Recognition of picornavirus internal ribosome entry sites within cells; influence of cellular and viral proteins

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ABSTRACT
The ability of different picornavirus internal ribosome entry site (IRES) elements to direct initiation of protein synthesis has been assayed in different cell lines in the presence and absence of viral proteases that inhibit cap-dependent protein synthesis. Reporter plasmids that express dicistronic mRNAs, containing different IRES elements, with the general structure CAT/IRES/LUC, have been assayed. In each plasmid, the CAT sequence encodes chloramphenicol acetyl transferase and the LUC sequence encodes luciferase. The poliovirus (PV) 2A protease and the foot-and-mouth disease virus (FMDV) Lb protease induce the cleavage of the translation initiation factor eIF4G and hence inhibit the activity of the cap-binding complex, eIF4F. In human osteosarcoma (HTK-143) cells, each of the various IRES elements functioned efficiently. In these cells, the co-expression of the viral proteases severely inhibited the expression of CAT, but the proteases had little effect on the activities of the various IRES elements. In contrast, in baby hamster kidney (BHK) cells, the efficiencies of the different IRES elements varied significantly, whereas, in normal rat kidney (NRK) cells, each of the IRES elements was relatively inefficient. In both BHK and NRK cells, the activities of those IRES elements that functioned inefficiently were strongly stimulated by the co-expression of the PV 2A or FMDV Lb proteases. This stimulation was independent of the loss of cap-dependent protein synthesis and was not achieved by the co-expression of the C-terminal fragment of eIF4G. The results suggest that the PV 2A and FMDV Lb proteases induce the cleavage of another cellular protein, in addition to eIF4G, which influences IRES function.

Keywords: cap-independent protein synthesis; IRES; translation initiation factor eIF4G; viral proteases

INTRODUCTION
Picornaviruses have a positive-sense RNA genome that functions as an mRNA within infected cells to produce a polyprotein that is proteolytically cleaved to the various polypeptides required for capsid assembly and RNA replication (Rueckert, 1996). Picornavirus RNAs have certain features that differ from most mammalian mRNAs; the viral RNAs lack the 5’-terminal cap-structure (m7GpppN) found on all cytoplasmic mRNAs and their 5’ noncoding regions contain extensive secondary structure and multiple AUG codons. These features are unfavorable for cap-dependent initiation of protein synthesis (Kozak, 1989). It is now established that picornavirus RNA is translated by a cap-independent mechanism. Translation of picornavirus RNA is dependent on complex RNA elements (about 450 nt) termed internal ribosome entry sites (IRESs) located within the extensive 5’ noncoding regions of these viral RNAs (650–1,300 nt) (reviewed in Jackson & Kaminski, 1995; Belsham & Sonenberg, 1996). The IRES-directed initiation of protein synthesis is maintained following the inhibition of host cell mRNA translation resulting from the cleavage of the translation initiation factor eIF4G (formerly termed p220 or eIF4α). This protein is a component of the cap-binding complex eIF4F, together with eIF4E (the cap-binding protein) and eIF4A (an RNA helicase). The cleavage of eIF4G is induced by the entero-/rhinovirus 2A proteases (Kraüsslich et al., 1987) and the FMDV L protease (Devaney et al., 1988). These proteases are unrelated to each other, but they each cleave eIF4G in a similar place, resulting in the loss of the N-terminal portion of the protein, which interacts with eIF4E (Lamphear et al., 1995; Mader et al., 1995).

