TRANSLATION OF SINDBIS VIRUS 26S mRNA DOES NOT REQUIRE INTACT EUKARIOTIC INITIATION FACTOR 4G

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Running title: Translation of SV 26S mRNA

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SUMMARY

The infection of BHK cells by Sindbis virus gives rise to a drastic inhibition of cellular translation, while under these conditions the synthesis of viral structural proteins directed by the subgenomic 26S mRNA takes place efficiently. In this report, the requirement for intact initiation factor eIF4G for the translation of this subgenomic mRNA has been examined. To this end, SV replicons that contain the protease of human immunodeficiency virus type 1 or the poliovirus $2A^{pro}$ replacing the sequences of SV glycoproteins have been constructed. BHK cells electroporated with the different RNAs synthesize protein C and the corresponding protease at late times. Notably, the proteolysis of eIF4G by both proteases has little effect on the translation of the 26S mRNA. In addition, recombinant viable SVs were engineered that encode HIV-1 PR or poliovirus 2A protease under the control of a duplicated late promoter. Viral protein synthesis at late times of infection by the recombinant viruses is slightly affected in BHK cells that contain proteolyzed eIF4G. The translatability of SV genomic 49S mRNA was assayed in BHK cells infected with a recombinant virus that synthesizes luciferase and transfected with a replicon that expresses poliovirus $2A^{pro}$. Under conditions where eIF4G has been significantly hydrolysed the translation of genomic SV RNA was deeply inhibited. These findings indicate a different requirement for intact eIF4G in the translation of genomic and subgenomic SV mRNAs. Finally, the translation of the reporter gene that encodes green fluorescent protein, placed under the control of a second duplicate late promoter, is also resistant to the cleavage of eIF4G. In conclusion, despite the presence of a cap structure in the 5' end of the subgenomic SV mRNA, intact eIF4G is not necessary for its translation.

Keywords: Alphavirus translation; Sindbis virus; eIF4G; Regulation of translation; Translation initiation factors.

INTRODUCTION

Sindbis Virus (SV) belongs to the *Togaviridae* family and is a prototype member of the *Alphavirus* genus. The SV genome is a single stranded RNA of positive polarity of about 11.7Kb. The two-thirds located at the 5' end of the genome encode for the non-structural proteins (nsP1-4), while the rest of this RNA codifies the structural proteins. The nucleocapsid is composed of 240 units of capsid protein (C) wrapped around one copy of the genomic RNA and is surrounded by a lipidic envelope that contains the glycoproteins E1 and E2. After virus entry, the genomic RNA is initially engaged in translation, directing the synthesis of the early proteins nsP1-4. These proteins are necessary to replicate and transcribe the SV RNAs. Viral transcription uses the minus-strand RNA complementary to the genome as a template to synthesize more copies of genomic 49S RNA and subgenomic 26S messenger RNA (mRNA) [1; 2]. Both mRNAs contain a cap structure at the 5' end and a poly(A) tail at their 3' end [3; 4]. The proteins (C-E3-E2-6K-E1) encoded by the subgenomic mRNA are synthesized as a polyprotein that is proteolytically processed. Once the C protein is made, it is liberated to the cytoplasm by autocatalytic activity [5]. Translation of the 26S mRNA continues, associated to the endoplasmic reticulum membranes, giving rise to the synthesis of the three glycoproteins E3, E2 and E1 and the viroporin 6K [1; 2; 6]. All the cleavages between the glycoproteins and 6K are accomplished by cellular proteases present in the vesicular system, during their trafficking to the plasma membrane where virus budding takes place [1; 2].

The SV lytic cycle exhibits two well-defined stages. During the early phase cellular translation and the synthesis of nsPs from the genomic RNA takes place. About 2-4 hours after SV infection the pattern of protein synthesis drastically changes in such a way that the structural proteins are mostly synthesized [7]. Thus, SV infection constitutes one of the best models to study the regulation of translation in animal virus-infected cells. The aim of the present work was to

gain an understanding of the requirements for translation of SV subgenomic mRNA under conditions that hamper the translation of either cellular and SV genomic mRNAs [3; 4; 7]. To this end, the requirement for a canonical translation initiation complex to translate this subgenomic mRNA was assayed. Since eIF4G plays a key role in the regulation of the initiation of protein synthesis in many virus-cell systems analysed, we have studied the relevance of this factor for the initiation of translation of the SV RNAs [8; 9; 10].

eIF4G is a large modular polypeptide that interacts with different cellular and viral proteins. There are two isoforms of eIF4G in eukaryotic cells, known as eIF4GI and eIF4GII, which exhibit similar biochemical activities [9]. The eIF4G interacts with eIF4E (cap binding protein) [11] and eIF4A (RNA helicase) [12; 13], forming the eIF4F complex. In addition, eIF4G can bind to the 43S preinitiation complex by interacting with eIF3 [14]. Recently it was reported that eIF4G also interacts with PABP (PolyA binding protein) [15; 16; 17], thus promoting the circularization of mRNA. All these features make eIF4G essential for the correct assembly of the translation initiation machinery. Besides, eIF4G can also interact with other translation regulatory proteins such as nuclear cap binding protein CBP80, the decapping enzyme Dcp1, the eIF4E kinase Mnk1 and heat-shock proteins such as hsp27 [9]. Moreover, viral proteins such as NSP3 from rotavirus, influenza virus NS1 [18; 19] and the 100 KDa adenoviral late protein [20], also bind to eIF4G. Notably, eIF4G associated with eIF4A can directly interact with the internal ribosome entry site (IRES) from both encephalomyocarditis virus (EMCV) or foot and mouth disease virus (FMDV) [21].

Picornaviral proteases have the ability to bisect the two forms of eIF4G, while some retroviral proteases selectively cleave eIF4GI, leaving eIF4GII intact to a large extent. Furthermore, picornavirus proteases have just one cleavage recognition site in eIF4G, dividing the factor in two moieties, while the proteases from retroviruses hydrolyze eIF4G at two different sites, yielding three cleavage products [22; 23; 24; 25; 26; 27]. The proteolysis of eIF4G impairs the translation of newly made cellular mRNAs, but translation of the mRNAs already engaged in translation are much less affected [28; 29; 30].Curiously, some mRNAs from viruses that do not hydrolyze eIF4G during their infections can be efficiently translated when

eIF4G has been cleaved. This is the case of the EMCV RNA, that contains an IRES element in its leader sequence [31; 32]. In addition, the expression of poliovirus $2A^{pro}$ in cells transfected with a plasmid encoding 2A proteolyzed eIF4G efficiently, impairing the translation of typical capped virus mRNAs from the vesicular stomatitis virus (VSV) or the recombinant vaccinia virus T7 [33]. Besides, inducible expression of poliovirus $2A^{pro}$ from a stable HeLa cell line led to eIF4G cleavage and strongly inhibited cellular and vaccinia virus protein synthesis (VV) [34]. In contrast, evidence has been provided that some vaccinia RNAs have a low requirement for intact eIF4F [35; 36]. Moreover, it has been reported that adenovirus and VSV infection induce a progressive dephosphorylation of eIF4E impairing cap-dependent translation, while viral mRNAs continue to be translated [20; 37]. Although alphavirus infection does not lead to cleavage of eIF4G, it was of interest to test whether or not this factor was required to translate SV mRNAs. Here we report that the SV subgenomic mRNA is translated in BHK cells that contain eIF4G cleaved by poliovirus $2A^{pro}$ or the protease of human immunodeficiency virus type 1 (HIV-1 PR).

