salts ranged between 70 and 100 mmol/l. However, for FMDV IRES-dependent translation the optima depended strongly on the anion used. While acetates caused only a weak stimulation of translation efficiency with maxima ranging between 150 and 180 mmol/l, chlorides lead to a strong stimulation with maxima ranging between 120 and 150 mmol/l. Competition experiments revealed that the concentration of chlorides had a greater influence on the discrimination between cellular and viral RNA translation than the total ionic strength. Taken together, the data support a model in which a specific increase in the chloride concentration rather than a general increase in the ionic strength is responsible for the shutoff effect induced by some picornaviruses.

Key words: in vitro translation; initiation; elongation; picornavirus; shutoff; IRES

Introduction

Picornaviruses are widespread in nature and cause important diseases in man (e.g. poliomyelitis) and animals (e.g. FMD). They contain a single-stranded, positive-sense RNA genome that functions as an mRNA within infected cells to produce a single polyprotein, which is subsequently proteolytically cleaved to yield structural and non-structural virus proteins (Rueckert, 1996). In contrast to most cellular mRNAs, which are capped and translated by the classical scanning mechanism (Kozak, 1978, 1989), picornavirus RNAs are uncapped and initiate translation by internal ribosome binding (Pelletier and Sonenberg, 1988; Jang *et al*., 1988). The IRES was demonstrated to be a cis-acting element that directs *in vivo* the binding of ribosomal subunits and cellular protein factors to the viral RNA in order to accomplish internal translation initiation (for recent reviews see Jackson and Kaminski, 1995; Belsham and Sonenberg, 1996, 2000). On the basis of their sequence and structure, IRESes are usually divided in two types: the cardio- and aphthovirus type (I) and the entero- and rhinovirus type (II). Within each type, there is moderate conservation of primary structure (nucleotide sequence) of the IRES and even stronger conservation of deduced secondary structure. However, there is almost no conservation between the two types.

For many picornaviruses infection of cells results in a shutoff of host protein synthesis. Several functioning

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Abbreviations: 4E-BP1 = eIF4E-binding protein 1; CAT = chloramphenicol acetyltransferase; eIF4E = eukaryotic translation initiation factor 4E; eIF4G = eukaryotic translation initiation factor 4G; EMCV = Encephalomyocarditis virus; FMDV = Foot-and-mouth disease virus; HRV = Human rhinovirus; IRES = internal ribosome entry site; Luc = luciferase

mechanisms for this phenomenon were proposed. It is now well established that picornaviruses with the IRES of the II type and FMDV support the shutoff by cleavage of the initiation factors eIF4GI and eIF4GII (Gradi *et al*., 1998; Svitkin *et al*., 1999; Zamora *et al*., 2002) by either the 2A proteinase of rhinoviruses or enteroviruses or by the L proteinase of aphthoviruses (Glaser *et al*., 2001). Another contribution to the shutoff phenomenon, at least in these viruses, comes from the cleavage of the poly(A)-binding protein (PABP) (Joachims *et al*., 1999; Kerekatte *et al*., 1999; Kuyumcu-Martinez, 2002). However, cleavage of protein factors alone is not sufficient to explain shutoff for all picornaviruses, because no degradation of host factors occurs during cardiovirus infection (Abreu and Lucas-Lenard, 1976; Jen and Thach, 1982; Zoll *et al*., 1996). Another mechanism that seems to be involved in the shutoff of some but not all picornaviruses is dephosphorylation of the 4E-BP1 factor (Gingras *et al*., 1996, Svitkin *et al*., 1999), a process in which protein 2A seems to be involved directly or indirectly (Svitkin *et al*., 1998). The underphosphorylated form of 4E-BP1 sequesters the cap-binding initiation factor eIF4E into an inactive eIF4E–4E-BP1 complex and thus inhibits the cap-dependent translation (Haghighat *et al*., 1995, Niedzwiecka *et al*., 2002). Another mechanism, which is not mutually exclusive, is the dephosphorylation of eIF4E itself (Kleijn *et al*., 1996). All these mechanisms are based on virus-induced alterations of cellular translation factors. A principally different mechanism of shutoff has been proposed by Carrasco (Smith and Carrasco, 1976; Carrasco, 1977). The Carrasco's "membrane leakage" model can be summarized as follows: the entry of the virus is associated with permeabilization of the host cell, distorting the gradient of monovalent ions which the membrane maintains: whereas sodium and chloride leak in, potassium leaks out from the cytoplasm. Whereas the resulting increased concentration of monovalent ions, inside the cell inhibits the initiation of host protein synthesis, the initiation of translation of viral mRNAs is stimulated. Later it has been shown that host cells indeed are permeabilized by picornaviruses (Carrasco, 1981, Almela *et al*., 1998) and that the resulting perturbation of monovalent cation balance favors, at least for Encephalomyocarditis virus (EMCV), translation of viral RNA at the expense of host mRNA (Alonso and Carrasco, 1982; Lacal and Carrasco, 1982).