Within the picornaviruses, two major classes of IRES element have been identified. Poliovirus (PV) (and other enteroviruses) together with the rhinoviruses contain one class of element, whereas the cardioviruses [e.g., encephalomyocarditis virus (EMCV)] and foot-and-mouth disease virus (FMDV) share another type of IRES

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element. There is little sequence or secondary structure similarity between these two classes of IRES. Even within a class, sequence identity between different members can be less than 50%, but the secondary structure predictions are very similar. Both classes of IRES element can function in the presence of cleaved eIF4G (although no cleavage of eIF4G occurs in EMCV infected cells). However, these classes of IRES do differ in their biology. The PV and rhinovirus elements work poorly in the rabbit reticulocyte lysate (rrl) translation system unless it is supplemented with HeLa cell proteins (Brown & Ehrnfeld, 1979; Dorner et al., 1984), whereas the cardiovirus/FMDV elements work very efficiently in unsupplemented rrl. Single point mutations within the PV IRES can strongly attenuate the virus apparently by restricting the cell types in which the virus can replicate (Agol et al., 1989; LaMonica & Raccanelli, 1989). The effect of these mutations is to reduce the translational activity of the IRES in certain systems (Svitkin et al., 1985, 1988). The activity of the hepatitis A virus IRES, which is quite distinct from the two major groups of picornavirus IRES elements, has been shown to be strongly stimulated by liver extracts (Glass & Summers, 1993). Taken together, these observations indicate that recognition of the IRES may be a major determinant of cell tropism for picornaviruses. IRES function must require either different amounts of translation initiation factors or, more likely, some additional proteins compared to cap-dependent initiation of protein synthesis (see Belsham & Sonenberg, 1996). The usual assay for IRES function is the analysis of artificial dicistronic mRNAs, in which the IRES element is located between the two open reading frames (ORFs). The upstream cistron acts as an indicator of cap-dependent translation and the expression of the downstream cistron indicates the activity of the IRES. Hambidge and Sarnow (1992) first reported an apparent stimulation of the PV IRES activity in PV infected-cells. This activation occurred before significant loss of cap-dependent protein synthesis was observed, but was dependent on a functional 2A protein. Recently, several laboratories have shown that both the PV 2A protease and the FMDV Leader (L) protease can stimulate the function of polio-/rhinovirus IRES elements in cell-free extracts, but little effect on the activity of the cardio-/aphthovirus IRES elements has been detected in these assays (Ziegler et al., 1995a, 1995b; Ohlmann et al., 1996; Borman et al., 1997a). The C-terminal fragment of eIF4G is sufficient to meet the requirement of the IRES for eIF4G (Ohlmann et al., 1996, 1997; Borman et al., 1997a).

Borman et al. (1997b) have recently presented data indicating that the recognition of different IRES elements varies within different tissue culture cell lines and showed that co-expression of the PV 2A protease resulted in a large stimulation of the activity of the polio-/rhinovirus IRES elements within mouse neuroblastoma cells. IRES elements that retained significant activity in these cells were little affected by the co-expression of the 2A protease.

We report here results consistent with these observations and further demonstrate that, in other cells, in which both of the major classes of picornavirus IRES elements function poorly, the apparent activities of both classes are strongly stimulated by the co-expression of the proteases that induce eIF4G cleavage. This stimulation is not related simply to the loss of cap-dependent protein synthesis and may be induced by either the PV 2A or FMDV L proteases.