RESULTS

Cleavage of eIF4G by HIV1 PR and poliovirus $2A^{pro}$ in BHK cells. Translation of the SV subgenomic mRNA. The aim of this work was to analyze the translation of the SV subgenomic mRNA under conditions where eIF4G has been proteolitically degraded by two viral proteases, HIV-1 PR or poliovirus $2A^{pro}$. These proteases cleave eIF4G in different manners (see above). Under these conditions, cap-dependent translation mediated by eIF4E does not occur [9; 10; 29; 30]. Initially, different constructs were engineered, based on an SV replicon that bears the capsid protein (C) followed by the protease gene (Figure 1(b)). These replicons lack the rest of the SV late sequences and efficiently express the gene placed after C [6]. Since this capsid protein is endowed with autoproteolytic activity, the translation efficiency of this mRNA can be estimated by measuring the synthesis of the C protein. Two different replicons were obtained, bearing either HIV-1 PR (Rep C-PR) or the poliovirus $2A^{pro}$ gene (Rep C-2A) (Figure 1(b)). BHK cells were electroporated with the *in vitro* transcribed RNAs from plasmids encoding Rep C and Rep

C-PR. After 16 hpe the integrity of eIF4G was estimated by western blotting, and protein synthesis was analyzed by SDS-PAGE. Previous analyses of eIF4G using specific antibodies have revealed the existence of two proteins of ~220 and ~150 KDa respectively in BHK cells. As already described, eIF4G exhibits different mobility patterns in SDS-PAGE in mammalian cells, possibly due to post-translational modifications [24; 29]. Most probably, the protein of 150 KDa corresponds to a full-length eIF4G which has not undergone the putative post-translational modification. Alternatively, it has been proposed that it could be a breakdown product of eIF4G [29]. Both polypeptides of 220 and 150 KDa disappeared in $2A^{pro}$ and in HIV-1PR expressing cells (Fig.2(a) and (b), upper panels) [31; 38]. In cells electroporated with Rep C-PR there is about 70% of eIF4GI cleavage as measured by densitometry of the 220 KDa band (Figure 2(a), upper panel). In agreement with previous reports, eIF4GII remained uncleaved in these cells (Figure 2(a), middle panel) [26; 32]. We could only detect the C-terminal proteolytic fragment with the anti-eIF4GI and anti-eIF4GII antibodies in BHK-21 cells [31]. The presence of saquinavir (SQ), a specific inhibitor of the HIV protease, prevented eIF4GI cleavage (Figure 2(a), upper panel), while SQ itself had no effect on the expression of Rep C (Figure 2(a), lower panel). Since the percentage of electroporated cells in this experiment was about 70%, as estimated by the remaining cellular translation as well as the percentage of cell rounding (see below), the amount of uncleaved eIF4G may correspond to non-electroporated cells that do not express HIV-1 PR (Figure 2(a)). Notably, the synthesis of C protein from cells electroporated with Rep C-PR was similar in the absence or presence of SQ, i.e. the level of C synthesis was the same when eIF4GI was intact or had been cleaved (Figure 2(a)). As previously observed in our laboratory, C protein is more efficiently synthesized when Rep C is used, as compared to replicons that bear another gene located after the C sequence, even when the SV 6K gene is placed [6].

The HIV-1 PR uses eIF4GI as a substrate, while eIF4GII is poorly recognized [26; 32]. By contrast, poliovirus $2A^{pro}$ can bisect both forms of this initiation factor [23]. Hence, it was of interest to test the effect of $2A^{pro}$ activity on the translation of the SV subgenomic mRNA. To this end, cells were electroporated with transcription buffer or with the RNAs obtained from Rep C, Rep C-2A, Rep L2A and Rep C-2C [6; 39]. The protease synthesized from Rep C-2A

contains four extra aminoacids at its N-terminus that do not hamper its proteolityc activity as compared with the native $2A^{pro}$ produced from Rep L2A (Figure 2(b), lower and middle panels). Over 90% cleavage of both forms of the initiation factor was seen at 8 hpe (data not shown) and at 16 hpe (Figure 2(b), upper and middle panels). Under these conditions significant amounts of C protein synthesis were still observed (Fig.2(b), lower panel). As a control, a replicon that encodes poliovirus 2C (Rep C-2C) was employed (Fig.1(b)). The levels of C synthesis with Rep C-2A were 2-fold higher as compared to Rep C-2C, irrespective of the amount of intact eIF4GI and eIF4GII present in cells (Figure 2(b)). Similar to Rep C-PR (Figure 2(a), lower panel), C expression from Rep C was 3-fold higher than from Rep C-2A (Figure 2(b), lower panel) and 2-fold higher as compared with Rep C-6K (Data not shown). These differences in the expression of the replicons were reproduced in three independent experiments. These findings support the notion that the translation of the SV subgenomic mRNA can occur even when both forms of eIF4G have been proteolyzed.

The levels of subgenomic mRNAs were examined in transfected cells (Fig.3(a)) to determine if the different amounts of C synthesis obtained from Rep C, Rep C-PR and Rep C-2A were the reflection of a partial inhibition of translation. For this purpose, real-time RT-PCR was carried out to quantitate the number of SV RNA molecules in $2x10^5$ cells. After transfection and RNA extraction, real- time RT-PCR revealed that the amount of SV subgenomic RNA was 10-fold higher than SV genomic RNA from Rep C-expressing cells (data not shown). The level of SV subgenomic mRNA obtained from BHK cells transfected with Rep C-PR was about 60% as compared to the subgenomic mRNA synthesized from cells transfected with Rep C (Figure 3(a)). In the case of Rep C-2A, the level of SV subgenomic RNA was about 40% compared with Rep C (Figure 3(a)). The amount of genomic RNA was much more diminished than subgenomic mRNA in Rep C-2A-expressing cells (Figure 3(a)). Taking into account the RNA levels, the normalization of translation data revealed that C synthesis was 70% in Rep C-PR and Rep C-2A compared to the control Rep C (Figure 3(b)).