In this study the influence of different monovalent salt concentrations on the efficiency of cellular and FMDV IRES-dependent translation was investigated in a rabbit reticulocyte cell-free system. The obtained data support the "membrane leakage" model for cardioviruses and aphthoviruses. Moreover, they suggest a special role for chloride ions in this shutoff mechanism. Evolutionary aspects of the shutoff phenomenon induced by picornaviruses are discussed.

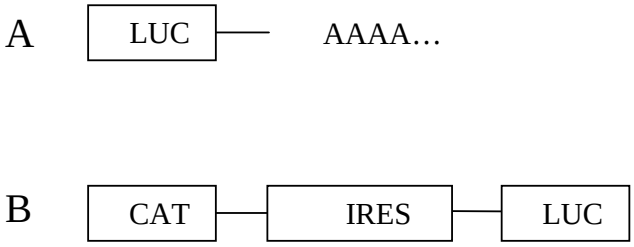


Fig. 1
RNA species used in this study
A. Luc RNA. B. CAT-FMDV-Luc RNA.

Materials and Methods

Chemicals. Restriction enzymes were purchased from MBI Fermentas. Salts were purchased from Sigma-Aldrich. Plasmid preparations were performed using the "Plasmid Midi Kit" (Qiagen) according to the manufacturer's instructions.

RNA species. Monocistronic polyadenylated Luc RNA (Fig. 1A) was supplied by Promega. Bicistronic CAT-FMDV-Luc RNA (Fig. 1B) originated from the pD128 vector (Niepmann *et al*., 1997): The vector DNA was linearized with *Hpa*I, purified with proteinase K, extracted with phenol/chloroform extraction and ethanol precipitated.

Run-off transcription was performed using SP6 RNA polymerase (MBI Fermentas). The efficiency of RNA synthesis was checked by agarose gel electrophoresis and photometry.

In vitro translation of run-off transcripts was accomplished using the Rabbit Reticulocyte Lysate System (Promega). The standard lysate contains 30 mmol/l K^+ (Hempel *et al*., 2001) and other compounds added by the manufacturer, e.g. 113 mmol/l potassium acetate.

In contrast to the manufacturer's instructions 5 µl of lysate was used in a 10 µl standard assay. By use of pooled lysates reproducible translation results could be achieved with SD of ≤3% within 1–2 weeks after mRNA preparation (Hempel *et al*., 2001). Standard translation conditions comprised 1.6 U/µl RNasin, 20 µmol/l amino acids without methionine and 0.4 mCi/ml [^{35}S]methionine. Various monovalent salts were added as indicated. 40 ng/µl RNA in each reaction was used to initiate the translation. Since at that high concentration capped and uncapped RNAs show comparable translation efficiencies (Beckler, 1992), all translation reactions were carried out with *in vitro* generated uncapped RNA. The RNA was heated to 95°C for 2 mins prior to addition to the reaction mixture. The translation reaction proceeded at 30°C for 1 hr.

Luciferase activity assay. 20 µl of the "Luciferase Assay Reagent" (Promega) was mixed with 1 µl of an appropriately diluted *in vitro* translation reaction mixture. The light produced was measured immediately in a luminometer (Lumat type LB9501, Berthold).

Analysis of radiolabeled proteins. In vitro translation reaction mixtures were analyzed by polyacrylamide gel electrophoresis in the presence of tricine-sodium dodecyl sulfate (SDS-PAGE, Schäg-