RESULTS

Dicistronic reporter constructs containing IRES elements of both major classes and four different genera of picornaviruses were produced (see Fig. 1A). The cardio-/aphthoviruses are represented by FMDV and EMCV, whereas the entero-/rhinoviruses are represented by PV (all three serotypes), CB4, and HRV14. All the reporter plasmids are of the form CAT/IRES/LUC and were constructed from a single parental vector. The dicistronic mRNA is expressed by transcription from the bacteriophage T7 promoter. The activity of these reporter plasmids was determined by transfection into human osteosarcoma (HTK-143) cells previously infected with the recombinant vaccinia virus vTF7-3, which expresses the T7 RNA polymerase. After 20 h, cell extracts were prepared and analyzed for the expression of CAT, as an indicator of cap-dependent translation, and LUC to monitor IRES function, as we have described previously (Belsham, 1997; Roberts & Belsham, 1997). The plasmids were analyzed alone and in the presence of the plasmid pAΔS02, encoding the PV 2A protease, which induces cleavage of eIF4G and hence inhibition of cap-dependent protein synthesis. As expected, CAT was efficiently expressed from all of the plasmids when assayed alone (Fig. 1B), but co-expression of the PV 2A protease greatly inhibited CAT expression. No luciferase expression was apparent in western blot analysis from pGEM-CAT/LUC, which lacks any IRES element (although luciferase activity can be detected readily using the highly sensitive enzymatic assay). However, each of the IRES-containing plasmids produced high levels of LUC both in the presence and absence of the PV 2A protease (Fig. 1B). This result is consistent with previous data (e.g., Belsham & Brangwyn, 1990; Medina et al., 1993; Stone et al., 1993) indicative that the picornavirus IRES-directed translation does not need intact eIF4G. The HRV14 IRES appeared less efficient than the others (although the level of LUC produced was still about 100-fold higher than that observed with the pGEM-CAT/LUC vector alone) and its activity increased in the presence of 2A (see also below), whereas, for the other elements, a drop in the level of LUC expression (by
about 50%) was observed. This decrease in expression may reflect a drop in the level of transcripts produced under these conditions as a result of competition between the plasmids; similar effects have been observed previously with co-transfection (e.g., Roberts & Belsham, 1997).

In contrast, when the same reporter plasmids were assayed in baby hamster kidney (BHK) cells, there were significant differences in the apparent efficiencies of the various IRES elements (Fig. 2A). The cardio-/aphthovirus elements produced high levels of LUC, but the entero-/rhinovirus elements produced much lower levels of LUC when assayed alone. However, in these cells, when the PV 2A protease was co-expressed, a large stimulation in the activity of the PV1, HRV14, and CB4 IRES elements was observed (Fig. 2A). A summary of multiple individual determinations of this effect is given in Table 1. These data are consistent with and extend previous observations from Borman et al. (1997b). A smaller increase in the activity of the EMCV IRES in the BHK cells in the presence of 2A was also noted, whereas the FMDV IRES was not greatly affected (see Table 1). When the reporter plasmids were cotransfected with plasmid pAΔ802fs (which produces an inactive 2A protein, Kaminski et al., 1990), no increase in LUC expression was observed and CAT expression was maintained (data not shown). Thus, in the absence of 2A, very different levels of LUC were produced from the different IRES elements, but, when assayed in the presence of PV 2A, quite similar levels of LUC expression were observed in each case (Fig. 2A) (again except for the HRV 14 IRES, which, although stimulated by the PV 2A, was still relatively less active). The stimulation of IRES activity occurred in parallel with the loss of cap-dependent synthesis of CAT, as observed in the HTK-143 cells (compare Fig. 1B with Fig. 2A).
In order to better characterize the stimulation of IRES activity in cells expressing the PV 2A protease, experiments have been performed aimed at distinguishing between potential mechanisms. Initially, the ability of the FMDV Lb protease to mimic the effect of the PV 2A protease was tested. The PV 2A and FMDV Lb proteases are unrelated; the FMDV Lb is a member of the papain-like family of cysteine proteases, whereas the entero-/rhinovirus 2A proteins are members of the trypsin-like family of proteases. However, they each induce the cleavage of eIF4G at sites just seven amino acids apart [on the C-terminal sides of residue 479 for...
TABLE 1. Luciferase expression in vTF7-3 infected BHK cells was quantitated as a measure of IRES activity from the reporter plasmids alone or in the presence of plasmids expressing PV 2A or FMDV Lb (as in Fig. 2).a

<table>
<thead>
<tr>
<th>IRES</th>
<th>+ PV 2A</th>
<th>+ FMDV Lb</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMCV</td>
<td>287 (13)</td>
<td>240 (5)</td>
</tr>
<tr>
<td>FMDV</td>
<td>129 (13)</td>
<td>90 (4)</td>
</tr>
<tr>
<td>PV1</td>
<td>695 (14)</td>
<td>381 (6)</td>
</tr>
<tr>
<td>HRV14</td>
<td>863 (8)</td>
<td>265 (3)</td>
</tr>
<tr>
<td>CB4</td>
<td>572 (8)</td>
<td>203 (3)</td>
</tr>
</tbody>
</table>

aValues are means of multiple determinations as indicated in parenthesis.