Cleavage of eIF4G profoundly blocks the translation of newly-made mRNAs, while protein synthesis of cellular mRNAs already engaged in translation is not greatly inhibited [28; 29;

[30]. To analyze whether the first event of translation of the subgenomic 26S mRNA can take place or not in cells containing cleaved eIF4G, we carried out a run-off assay (scheme of the protocol in Figure 4(a)). When cells are incubated in hypertonic medium run-off of polysomes occurs, thus blocking initiation of translation, while elongation still occurs. A return to normal medium leads to initiation of translation on mRNA in treated cells [30]. Cells were electroporated with Rep C, Rep C-2A or mRNA transcribed from pTM1-2A containing EMCV IRES, followed by a $2A^{pro}$ sequence (EMC IRES-2A) (scheme in Figure 1(d)). As a control, cells electroporated with transcription buffer in the absence of RNA were used. Over 95% of eIF4GI and eIF4GII were cleaved in $2A^{pro}$-expressing cells at 16 hpe (Figure 4(b), upper and middle panels). Under these conditions, C synthesis in cells electroporated with Rep C was 3-fold higher than in Rep C-2A- expressing cells (Figure 4(b), lower panel). This result was similar to that shown in Figure 2B. At 16 hpe NaCl was added to a final concentration of 300 mM, and the cells were incubated for 2h. Under these hypertonic conditions, protein synthesis was blocked to a great extent (Fig.4(b), lower panel). Upon return to normal medium, cellular protein synthesis was quickly reestablished in cells electroporated with transcription buffer (Fig.4(b), lower panel). As expected, cellular mRNAs cannot initiate their translation when eIF4G was hydrolyzed by $2A^{pro}$ produced from EMC IRES-2A (Figure 4(b)). In contrast, the translation of C protein from the SV subgenomic mRNA was restored after return to normal medium. Thus, the (3:1) ratio observed for C synthesis from Rep C and Rep C-2A was recovered even when both eIF4GI and eIF4GII were proteolyzed by $2A^{pro}$ (Fig.4(b)). This finding indicates that the first initiation event directed by subgenomic mRNA takes place when eIF4G has been cleaved.

Recombinant viable SVs that express HIV-1 PR or poliovirus $2A^{pro}$. Once we found that the late SV mRNA could be translated in BHK cells containing cleaved eIF4G, we decided to construct recombinant SVs that express the two different viral proteases as depicted in Figure 1(c). We expected these viruses to be viable since they contain all non structural and structural SV genes. The protease gene is placed under the control of a duplicated late promoter. The heterologous protein is less efficiently produced from these recombinant SVs than in the previous constructs using replicons [40]. Unlike the replicons, in this case the subgenomic 26S

mRNA remains intact, directing the synthesis of all SV late proteins, while the heterologous protease is synthesized from another subgenomic mRNA. Once the different plasmids were obtained, the transcribed RNAs corresponding to wt SV, SV-PR and SV-2A were electroporated and protein synthesis and the integrity of the two isoforms of eIF4G were examined at 8 hpe in three independent experiments. HIV-1 PR was analyzed by western blotting in cells treated or not treated with SQ (Figure 5(b), lower panel). The expression of HIV-1 PR in this system led to over 85% cleavage of eIF4GI, while SQ blocked this proteolysis (Figure 5(a), upper panel). The synthesis of C protein in HIV-1 PR-expressing cells with SQ was about 25-35% higher as compared to that observed in the absence of the inhibitor, and was similar to wt SV (Fig.5(b), upper panel). The expression of poliovirus $2A^{pro}$ from the corresponding recombinant SV caused a drastic cleavage of both eIF4GI and eIF4GII (Figure 5(a)). The production of SV C protein was nearly 50-60% as compared to wt SV (Fig.5(b), upper panel). A background of about 10-20% cellular protein synthesis was seen in cells electroporated with SV-PR (without SQ) and SV-2A (Fig.5(b), upper panel), most probably corresponding to non-electroporated cells.

The levels of SV RNAs were then analyzed by real time RT-PCR as described above. As with to Rep C, the amount of SV subgenomic mRNA in wt SV electroporated cells was 10-fold higher as compared with SV genomic RNA (data not shown). Both SV-PR and SV-2A exhibited a decrease of about 40-50% of subgenomic mRNA compared to controls wt SV and SV-PR in the presence of SQ (Fig.5(c)). Notably, the amount of SV genomic RNA was greatly diminished in SV-PR and SV-2A transfected cells (Figure 5(c)). Thus, the presence of SQ abrogates the inhibition of SV RNAs (Fig.5(c)). The normalization of translation of SV structural proteins, taking into consideration the values of SV subgenomic RNA, reflected the fact that the expression from the two recombinant viruses was similar to wt SV, even when both forms of eIF4G were cleaved by the two viral proteases (Figure 5(d)).

Next, citotoxicity of the recombinant SVs was analyzed. The expression of HIV-1 PR or $2A^{pro}$ in BHK cells enhanced cell rounding to about 80% compared with wt SV (data not shown). Moreover, the titer and the morphology of the plaques were them analyzed. The virus titer obtained for SV-PR was one order of magnitude lower in the absence (10^7 pfu/ml) than in

the presence (10^8 pfu/ml) of SQ and the plaques were smaller and irregular when the protease inhibitor was absent (data not shown). These findings are consistent with the reduction of SV genomic RNA observed in SV-PR and SV-2A-infected cells (Figure 5(c)), indicating that the inefficient replication of genomic RNA in the presence of HIV-1 PR or $2A^{pro}$ in the later phase of SV infection impaired or diminished virus yield. The remaining cellular protein synthesis obtained in SV-PR, in the absence of SQ and SV-2A (Figure 5(b), upper panel), may correspond to non electroporated cells that continue uninfected.

Culture supernatants were also employed to infect BHK and COS-7 cells in order to analyze SV protein synthesis and eIF4G cleavage. Infection occurred with the recombinant SV-PR as evidenced by the synthesis of viral proteins, although the cleavage of eIF4G was low (about 10-20%) (data not shown). These findings point to the idea that SV-PR readily loses its ability to express the protease gene when it replicates, even in the presence of SQ.