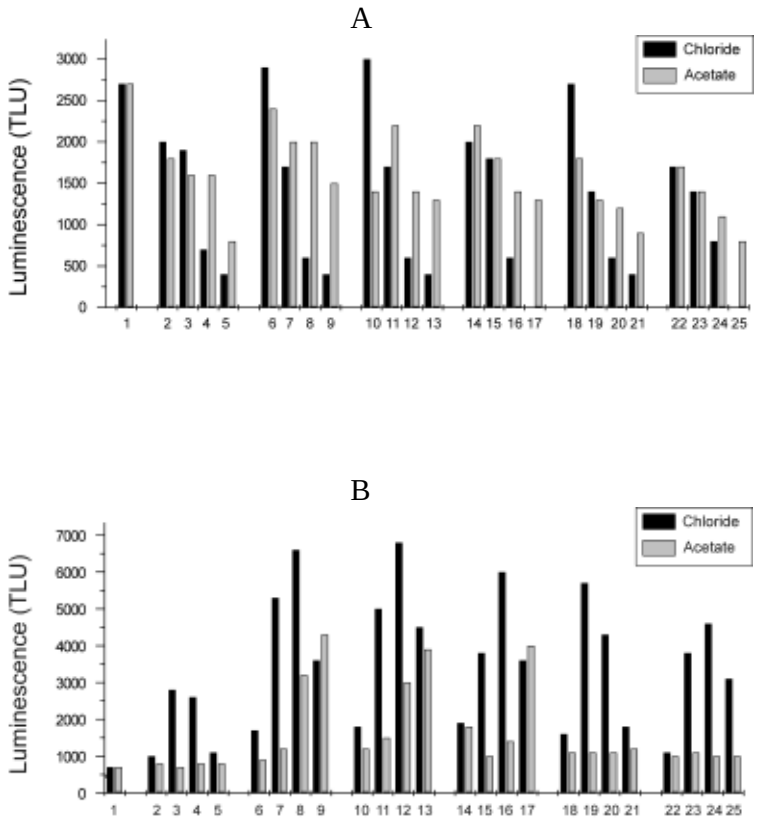


Fig. 2
Monovalent ion requirements for Luc RNA (A) and CAT-FMDV-Luc RNA (B)
(Lysates instructed with Luc RNA containing 120 mmol/l RbCl and 120 mmol/l NH_4Cl were not measured.)

1: Standard; 2–5: 30, 60, 90, 120 mmol/l Li^+; 6–9: 30, 60, 90, 120 mmol/l Na^+; 10–13: 30, 60, 90, 120 mmol/l K^+; 14–17: 30, 60, 90, 120 mmol/l Rb^+; 18–21: 30, 60, 90, 120 mmol/l Cs^+; 22–25: 30, 60, 90, 120 mmol/l NH_4^+.

ger and von Jagow, 1987), loading 2 µl of sample per track. The gels were exposed to "BAS-IIIs" plates (Fuji) overnight and scanned using the Fujix type BAS 1000 (Fuji) phosphorimager. Relative band intensities were measured using the enclosed software. In parallel 2 µl aliquots of the translation reaction mixture were taken for assay of incorporation [^{35}S]methionine into trichloroacetic acid precipitable protein. These measurements revealed the same relative translation efficiencies compared to those obtained by the enzymatic assay.

Results

Two RNA species were used in the translation experiments (Fig. 1). Luc RNA served as a model for cellular mRNA translated via the classical scanning mechanism (Kozak, 1989). CAT-FMDV-Luc RNA contained two cistrons: the CAT cistron was used as another model of host mRNA, while the Luc cistron served as model of FMDV RNA translated by an IRES-dependent mechanism.

Monovalent ion requirements for optimal translation of cellular and FMDV IRES-dependent Luc cistron are totally different

Fig. 2 depicts the detailed analysis of translation efficiency of the two luciferase cistrons after addition of several monovalent ions in different concentrations. The concentrations of added salts given here represent concentrations in addition to the endogenous potassium acetate level in the translation cocktail. Translation efficiencies were measured as luciferase activities in a standard assay. For Luc RNA all the salt additions except for 30 mmol/l NaCl, 30 mmol/l KCl, and 30 mmol/l CsCl lowered the level of translation of luciferase, suggesting an inhibitory effect of these salts (Fig. 2A). This effect was only slightly dependent on the cation species as all cations, at least at the concentrations of $\geq$60 mmol/l, led to similar results. However, significant differences in the translational efficiency were observed depending on the