FMDV Lb (Kirchweger et al., 1994) and residue 486 for 2A (Lamphear et al., 1993)]. When the reporter plasmids were co-transfected with pLb, qualitatively similar effects were observed on the expression of CAT and LUC, as seen with the PV 2A (Fig. 2B). The expression of CAT was severely inhibited, but the expression of LUC was significantly stimulated in each case from the EMCV, PV1, HRV14, and CB4 IRES elements (see Fig. 2B and Table 1). Thus, when either of these unrelated proteases are expressed, the activity of the IRES elements is stimulated. To confirm that the 2A and L proteases were inducing the cleavage of eiF4G in these assays, extracts prepared from BHK cells expressing the products from pAΔ802 and pLb were analyzed by western blotting with anti-eiF4G antisera. The very similar C-terminal cleavage products generated by the activity of the proteases were apparent (data not shown).

The generality of the effect of PV 2A on the IRES activity in BHK cells was investigated using a larger collection of reporter constructs, each containing an IRES element from the enterovirus group. Five different PV IRES elements (PV1, PV2 Lansing, PV2 Sabin, PV2117, PV3), as well as the HRV14 and CB4 IRES elements, were shown to produce higher levels of LUC in the presence of PV 2A, whereas the expression of CAT was strongly inhibited (data not shown). The different enterovirus IRES elements produced a spectrum of different levels of LUC when assayed alone, but, in the presence of 2A, the level of LUC expression achieved in each case was much more comparable (again the HRV14 element was the least active). The attenuating mutations (see Pollard et al., 1989) present in the Sabin strain of type II PV had no significant effect on the activity of this IRES, in the presence or absence of PV 2A, compared to the IRES from its neurovirulent partner 117 in this system.

One possible explanation for the stimulation of IRES activity when cap-dependent protein synthesis is inhibited may be that the IRES element has less competition for the cellular translation machinery under these conditions. To address this point, the inhibition of cap-dependent protein synthesis, without the expression of either of the proteases, was achieved and the effect on IRES activity examined. Each of the reporter plasmids was co-expressed with the translational repressor 4E-BP2 (Pause et al., 1994). This protein binds to eiF4E and is believed to block its interaction with eiF4G (as shown for 4E-BP1, Haghighat et al., 1995). From Figure 3, it is apparent that the 4E-BP2 inhibited the cap-dependent expression of CAT, as expected, but had little effect (positive or negative) on the expression of the LUC in BHK cells, as observed previously in HTK-143 cells (Pause et al., 1994) (similar results were also observed using a plasmid overexpressing 4E-BP1, data not shown). As observed in Figure 2A, the co-expression of PV 2A, in the same experiment, strongly stimulated the activities of the various IRES elements in the BHK cells, while strongly inhibiting the expression of CAT (Fig. 3).

Because previous studies have indicated that the C-terminal portion of eiF4G is sufficient to meet the requirement of IRES elements for eiF4G (Ohlmann et al., 1997) and may stimulate IRES activity (Borman et al., 1997a) in vitro, we sought to examine whether expression of the C-terminal fragments of eiF4G modified IRES activity within cells. Pestova et al. (1996) have described plasmids encoding the P100 C-terminal portion of eiF4G (amino acid residues 457–1396, termed 4G p100), corresponding to the major C-terminal fragment generated by the action of the enterovirus 2A and FMDV L proteases, and also smaller regions of this fragment of eiF4G corresponding to residues 457–932 (termed 4G M) and 920–1396 (termed 4G C). The expression of these eiF4G fragments in the transient expression assay was tested using anti-eiF4G antiserum in a western blot assay (Fig. 4A). Each of the three fragments of eiF4G was expressed, but the signal for the fragment 457–932 (4G M) was weaker. It is probable that the 4G M fragment is recognized fairly weakly by the antiserum because the antibodies were generated against the fragment 920–1396 (4G C), expressed in Escherichia coli, and hence the extent of common sequence is very limited (note that both fragments also include a His-tag). The effect of the eiF4G fragments on the activity of the different IRES elements in BHK cells was determined. None of these fragments when co-expressed with the reporter constructs produced any stimulation (or inhibition) of IRES activity (Fig. 4B). It should be noted that the endogenous intact eiF4G was still present in these assays.