Effect of eIF4G cleavage on the translation of genomic SV RNA. The SV non-structural proteins (nsPs) are synthesized during the early phase of infection upon translation of the genomic 49S RNA [2; 7]. To assay the requirement of intact eIF4G for the translation of genomic RNA, it is necessary to cleave eIF4G very early during SV infection. To this end, we have employed two strategies to cleave eIF4G efficiently. One of them was based on the transfection of the SV replicon containing the poliovirus leader sequence followed by the $2A^{pro}$ gene that replaces the region corresponding to the SV subgenomic RNA (Rep L2A) (Fig.1(b)). Synthesis of poliovirus $2A^{pro}$ may occur either by internal initiation on genomic RNA, or after transcription of the corresponding subgenomic RNA. The other strategy made use of the electroporation of EMC IRES-2A RNA (Figure 1(d)). $2A^{pro}$ expression from EMC IRES-2A is low, but it led to the cleavage of 80-100% eIF4GI and eIF4GII after 2 hpe (Figure 7(a)). To quantitate the translation of the genomic SV RNA, recombinant virus Toto1101/Luc (SV-Luc) containing the luciferase gene placed inside the nsP3 sequence was employed (Figure 1 (c)) [41].

BHK cells electroporated with transcription buffer, EMC IRES-2A, Rep C or Rep L2A were subsequently infected with SV-Luc at 1 hpe. As described previously, the expression of

$2A^{pro}$ does not block subsequent infections with poliovirus or EMCV [34]. $2A^{pro}$–expressing cells from Rep L2A or EMC IRES-2A exhibited an efficient cleavage of both eIF4GI and eIF4GII in such a way that at 4 hpi no intact eIF4G was observed (Figure 6(a)). After 2 hpi, a profound inhibition of host protein synthesis was found in these cells (Figure 6(b)). At 6 hpi the SV-Luc structural proteins were measured. The synthesis of C protein was lower in cells electroporated with transcription buffer than in those electroporated with Rep C since, in this last case, C was generated from Rep C and SV-Luc. PE2 precursor and E1 glycoprotein were only produced from SV-Luc, so they were synthesized in a similar fashion in both cases (Figure 6(b)). Notably, SV-Luc structural proteins were deeply inhibited in 2A-expressing cells (Figure 6(b)). Transcription to yield subgenomic mRNA require the synthesis of non structural proteins to form the replicative complexes. Thus, the inhibition of the SV structural proteins in $2A^{pro}$-expressing cells may be due to the blockade of genomic RNA translation. Luciferase activity was determined in each case to quantitate genomic RNA translation. A significant inhibition of luciferase synthesis (about 60-80%) was found throughout the time course in $2A^{pro}$-expressing cells as compared to cells electroporated with transcription buffer or Rep C (Figure 6(c)). These results have been reproduced in three independent experiments.

Next, the inhibition of protein synthesis was calculated, considering the amount of genomic RNA present. To quantitate this RNA in SV-Luc infected cells real time RT-PCR was employed using oligonucleotides that hybridize with the nsP2 gene. In cells superinfected with an SV replicon and SV-Luc the genomic RNA level was higher than in cells only infected with SV-Luc, since the nsP2 gene is contained in both constructs (Figure 6(d)). On the other hand, from 3-5 hpi genomic RNA is not only employed in translation and RNA replication, but it is also encapsidated to form viral particles [2; 41]. However, SV-Luc did not produce an effective infection when $2A^{pro}$ was co-expressed in the early phase of the viral cycle since structural proteins were inhibited (Figure 6(b)). Therefore, the genomic RNA level at 2 hpi was taken to normalize the luciferase activity data in control infected cells. The amount of SV-Luc genomic RNA present in cells electroporated with EMC IRES-2A was similar to control cells at 2 hpi (Figure 6(d)). However, it progressively decreased in 2A-expressing cells as compared to those

electroporated with transcription buffer throughout the time course, possibly due to the inhibition of nsPs synthesis (data not shown). Relative luciferase activity was corrected taking into account the values of SV-Luc genomic RNA. At 2 hpi genomic RNA translation was about 30% when both forms of eIF4G were cleaved (Fig.6(e)). In addition, nsP1 was analyzed by western blotting, employing specific antiserum. The amount of nsP1 that accumulated in non-infected cells electroporated with Rep L2A was approximately 35-40% as compared with Rep C-expressing cells at 6 hpe (Figure 6(f)).

To analyze if the first translation initiation event directed by genomic RNA takes place even to a lesser degree, cells were electroporated with EMC IRES-2A or transcription buffer as a control and were infected at 1, 2, 4 or 6 hpe with SV-Luc. As expected, eIF4G was hydrolyzed by $2A^{pro}$ in a time-dependent manner and was almost totally cleaved at 4 hpe (Figure 7(a)). To analyze genomic RNA translation, luciferase activity was measured at 3 hpi in each case (Figure 7(b)). In accordance with the results shown in Figure 8, luciferase activity obtained when SV-Luc was added at 1 hpe was 25-35% in 2A-expressing cells, as compared to the control. Notably, when cells were infected with SV-Luc at 2, 4 or 6 hpe, a decrease of about 85-90% was observed in genomic RNA translation in cells electroporated with EMC IRES-2A (Figure 7(c)). A significant correlation between inhibition of SV-Luc genomic translation and eIF4G proteolysis was found (Figure 7(a) and (c)). Therefore, the genomic SV mRNA, as occurs with cellular mRNAs, is translated in a cap-dependent manner, while 26S mRNA can be translated when the cap binding protein eIF4E does not form part of the eIF4F complex.

Effect of eIF4G cleavage on the translation of RNAs from a recombinant SV-bearing GFP protein. Next, we wanted to analyze the requirement of intact eIF4G for the translation of SV-GFP RNAs. This SV recombinant expresses a heterologous gene placed under the control of a duplicated late promoter (Figure 1(c)), and produces two types of subgenomic mRNAs. One is the canonic subgenomic mRNA, and another contains the 26S leader sequence followed by a heterologous protein coding sequence (GFP) (Figure 8(a)). To accomplish efficient cleavage of eIF4G, BHK cells were electroporated with EMC IRES-2A; as controls, EMC IRES-2C (Figure

8(d)) or a transcription buffer were used. Cells were infected 2 h before or 1 h after electroporation with *in vitro* synthesized RNAs. Both forms of eIF4G were proteolyzed before genomic translation (Figure 8(c), left upper and middle panels) or subgenomic RNAs translation (Figure 8(c), right upper and middle panels). When eIF4G was cleaved before SV-GFP infection, the viral late proteins and GFP synthesis were radically inhibited (Figure 8(c), left lower panel). Notably, normal levels of structural protein synthesis occurred when eIF4G was proteolyzed immediately before subgenomic translation (Figure 8(c), right lower panel). In this instance, the levels of SV structural proteins or GFP synthesis from SV-GFP were similar, irrespective of the amount of intact eIF4G present in BHK cells (Figure 8(c), right lower panel). These results were reproduced in three independent experiments and indicate that late protein synthesis is hampered in $2A^{pro}$ expressing cells when eIF4G is cleaved before SV-GFP infection by inhibition of genomic RNA translation. However, the translation of 26S and the second subgenomic mRNAs occurred in the absence of intact eIF4G when cells were electroporated at 2 hpi. The translation of the two different subgenomic mRNAs points to the relevance of the subgenomic 26S leader sequence in providing independence for intact eIF4G.