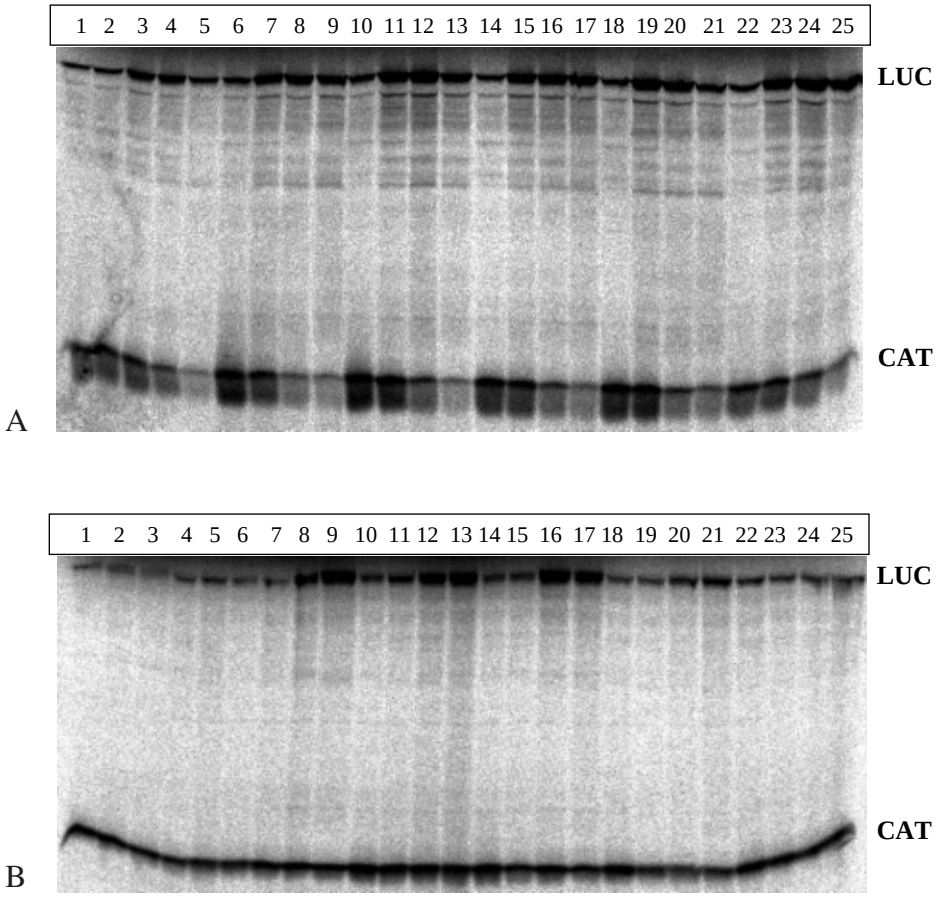


Fig. 3
Fluorograms showing the effect of different salt conditions on the translation efficiency of FMDV IRES-dependent translation (Luc) and translation via the classical scanning mechanism (CAT) in an *in vitro* translation system

A. Chlorides. 1: Standard; 2–5: 30, 60, 90, 120 mmol/l LiCl; 6–9: 30, 60, 90, 120 mmol/l NaCl; 10–13: 30, 60, 90, 120 mmol/l KCl; 14–17: 30, 60, 90, 120 mmol/l RbCl; 18–21: 30, 60, 90, 120 mmol/l CsCl; 22–25: 30, 60, 90, 120 mmol/l NH_4Cl.
B. Acetates. 1: Standard; 2–5: 30, 60, 90, 120 mmol/l LiAc; 6–9: 30, 60, 90, 120 mmol/l NaAc; 10–13: 30, 60, 90, 120 mmol/l KAc; 14–17: 30, 60, 90, 120 mmol/l RbAc; 18–21: 30, 60, 90, 120 mmol/l CsAc; 22–25: 30, 60, 90, 120 mmol/l NH_4Ac.

anion used. While up to addition of 60 mmol/l salt chlorides led to similar or even higher translation efficiencies than acetates; at higher concentrations (≥90 mmol/l) chlorides led to significantly lower protein yields. At these high concentrations translation was much more inhibited by the chlorides than by acetates. There was a remarkable drop in the translation efficiency with an increase in the chloride concentration from 60 to 90 mmol/l.