From a screen of other cell lines, it was found that each of the picornavirus IRES elements, including FMDV and EMCV, were relatively defective when assayed alone in normal rat kidney cells (NRK) (see Fig. 5). Co-expression of PV 2A strongly stimulated the activity of each of the IRES elements (although the activity of the HRV14 IRES is low even in the presence of 2A). A summary of the results from similar experiments is pre-
sented in Table 2. The expression of CAT was similar from each of the constructs when assayed alone and was inhibited by the co-expression of the PV 2A. Stimulation of the activity of each of the IRES elements in NRK cells was also observed when the FMDV Lb was co-expressed (see Table 2), concomitant with the loss of CAT expression (data not shown).

DISCUSSION

The results in this study clearly demonstrate that, in BHK and NRK cells, the co-expression of the PV 2A or FMDV Lb proteases with IRES-containing mRNAs strongly stimulated the activity of IRES elements while cap-dependent protein synthesis was severely blocked. However, it is apparent that this stimulation of IRES activity was dependent on the cellular environment. In human HTK-143 cells, in which all the picornavirus IRES elements show high basal activity, there was little or no effect of the co-expression of these proteases on IRES activity. However, in NRK cells, in which all the IRES elements were much less active, each of the IRES elements, including those of EMCV and FMDV, were stimulated by the proteases. In BHK cells, an intermediate situation was observed: the FMDV IRES was highly active in these cells alone and its activity was only very modestly enhanced (30% increase) by the 2A protease, whereas the PV and CB4 IRES elements, which show low activity in these cells, were more strongly stimulated (to 600–700% of the level obtained in the absence of 2A). The activities of the different IRES elements in the presence of the proteases were similar. In cell-free translation studies, it has been shown that PV 2A and the FMDV Lb stimulated the PV and rhinovirus IRES elements, but not significantly the cardiovirus or FMDV IRES elements (Ziegler et al., 1995a, 1995b; Ohlmann et al., 1996, 1997; Borman et al., 1997a). The latter result contrasts with the significant stimulation of each of the IRES elements by these proteases within NRK cells, and to a lesser extent in BHK cells, demonstrated here. This difference may reflect the properties of rabbit cells or be due to the different properties of the in vitro and in vivo assay systems. We have observed, using immunoblotting experiments, that extracts from human HTK-143 cells produce much higher signals for the polypyrimidine tract binding protein (PTB) and La than either BHK or NRK cells relative to actin (data not shown). However, we have not excluded the possibility that this reflects differential recognition of the PTB proteins, from different species, by the antibodies. Both La and PTB have been implicated in the function of IRES elements (reviewed in Belsham & Sonenberg, 1996), but neither appears to represent a complete explanation for the recognition of IRES elements within cells.
The stimulatory effect of FMDV Lb and PV 2A on IRES function could be accounted for by several different mechanisms. These include: (1) a direct interaction between the L/2A proteases and the IRES elements; (2) loss of competition from capped mRNAs, resulting in increased availability of the C-terminal region of eIF4G for the IRES; (3) stimulation of the IRES activity by the eIF4G cleavage products (i.e., requiring a direct interaction of the eIF4G products with the IRES); or (4) loss of an inhibitor of IRES function and/or the generation by proteolysis of an activator of IRES activity.