The leader sequence of the subgenomic SV mRNA contains 49 nt from the cap structure until the initiator AUG codon. To map the regions in this sequence that confers high translatability to this mRNA, three deletion variants in the leader sequence placed before eGFP gene were constructed (Figure 8(b)). The first 11nt from the 5'-end must remain in the three constructs, because they are necessary for efficient transcription [42]. Thus, one of the constructs lacks nucleotides from 11 to 31 (SV-GFPΔ11-31), another lacks nt 31-49 (SV-GFPΔ31-49) and the third one lacks nt 11-49 (SV-GFPΔ11-49) (Figure 8(b)). Notably, the synthesis of SV structural proteins was similar in all three SV-GFP variants, but GFP synthesis was significantly decreased in SV-GFPΔ11-49. The ratio between C and GFP expression diminished about 20-30% from SV-GFPΔ11-31 and 50-60% from SV-GFPΔ11-49 compared with SV-GFP or SV-GFPΔ31-49 (Figure 8(d), lower panel). Curiously, GFP was still synthesized even when large region of the leader region was deleted (Figure 8(d), lower panel). These results were

reproduced in two independent experiments and indicate that only a profound modification of the leader sequence of subgenomic SV mRNA leads to inhibition of GFP translation.

To test the dependence on eIF4G intactness of the different leader deletion variants, BHK cells were infected with SV-GFPΔ11-49, SV-GFPΔ11-31 or SV-GFPΔ31-49 at a multiplicity of 10 pfu/cell. 2 hpi, cells were electroporated with EMC IRES-2A, EMC IRES-2C or transcription buffer as a control. As shown in figure 8(d), upper and middle panel), both forms of eIF4G were significantly proteolyzed. Of interest, translation of the different deletion variants still occurred even when eIF4G was bisected (Figure 8(d), left panels). This result suggest that the presence of the initial 11 nt suffices to confer eIF4G independence for the translation of the SV subgenomic mRNA.

DISCUSSION

The majority of late viral mRNAs have the ability to be translated under conditions in which host cell protein synthesis is deeply inhibited [8]. This is the case of the translation of SV subgenomic mRNA. The alphavirus 26S mRNA contains a particular structure that confers on it a high translatability under conditions that are detrimental for cellular protein synthesis [3; 4; 43]. There are at least two sequences that could be involved in this feature. One of them is the UTR sequence placed at the 5' end. This sequence from SFV confers on chimaeric mRNAs that encode a reported gene, the capacity to be translated in the presence of low amounts of initiation factors [44]. In the case of the UTR sequence of the SV subgenomic mRNA, which contains 49 nt, also provides a good translatability to chimeric mRNAs bearing the GFP gene as shown in this paper. Another sequence implicated in subgenomic mRNA translatability is included in the C gene. SV subgenomic mRNAs which contain the first 226 nt from the capsid gene are translated 10-fold more efficiently than those lacking this sequence [3; 4]. The first 170 nt downstream of the translation initiation codon may be folded into an extensively base-paired structure. This hairpin structure could recruit some initiation factors present at low concentrations; alternatively, it could pause the 40S ribosome subunit at the AUG initiation codon [4]. We now provide evidence that, indeed, the SV 26S mRNA does not require the eIF4F complex. For these assays, we have

developed two effective protease expression systems to hydrolyze eIF4G. The first one is based on the use of SV replicons or recombinant viable SV to obtain good expression of the proteases in a high percentage of BHK cells. The second system utilizes in vitro transcribed mRNAs, which contain the EMCV IRES followed by the poliovirus 2A gene. This mRNA is electroporated into cells, leading to a low expression of this protease.

Our present results indicate that the SV subgenomic mRNA can be translated when eIF4G is proteolyzed by $2A^{pro}$ or HIV-1 PR. These findings suggest that eIF4E, at least when forming part of the eIF4F complex, is not required to initiate SV subgenomic mRNA translation. It has been described that the interaction between eIF4G and PABP is essential for the correct recruitment and assembly of the translation machinery [45]. The hydrolysis of eIF4G by these two viral proteases separates the PABP-binding domain in eIF4G impairing its interaction. Moreover, the proteolisis of eIF4G by HIV-1 PR separates the Mnk-1 interaction domain of eIF4GI [27]. This kinase phosphorylates eIF4E, increasing its cap-binding activity, thus enhancing subsequently cap-dependent translation [9].

The dependence of cellular mRNA translation on eIF4G is evident when newly-formed mRNAs are examined. Once cellular mRNAs are bound to the protein synthesizing machinery, subsequent initiation events may not require the participation of an intact eIF4F complex [28; 29; 30]. When cellular mRNAs are stripped of ribosomes by inducing the run-off of translation with hypertonic medium, the mRNAs cannot participate in initiation if eIF4G has been proteolyzed [30]. This is not the case for SV subgenomic mRNA, since it can interact with ribosomes and initiation factors to accomplish the first initiation event when eIF4G has been cleaved. The capacity of the subgenomic mRNA to be translated after eIF4G proteolysis and treatment with hypertonic medium, clearly indicates that intact eIF4F is not required to build up the initiation complex directed by this mRNA. Comparison of the translation of SV genomic and subgenomic mRNAs points to their different ability to participate in translation in cells lacking intact eIF4G. Thus, the finding that protein synthesis directed by SV genomic mRNA is inhibited by about 60-80% when eIF4G is hydrolyzed by poliovirus $2A^{pro}$, indicates that genomic RNA is more similar to cellular mRNAs in its translation behaviour than subgenomic mRNA. Moreover,

when eIF4G is proteolyzed before SV-Luc and SV-GFP infection, the structural SV protein synthesis is blocked, indicating a reduction in non-structural protein synthesis when $2A^{pro}$ is co-expressed. These data point to the different behaviour between early and late SV RNAs, as regards the requirement for eIF4G. However, normal levels of structural proteins are synthesized when eIF4G is proteolyzed before subgenomic translation. In this instance, the amount of SV C or GFP synthesis from SV-GFP is similar, irrespective of intact eIF4G. The observation that one RNA with a subgenomic leader sequence placed upstream of the GFP gene can be efficiently translated in SV infected cells when eIF4G is cleaved by $2A^{pro}$, provides evidence that a short sequence could contribute to cap independent translation.