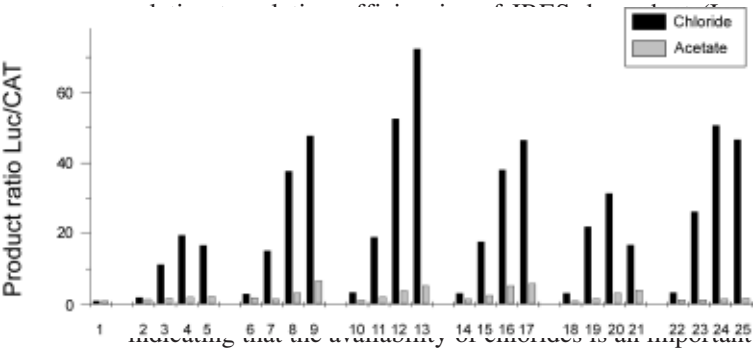
For the Luc translation controlled by FMDV-IRES a totally different picture was observed (Figs. 2B and 3). In contrast to the results obtained with Luc RNA, this kind of translation was lowest under standard conditions. All chlorides strongly stimulated translation with optima ranging from 60 to 90 mmol/l of added salt. Most cations caused similar translation enhancements. The physiological cations Na^+ and K^+ gave rise to the strongest stimulation effect. Only lithium chloride proved to be clearly less efficient in supporting *in vitro* translation. Acetates up to the added concentration of 60 mmol/l caused no significant alterations in the translation efficiency. At high concentrations (addition of 90 mmol/l and 120 mmol/l) acetates can be divided into two groups: Whereas lithium acetate, cesium acetate, and ammonium acetate had no significant effect on translation efficiency even at these high concentrations, sodium acetate, potassium acetate and rubidium acetate stimulated the *in vitro* translation considerably. At 120 mmol/l the stimulation effect of these acetates and the corresponding chlorides were in a similar range.

Fig. 4
Relative Luc/CAT product ratio in dependence on different salt conditions in an *in vitro* translation system

Gel band intensities of luciferase and CAT, respectively, under standard conditions (lane 1) were defined as 100%. The intensities of other bands were related to these standards.

Chlorides discriminate much more strongly between FMDV IRES-dependent and cellular RNA translation than acetates

Since the bicistronic CAT-FMDV-Luc RNA construct contains two differentially translated cistrons, it can be used as an one-molecule competition system for comparing the



indicating that the availability of chlorides is an important prerequisite for preferential translation of FMDV IRES-dependent RNA. This effect was most pronounced at the added chloride concentrations of 90–120 mmol/l. Under these conditions the product ratio shifted up to 70-fold towards the IRES–dependent product in comparison to standard conditions. Again, there took place the cation effect: KCl led to the highest and LiCl to the lowest luciferase/CAT ratio. Addition of acetate salts affected the product ratio only slightly. Only at high concentrations a small shift (at most a 5-fold) towards the IRES-dependent product could be observed.

Discussion

Protein synthesis shows a strong requirement for K^+, and in *in vitro* translation experiments this requirement is normally met by the addition of 70–120 mmol/l KCl or higher concentrations of potassium acetate (Beckler, 1992; Hempel *et al*., 2001; Jackson, 1991; Pelham and Jackson, 1976; Smith and Carrasco, 1976; Weber *et al*., 1977). The optimum for Luc RNA lies within this range as can be deduced from the following considerations: as the rabbit reticulocyte lysate contained about 140 mmol/l K^+ (see *Materials and Methods*) and the translation reaction mixture contained 50% of the lysate, the final basic concentration of K^+ was 70 mmol/l. For all the salts tested optimal concentrations of monovalent cations laid between standard conditions and those increased by 30 mmol/l , i.e. betweeen 70 mmol/l and 100 mmol/l. Translation efficiency of Luc RNA was more sensitive to suboptimal chloride concentrations than to suboptimal acetate concentrations, because by exceeding the optimum concentrations chlorides exhibited a stronger inhibition effect than the respective acetates.

The FMDV IRES-dependent Luc cistron required totally different milieu conditions for an optimal translation efficiency than the classically translated Luc cistron of the Luc RNA. Except for lithium acetate, cesium acetate and ammonium acetate, any salt tested stimulated the translation efficiency. This was particularly true for chlorides at additions of 60–90 mmol/l. The finding that lithium acetate, cesium acetate and ammonium acetate exhibited no significant influence on translation efficiency indicates that neither the corresponding cations (Li^+, Cs^+, NH_4^+) nor the anion (acetate) of these salts had any influence on FMDV IRES-dependent translation. If this is true, the only salts of which both cation and anion stimulate translation efficiency will be sodium chloride, potassium chloride and rubidium chloride, while all acetates will show a pure cation effect and lithium chloride, cesium chloride and ammonium chloride will show a pure chloride effect. Since lithium chloride, cesium chloride and ammonium chloride led to

significantly different translation efficiencies, the influence of chlorides might be modulated by different cations. Up to addition of 60–90 mmol/l all chlorides stimulated translation independently of its counterion. However, by exceeding this critical concentration of chloride ions the translation efficiency decreased. This relative inhibition was probably not due to the rise in cation concentration, because with sodium acetate, potassium acetate or rubidium acetate the translation efficiency increased due to addition of these salts up to 120 mmol/l. Therefore, the optimum concentrations for cations seem to be higher than for anions.