Genetic evidence for a direct interaction between PV 2A and the PV IRES was obtained by Macadam et al. (1994), who showed that revertants of ts mutants of PV, in which the mutation was located in the IRES, mapped to 2A. However, the changes were scattered over the predicted structure of this protease rather than defining a specific binding site. Furthermore, because it has been shown that the PV 2A and also the FMDV L protein can each stimulate different IRES elements, which they never normally encounter, it seems more likely that the stimulation in activity observed is attributable to a common function of the PV 2A and FMDV L proteases. To date, the only known shared function is the cleavage of eIF4G; however, this does not rule out other shared substrates for these two proteins. It has been reported previously that the FMDV Lb protease, at high concentrations, can cleave a variety of different substrates within cell extracts (Ziegler et al., 1995b).

An active protease (either PV 2A or FMDV Lb) is required to observe stimulation of IRES activity; this may reflect a need to degrade a particular cell protein, i.e., an IRES inhibitor. Alternatively, the generation of the cleavage products, which may have their own stimulatory activities, may be the critical event. Previous data have shown that the C-terminal cleavage product of eIF4G is able to meet the requirement of the cardiovascular IRES elements for eIF4G (Ohlmann et al., 1997) and may even stimulate the HRV IRES (Borman et al., 1997a), but no effect of the N-terminal cleavage product has been detected. We have found no support for a direct stimulatory effect of the eIF4G C-terminal cleavage products on IRES function (Fig. 4B); however, it is important to note that, in these experiments, the intact eIF4G will still be present within the cell and the N-terminal region of eIF4G has not been tested. In contrast, Yamanaka et al. (1997) have reported a stimulation of EMCV IRES activity by the co-expression of the eIF4G C-terminal region in COS7 cells. Their result is in contrast to the lack of effect of eIF4G cleavage seen using in vitro studies and to our results within cells, but we have no explanation for this discrepancy.

Borman et al. (1997a) reported that the intact eIF4G was inhibitory to the action of the HRV2 IRES in vitro, but we have no evidence to support this in general terms. Indeed, it is apparent that in HTK-143 cells, the cleavage of eIF4G by the co-expression of PV 2A did not produce a general stimulation of IRES activity, but
each of the IRES elements displayed high activity. Furthermore, after a picornavirus enters the cell, it is imperative that the input RNA is able to initiate translation efficiently in the presence of intact eIF4G because it is only after the initial translation of the viral RNA that the viral proteases are generated so that the eIF4G can be cleaved. We have shown that no stimulation of IRES activity occurred when cap-dependent protein synthesis is blocked by the translational repressor 4E-BP2 (Fig. 3) in the absence of any viral protease. Because 4E-BP2 blocks the function of eIF4F (Pause et al., 1994) but by a different mechanism than achieved by the cleavage of eIF4G, these observations encourage the view that the FMDV L and PV 2A proteases stimulate IRES activity independently of their effect on eIF4G, presumably by inducing the cleavage of another cellular protein. This conclusion is also consistent with the data of Hambidge and Sarnow (1992) who observed a 2A-dependent increase in PV IRES activity without any apparent effect on the rate of cellular cap-dependent protein synthesis. We have noted that the detection of the eIF4G cleavage products was much easier using the FMDV Lb protease than with the PV 2A (data not shown), although we normally find that the stimulation of IRES activity is more efficient with the PV 2A, a result consistent with the idea that the two processes are distinct.

Because the stimulation of IRES activity by PV 2A or the FMDV Lb proteases depended on the basal level of IRES activity within a particular cell type (cf. HTK-143 cells and NRK cells), it appears that the effect of the putative cleavage of another cell protein must be dependent upon other constraints, or stimulators, of IRES activity present within the cells. Thus, the activity of a particular IRES in a cell may be dependent on the relative level of stimulatory and inhibitory molecules and the influence of the proteases will be determined by their effect on these IRES modulatory proteins. In order to identify putative substrates for the entero-/

**TABLE 2.** Luciferase expression in vTF7-3 infected NRK cells was quantitated as a measure of IRES activity from the reporter plasmids alone or in the presence of plasmids expressing PV 2A (see Fig. 5) or FMDV Lb.\(^a\)