The findings obtained with SV-GFP deletion variants suggest that there is not an essential region between nucleotides 11-49 to be translated when eIF4G was hydrolyzed by $2A^{pro}$. However, the absence of the last 38 nt of the leader 26S sequence diminished GFP expression. In addition, the presence of 42 nt from the luciferase leader sequence after the first 11 nt of SV 26S mRNA severely impaired subgenomic mRNA translation (unpublish data). These results suggest that it is not just the length but the sequence of nucleotides 11-49 what is important for the efficient expression of GFP from SV-GFP. In this regard, VSV mRNAs also contain short, unstructured 5'UTRs (from 11 to 49 nt) and can be translated in absence functional eIF4F complex [37]. These features may contribute to the translatability of VSV RNAs and SV subgenomic RNA. Beside, a number of adenovirus late mRNAs contain the so-called tripartite sequence at their 5' ends. In accordance with the findings described here, these capped adenovirus mRNAs are also capable of being translated when eIF4G is cleaved by poliovirus infection [46; 47; 48]. The unstructured conformation of the leader region of some adenovirus mRNAs may confer the translation properties of the tripartite sequence [49].

As occurs with these adenovirus mRNAs, most probably the leader sequence of the 26S mRNA is not translated by internal initiation. Thus, SV structural proteins are not produced from genomic RNA in the early phase of SV infection [7; 48; 49]. Moreover, eIF2α is phosphorylated by PKR during SV infection. This modification inactivates eIF2 activity, contributing to the inhibition of cellular translation. Under these conditions, SV subgenomic

mRNA continues to be translated [50]. Therefore, the initiation of translation of the subgenomic SV mRNA could occur by a mechanism that differs from those described for cellular or picornavirus mRNAs.

The fact that SV subgenomic mRNA contains a cap structure raises questions about its participation in its translation. One possible function of the cap structure in this RNA is to increase the RNA stability in cooperation with the poly(A) sequence [51]. The possibility that the small ribosomal subunit interacts with the initiation AUG codon without participation of eIF4E and the cap structure remains open. Therefore, we can distinguish two different strategies followed by animal viruses to originate mRNAs with a high translatability. Both strategies are directed at decreasing the requirement for translation initiation factors. One type of these viral mRNAs corresponds to uncapped, IRES-containing mRNAs that possess long and highly structured 5' UTRs [9; 52]. Another kind of viral mRNA, which is highly translatable, contains capped short and unstructured leader sequences [37; 49]. Further understanding of the mechanism used to assemble the initiation translation complex directed by the SV subgenomic leader sequence may provide clues to help identify the factors that are involved in the discriminatory recognition between cellular and viral mRNAs.

Another point of interest in this work is the evidence that viable recombinant SV that express HIV-1 PR are feasible, particularly when SQ is present. These recombinant viruses induce a clear cytophatic effect and cell rounding, suggesting that the synthesis of HIV-1 PR or poliovirus $2A^{pro}$ suffices to provoke this cytotoxic effect. Moreover, in the absence of inhibitor, SV-PR renders lytic plaques with a different morphology than wt SV or SV-PR in the presence of the inhibitor. Hence, SV-PR could be employed as a simple and rapid approach to search for inhibitors against-HIV-1 PR or poliovirus $2A^{pro}$ in eukaryotic cells.

MATERIALS AND METHODS

Cell cultures. Baby hamster kidney (BHK-21) cells were grown at 37ºC in Dulbecco´s Modified Eagle´s medium (DMEM) supplemented with 5% fetal calf serum (FCS) and non-essential amino acids.

Plasmids. Construction of the SV replicons Rep C and Rep C-6K have already been described [6; 39]. Rep C-PR, Rep C-2A and Rep C-2C were made by inserting a PCR product encoding HIV-1 PR, or poliovirus $2A^{pro}$ or 2C respectively after the sequence of the C gene in the plasmid pH3'2J-C, employed as a shuttle vector using *Nde*I/*Bam*HI restriction sites [40]. Next, the fragment between the two sites (*Aat*II/*Xho*I) was transferred to the same sites in the vector pT7SVwt (wt SV), described previously [40]. Rep L2A was constructed by inserting the *Hpa*I/*Apa*I digested product containing the hybrid sequence from sindbis virus and poliovirus obtained by PCR in the same sites of pT7SVwt. To obtain this PCR product we designed four oligonucleotides: the first oligonucleotide hybridizes with the *Hpa*I sequence into the SV sequence; the second has the junction sequence between the Sindbis virus and poliovirus sequences in the opposite direction; the third has a complementary and inverted sequence related to the second oligonucleotide; and the fourth has the carboxyl-terminal sequence of $2A^{pro}$, a stop codon and, next, the sequence for *Apa*I. We made a PCR using the first two oligonucleotides and pT7SVwt as a template and another PCR using the last two oligonucleotides and the plasmid pSK-L2A as a template [31]. Then we used a mixture of these products as a template with the oligonucleotides that have the Hpa I and ApaI sites.

pToto1101/Luc (SV-Luc) was generously provided by Charles Rice (Rockefeller University, NY) [41]. pT7SV-HIV-1 PR (SV-PR) and pT7SV-$2A^{pro}$ (SV-2A) were generated by inserting a PCR product containing the corresponding protease gene digested with *Xba*I/*Bam*HI in the same sites of pH3'2J. The subgenomic promoter casette of pH3'2J1-HIV-1 PR and pH3'2J-$2A^{pro}$ was inserted into the SV cDNA clone pT7SVwt using the *Apa*I/*Xho*I restriction sites. pT7SV-GFP (SV-GFP) was obtained following a similar strategy as for Rep L2A (see above) using ApaI/XbaI restriction sites in pT7SVwt and pEGFP-N1 (Clontech) as a template. The SV-GFP mutants SV-GFPΔ11-49, SV-GFPΔ11-31 and SV-GFPΔ31-49, that contain certain deletions inside SV subgenomic leader sequence placed before eGFP gene (nucleotides

11 to 49, 11 to 31 or 31 to 49 respectively), were constructed using specific oligonucleotides and SV-GFP as a template.

pTM1-2A and pTM1-2C were described earlier [53].

Strains BH10 of HIV-1 and pT7(XLD) were used as a template for HIV-1 and poliovirus constructions respectively [54].