Both the classically translated RNA and FMDV IRES-dependent RNA exhibited sharp maxima for chloride ions. These maxima appear at considerably different concentrations. Therefore, it is almost impossible to create milieu conditions, in which both RNA species are translated efficiently.

This chloride effect obviously plays a much more important role in discriminating between the classical and FMDV IRES-dependent translation than the total concentration of monovalent ions: While addition of even 120 mmol/l acetates led to at most 5-fold increase in the ratio of FMDV IRES-dependent/classically translated product, replacement of acetate by chloride could increase this ratio up to 70-fold. These findings support the "membrane leakage" model. According to this model the host shutoff is correlated with the general increase of ionic strength in the host cell after virus-induced permeabilization of the cellular membrane. However, the results of this communication suggest a broadening of the model, to include the influence of the ion species on the discrimination between viral and host mRNA. Because the Cl^- optima are much sharper than those for Na^+ and K^+ it can be concluded that the successive increase in Cl^- after viral infection rather than the increase in Na^+ or the decrease in K^+ accounts for shutoff and subsequent synthesis of viral proteins. Weber *et al*. (1977) have speculated that high concentrations of Cl^- may interfere with protein-protein and protein-nucleic acid interactions involving ionic bonds. Interestingly, the IRESes of cardio/aphtho type need less host protein factors for efficient functioning than the IRESes of the entero/rhino type and might therefore be less sensitive to inhibition by Cl^- ions. On the other hand, the stimulatory effect of Cl^- ions for these IRESes was probably due to a specific interaction with a viral factor that is involved in the translation process.

Gingras *et al*. (1996) hypothesized that dephosphorylation of 4E-BP1 might have been used by all picornaviruses early in their evolution, but cleavage of eIF4G has evolved for some picornaviruses as a more efficient means to inhibit host protein synthesis. This hypothesis is supported by the fact that eIF4G is cleaved by different proteinases of different picornaviruses at different sites: In entero- and rhinoviruses the serine proteinase 2A is responsible for this processing step whilst in aphthoviruses the cleavage is performed by a thiol-type proteinase (Ryan and Flint, 1997). Thus, the important eIF4G cleavage activity has evolved convergently. However, cardioviruses have not the ability to cleave eIF4G. Therefore they alternatively might have developed the "membrane leakage" strategy to augment the host shutoff. *Vice versa*, the "membrane leakage" strategy is probably not used by entero- and rhinoviruses since the IRESes of this type are less efficient compared to the IRESes of the cardio- and aphthoviruses and at least poliovirus induces the host shutoff before alterations in concentration of monovalent ions are detectable (Lopez-Rivas *et al*., 1987). Apparently, the entero- and rhinoviruses on one side and the cardioviruses on the other side exclusively use one of two general mechanisms to augment the host shutoff: entero- and rhinoviruses have developed an "aggressive" host factor cleavage mechanism in order to be translated preferentially. On the other hand, cardio- and aphthoviruses have evolved a particularly efficient IRES to be preferred by the translation machinery in the "competition race" against cellular mRNA rather late in infection. The usage of different shutoff mechanisms is also reflected by the kinetic patterns of total protein synthesis. Whereas, e.g. in poliovirus-infected HeLa cells the decline in host translation occurs well before the onset of viral production, the decline in EMCV-infected cells occurs much later and concomitantly with the onset of viral translation (Jen *et al*., 1980). The late occurrence of changes in the protein patterns of EMCV-infected cells can be explained by a relatively long time needed to alterate the ionic milieu within the cell so that viral translation is favored and host translation disfavored.

Interestingly, the aphthovirus FMDV seems to use both the cleavage of eIF4G and the "membrane leakage" strategy to induce the shutoff. Possibly, before cardio- and aphthoviruses have separated into different genera they have developed an efficient IRES in combination with the "membrane leakage" strategy in order to augment the ancient shutoff effect caused by dephosphorylation of 4E-BP1. Later on the leader protein of aphthoviruses but not cardioviruses may have acquired an eIF4G cleaving activity to accelerate the inhibition of host mRNA translation early in infection. After development of more efficient shutoff strategies some picornaviruses may have lost the ancient 4E-BP1 dephosphorylation mechanism. Evidence for this hypothesis has been provided by Svitkin *et al*. (1999), who were unable to detect any 4E-BP1 dephosphorylation in the course of HRV-14 or HRV-16 infections.

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