<table>
<thead>
<tr>
<th>IRES</th>
<th>+ PV 2A</th>
<th>+ FMDV Lb</th>
</tr>
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<tbody>
<tr>
<td>EMCV</td>
<td>884</td>
<td>560</td>
</tr>
<tr>
<td>FMDV</td>
<td>424</td>
<td>240</td>
</tr>
<tr>
<td>PV1</td>
<td>2,533</td>
<td>574</td>
</tr>
<tr>
<td>HRV14</td>
<td>550</td>
<td>375</td>
</tr>
<tr>
<td>CB4</td>
<td>1,138</td>
<td>338</td>
</tr>
</tbody>
</table>

\(^a\)Values are means of two or three independent determinations.
rhinovirus 2A or FMDV Lb proteases, the biochemical search for IRES-stimulating proteins should be conducted on cell extracts from both uninfected and picornavirus-infected cells.

**MATERIALS AND METHODS**

**Plasmids**

DNA preparation and manipulations were performed using standard methods as described in Sambrook et al. (1989) or by manufacturers. Reporter plasmids (see Fig. 1A) were constructed using the pGEM-CAT/LUC plasmid described previously by van der Velden et al. (1995). IRES elements (blunt ended) were ligated into the unique BamHI I site (following treatment with the Klenow enzyme with all four dNTPs and then calf intestinal phosphatase) between the reporter genes. Picornavirus IRES elements from PV1 (Mahoney, nt 70–630), PV2 (Lansing, nt 1–730), PV2 (Sabin, nt 70–630), PV2 (117, a neurovirulent revertant of Sabin, nt 70–630), PV3 (nt 1–675), coxsackie B4 (CB4, obtained from pSKCB4, Stone et al., 1993), human rhinovirus 14 (HRV14, obtained from pSKHRV14, Stone et al., 1993), FMDV (from pSKRCla, Drew & Belsham, 1994), and EMCV (obtained from pSKEMCRB, Drew & Belsham, 1994) were used. Some of these reporter constructs have been described previously (see Pause et al., 1994; van der Velden et al., 1995). In each case, the orientation of the IRES was determined by restriction enzyme digestion and constructs producing the positive-sense form of the viral sequence selected.

Plasmids encoding PV 2A (pAΔ802, Kaminski et al., 1999), the FMDV L protease (pLb, Medina et al., 1993), the elf4E-binding protein 2 (4E-BP2, Pause et al., 1994), and fragments of elf4G (Pestova et al., 1996) have been described previously.

**Transient expression assays**

Plasmids (2 μg, or 0.5 μg of plasmids expressing PV 2A or FMDV Lb) were transfected into cells (35-mm dishes) using Lipofectin (8 μg) (Life Technologies) with OptiMEM following prior infection for 1 h with the recombinant vaccinia virus vTF7-3 (which expresses the T7 RNA polymerase; Fuerst et al., 1986). After 20 h, cell extracts were prepared using Promega lysis buffer (400 μL) and samples were assayed for CAT expression using the CAT-ELISA kit (Boehringer) and LUC expression using the Promega luciferase assay kit with a Bio-Orbit luminometer. Additionally, samples were analyzed by immunoblotting from 12% SDS-PAGE minigels (Laemmli, 1970) and probed with rabbit anti-CAT (1:5,000, 3prime-3prime, Inc) or rabbit anti-LUC (1:5,000, Promega) antibodies followed by peroxidase-labeled donkey anti-rabbit IgG (1:3,000, Amersham). Detection onto X-ray film was achieved using chemiluminescence reagents (Pierce). For detection of elf4G cleavage products, samples were analyzed on 7% SDS-PAGE minigels and, following transfer, were detected with a rabbit anti-elf4G serum [raised against the C-terminal fragment (residues 920–1396) and kindly provided by S.J. Morley, University of Sussex].

**REFERENCES**


**ACKNOWLEDGMENTS**

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IRES tropism


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