Transfection of BHK-21 cells. BHK-21 cells were electroporated with in vitro synthesized RNAs from the different plasmids. Subconfluent cells were harvested, washed with ice-cold phosphate-buffered saline (PBS), and resuspended in PBS at a density of about $2.5x10^6$ cell/ml. 50 µl aliquots of T7 RNA polymerase (Promega) transcription mixture with about 25 µg RNA from each different cDNA construct were added to 0.4 ml of cells, and the mixtures were transferred to 2 mm electroporation cuvettes (Bio-Rad). Electroporation was performed at room temperature by two consecutive 1.5- kV, 25- µF pulses using a Gene Pulser apparatus (Bio-Rad) as described [55]. Control BHK cells were electroporated with 50 µl transcription mixture in PBS. The cells were then diluted in growth medium and seeded onto culture plates. Viral protein synthesis was analyzed by metabolic labeling with [^{35}S] Met-Cys, followed by polyacrilamide gel electrophoresis (SDS-PAGE) and fluorography. Western blot analysis was carried out using an antibody against SV nsP1 (a gift from V. Stollar, Robert Wood Johnson Medical School, New Jersey) at 1:1,000 dilution. The integrity of translation initiation factors was analyzed by western blot using anti-eIF4GI antisera raised against peptides derived from the N- and C-Terminal regions of human eIF4GI [31] at a 1:1,000 dilution or with rabbit antisera against the C-terminal region of eIF4GII (a gift from N. Sonenberg, McGill University, Montreal, Canada) at a 1:500 dilution. Goat antiserum against HIV-1 PR was provided by the EU program EVA/MRC Centralised Facility for AIDS Reagents, NIBSC, UK and used at dilution 1:700. The amount of sample loaded in each experiment was tested by western blotting with anti-eIF4A at a 1:50 dilution (a gift from Dr. H. Trachsel, Institute for Biochemistry and Molecular Biology, University of Berne, Switzerland). Anti-rabbit, anti-mouse and anti-sheep immunoglobulin G antibodies coupled to peroxidase (Pierce) was used at a 1:10,000 dilution.

Viral infections. BHK-21 cells were infected with SV-Luc, SV-PR or SV-GFP at a multiplicity of infection of 10 pfu/cell. After one hour of adsorption the medium was removed and culture plates were incubated with fresh DMEM medium supplemented with 5% FCS. Sindbis virus (SV) wild-type (wt) and recombinants SV-PR, SV-2A, SV-Luc and SV-GFP were titered in BHK-21 cultures. In the SV-PR titration saquinavir (SQ) was added at a final concentration of 12 μM.

Analysis of mRNA by real-time RT-PCR. SV RNA levels in transfected cells were determined by real-time quantitative reverse transcription (RT)-PCR. For this purpose, total RNA was extracted from $2x10^5$ cells at the times indicated in each figure using the RNeasy commercial kit (Qiagen) following the manufacturer's recommendations. The isolated RNA was resuspended in 30 μl of nuclease-free water, and 3 μl was subjected to analysis. Real-time quantitative RT-PCR was performed with the LightCycler thermal cycler system (Roche Diagnostics) using the RNA Master SYBR Green I kit (Roche Diagnostics) as described by manufacturer. The primers nSP2-forward (5'-GGAGGGGCTCCAGGCGGACATCG-3') and nSP2-reverse (5'-GCTCCTCTTCTGTATTCTTGGCG-3') were used to quantitate the SV genomic RNA. The primers C-forward (5'-GAACGAGGACGGAGATGTCATCG-3') and C-reverse (5'-CAGCGCCACCGAGGACTATCGC-3') were employed to quantitate the total SV RNA. Subgenomic SV RNA was calculated as the difference between total SV RNA and SV genomic RNA. These primers were designed to amplify sequences of 250-300 nt to maximize the efficiency of the reaction. RT-PCR was carried out in 20 μl of LightCycler RNA Master SYBR Green I solution containing 3 mM manganese acetate and a 1 μM concentration of each primer. RT was performed at 61ºC for 20 min. After that, PCR amplification was initiated with incubation at 95ºC for 2 min, followed by 45 cycles of 95ºC for 5 s, 58ºC for 12 s, and 72ºC for 20 s. Data analysis was done using the Roche Molecular Biochemicals LightCycler software (version 3.3). The specificity of amplification reactions was confirmed by analyzing their corresponding melting curves.

Hypertonic medium treatment. To produce the ribosomal run-off from polysomes, 150 mM NaCl was added to cell cultures to reach a final concentration of 300 mM in DMEM 10%

FCS for 2 hours. Protein synthesis was then recovered by washing the cells twice with DMEM to remove the excess of NaCl in the culture medium [30]. After that, cell monolayers were incubated with DMEM supplemented with 10% FCS for 2 hours. Protein synthesis was estimated as described above at the times indicated in the figure legend.

Measurement of luciferase activity. BHK-21 cells were electroporated with the different *in vitro* synthesized RNAs. Control cells were electroporated with 50 µl transcription buffer in PBS. Then cells were infected with SV-Luc. At different hours postinfection, cells were lysed in a buffer containing 0.5% Triton X-100, 25mM glycylglycine (pH 7.8) and 1mM dithiothreitol. Luciferase activity was determined using a Monolight 2010 apparatus (Analytical Luminiscence Laboratory) as described previously [53].

Optical microscopy. BHK-21 cells were electroporated with wt SV, SV-PR or SV-2A and grown on glass cover slips in DMEM with 10% FCS. At 16 hours post-electroporation (hpe) cells were washed with PBS and fixed with 4% (w/v) paraformaldehyde in PBS for 20 min at room temperature. Finally, cells were washed and mounted in mowiol by inverting the coverslip. They were examined by microscopy using an Axiovert 200 inverted microscope (Zeiss) with a 20X0.6 Plan-Apochromat Ph2 objective.

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FIGURE LEGENDS

Figure 1. **Schematic representation of.** (a) full-length wt SV RNA genome. (b) SV Replicons containing SV C protein followed by heterologous gene, or poliovirus IRES after the SV subgenomic promoter (SG.P) and followed by $2A^{pro}$, as indicated. (c) Recombinant SV RNAs

that possess heterologous genes placed under the control of a duplicated subgenomic promoter and recombinant SV containing the luciferase gene placed inside the nsP3 sequence. (d) *in vitro* transcribed mRNAs from pTM1-2A and pTM1-2C that only contain the EMCV leader sequence and poliovirus 2A or 2C gene.

Figure 2**. Dependence of 26S mRNA translation on intact eIF4G using different SV replicons.** BHK cells were electroporated with transcription buffer (BHK), Rep C or Rep C-PR and grown in the presence or absence of 12 μM saquinavir (SQ) (a); or with transcription buffer, Rep C, Rep C-2A, Rep L2A or Rep C-2C (b). Proteins were labeled with [^{35}S]Met/Cys from 15 to 16 hpe and processed as described in Materials and Methods ((a) and (b), lower panels). Aliquots were analyzed by western blotting with specific antisera against eIF4GI (panel (a) and (b), upper panels) or eIF4GII (panel (a) and (b), lower panels). Percentage of Capsid protein synthesis or intact eIF4GI and eIF4GII were determined by densitometric scanning of the corresponding protein band. Ct, C-terminal fragments of eIF4GI or eIF4GII. C, capsid SV protein. Mr (KDa) molecular weight markers.

Figure 3. **Analysis of SV RNA levels by real time RT-PCR.** (a) The SV genomic and subgenomic RNAs were isolated at 16 hpe from cells transfected with the different SV replicons and quantitated as described in Materials and Methods. The data are presented as a relative comparison of Rep C-PR or Rep C-2A RNAs levels with Rep C. (b) Representation of 26S normalized mRNA translation considering the level of SV subgenomic mRNA in electroporated cells. The C synthesis values were corrected to the relative amount of SV subgenomic mRNA and calculated based on values obtained for Rep C.

Figure 4**. Effect of eIF4G cleavage on the reinitiation of SV protein synthesis after exposure to hypertonic medium.** BHK cells were electroporated with transcription buffer (BHK), Rep C or Rep C2A. From 16 to 18 hpe, the concentration of NaCl in the medium was increased to 300 mM. At 18 hpe isotonicity was restored. From 15 to 16 hpe, 17 to 18 hpe and 19 to 20 hpe, cell cultures were labeled for 1h. (a) Schematic representation of the protocol. (b)

upper panel: western blotting against eIF4GI. Middle panel: western blotting against eIF4GII. Lower panel: Protein synthesis analyzed by fluorography and autorradiography.

Figure 5**. Effect of eIF4G cleavage on the translation of 26S mRNA using recombinant SVs that express heterologous proteases.** Cells were electroporated with transcription buffer (BHK), wt SV, SV-PR or SV-2A RNAs, grown in the presence or absence of 12 μM saquinavir, labeled with [^{35}S]Met-[^{35}S]Cys from 7 to 8 hpe. Aliquots of the same samples were analyzed by western blotting with specific antisera against eIF4GI ((a), upper panel), eIF4GII ((a), lower panel) and HIV-1 PR (B, lower panel) and fluorography and autorradiografy ((b), upper panel). Percentage of capsid protein synthesis or intact eIF4GI and eIF4GII were determined by densitometric scanning of the corresponding protein band. (c) Analysis of SV RNA levels by real time RT-PCR. The SV genomic and subgenomic RNAs were isolated from transfected cells and quantitated as described in Materials and Methods. The data are presented as relative comparison of SV-PR, in the absence and in the presence of SQ, or SV-2A RNA levels as compared to wt SV. (d) Representation of normalized 26S mRNA translation considering the level of SV subgenomic mRNA in electroporated cells. C synthesis was corrected to the relative amount of SV subgenomic mRNA.

Figure 6. **Dependence of translation of genomic SV mRNA on the integrity of eIF4G.** Cells were electroporated with transcription buffer (BHK), EMC IRES-2A, Rep C or Rep L2A and infected at 1 hpe with SV-Luc. Cells were labeled with [^{35}S]Met-[^{35}S]Cys at different time points, as indicated. (a) Integrity of eIF4GI and eIF4GII was analyzed by western blotting. (b) Cellular protein synthesis was examined by fluorography and autoradiography. Actine, C protein, SV glycoprotein (PE2 and E1) synthesis, nsP1 accumulation and % of proteolysis of eIF4GI and eIF4GII were determined by densytometric scanning of the corresponding protein band. (c) Cells were collected in luciferase lysis buffer at different time points, as indicated. Luciferase activity was measured using a Monolight 2010 apparatus. (d) RNA was isolated at 2 hpi from SV-Luc infected cells as indicated in Materials and Methods. SV genomic RNA levels were analyzed by real time RT-PCR. The data represent the relative comparison with the RNA

isolated from cells electroporated with transcription buffer and infected with SV-Luc. (e) Normalization of % of luciferase activity at 2 hpi of cells electroporated with transcription buffer or EMC IRES-2A and infected at 1 hpe with SV-Luc. The luciferase activity values were corrected to the relative amount of SV-Luc genomic RNA and calculated based on values obtained for control cells infected with SV-Luc. (f) Cells were electroporated with transcription buffer, Rep C or Rep L2A. SV nsP1 accumulation was analysed by western blotting against SV nsP1. Luc, luciferase. RLU, relative light units.

Figure 7. **Effect of eIF4G cleavage on genomic SV translation initiation.** Cells were electroporated with transcription buffer or EMC IRES-2A. (a) One half of cells were collected at 1, 2, 4 and 6 hpe and eIF4GI and eIF4GII was analyzed by western blotting. The relative amount of intact eIF4G is represented. (b) The other half of the electroporated cells were infected at 1, 2, 4 or 6 hpe with SV-Luc and collected in luciferase lysis buffer at 3 hpi. Luciferase activity was measured as described in Figure 8. The result is showed as the relative luciferase activity in cells extracts (expressed in light units set to 100% of control reactions).

Figure 8. **Effect of eIF4G cleavage on the translation of GFP mRNAs using recombinants SV.** (a) Schematic representation of mRNAs synthesized from SV-GFP. (b) Schematic representation of the SV subgenomic leader sequence placed upstream of eGFP gene and the deletion variants. (c) Cells were electroporated with transcription buffer, EMC IRES-2A or EMC IRES-2C and infected with 10 pfu/cell of SV-GFP 2h before or 1h after electroporation. The cells were collected at time indicated (d) Cells were infected with 10 pfu/cell of SV-GFPΔ11-49, SV-GFPΔ11-31 or SV-GFPΔ31-49 and at 2 hpi were electroporated with transcription buffer, EMC IRES-2A or EMC IRES-2C. The protein synthesis and the integrity of initiation factors were analyzed at 16 hpe/18 hpi. (c) and (d) Upper panels: western blotting against eIF4GI; middle panels: western blotting against eIF4GII; lower panels: analysis of viral protein synthesis by fluorography and autoradiography. L49, genomic 49S leader sequence. L26, subgenomic 26S leader sequence. eGFP, enhanced green fluorescence protein.