Two isoforms of the translation initiation factor eIF4G, eIF4GI and eIF4GII, have been described in eukaryotic cells. The exact function of each isoform during the initiation of protein synthesis is still under investigation. We have developed an efficient and reliable method of expressing poliovirus 2Apro, which differentially proteolyzes eIF4GI and eIF4GII in a time- and dose-dependent manner. This system is based on the electroporation of an in vitro transcribed mRNA that contains the encephalomyocarditis virus internal ribosome entry site followed by the sequence of poliovirus 2Apro. In contrast to HeLa cells, expression of this protease in BHK-21 cells induces delayed hydrolysis kinetics of eIF4GI with respect to eIF4GII. Moreover, under these conditions the polyadenylate binding protein is not cleaved. Interestingly, translation of de novo synthesized luciferase mRNA is highly dependent on eIF4GI integrity, whereas ongoing translation is inhibited at the same time as eIF4GII cleavage. Moreover, reinitiation of a preexisting mRNA translation after polysome run-off is dependent on the integrity of eIF4GII. Notably, de novo translation of heat shock protein 70 mRNA depends little on eIF4GII integrity but is more susceptible to eIF4GII hydrolysis. Finally, translation of an mRNA containing encephalomyocarditis virus internal ribosome entry site when the two isoforms of eIF4G are differentially hydrolyzed has been examined.

The initiation of translation is a major target for the regulation of gene expression in eukaryotic cells. A number of initiation factors participate in this process leading to the interaction of the small ribosomal subunit with mRNA. The eukaryotic initiation factor 4F (eIF4F) plays a central role in the early steps of protein synthesis. eIF4F is composed of three polypeptides: eIF4E, eIF4A, and eIF4G (1, 2). eIF4E is the cap binding subunit. eIF4A is an RNA helicase which together with eIF4B unwinds the secondary structure present at the 5’ end of mRNAs. eIF4G is a scaffolding protein that physically links the cap structure and the poly(A) tail of mRNAs with the small ribosomal subunit by means of its interaction with eIF4E, poly(A)-binding protein (PABP), and eIF3. In addition, eIF4G interacts with other cellular and viral proteins involved in the regulation of translation (2). There are two isoforms of eIF4G in mammalian cells known as eIF4G1 and eIF4GII that are 46% identical in their primary amino acid sequence. It has been proposed that these two isoforms possess similar biochemical activities and are functionally interchangeable (2). eIF4G contains three domains of a similar size: (i) the N-terminal region, which contains the eIF4E and PABP binding sites, which are needed for cap and poly(A) recognition (3, 4); (ii) the middle portion, which participates in the recruitment of the 43S preinitiation complex on interaction with eIF3 (5); (iii) the C-terminal domain, which binds Mnk1, a mitogen-activated protein kinase that enhances cap-dependent translation by phosphorylation of eIF4E (6, 7). Two eIF4A interaction sites have been found in eIF4G, located in the middle and C-terminal domain, respectively (8). Thus, eIF4G is essential for coordinating a number of components of the translation machinery to assemble the initiation complex.

Infection of mammalian cells with most cytopathic animal viruses induces a marked inhibition of host transcription and translation. Many viruses have evolved mechanisms that employ viral proteases to manipulate the host translational machinery to maximize the selective translation of viral mRNAs compared with endogenous host transcripts (9). Although it was found some time ago that picornavirus infection induces a marked shutoff of host protein synthesis, the mechanisms involved are still being investigated. Previous reports revealed that hydrolysis of eIF4GI, which comprises most of the total eIF4G (10, 2), is not sufficient to fully inhibit host translation (11–13). Proteolysis kinetics of eIF4GII were delayed with respect to cleavage of eIF4GI in poliovirus (PV)- and human rhinovirus-infected cells. In this regard disappearance of intact eIF4GII correlated with the abrogation of host translation in PV- and rhinovirus-infected and apoptotic cells (14–16). Cleavage of both eIF4G1 and eIF4GII strongly blocked the initiation of de novo synthesized mRNAs (17). In addition, PV and Coxackievirus 2Apro and 3Cpro are able to cleave PABP during infection (10). More recently, it has been reported that...
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hydrolisis of PABP by 3Cpro in the absence of eIF4G degradation blocks the translation of endogenous mRNAs in HeLa cell extracts (18). 3Cpro preferentially hydrolized the PABP associated with the translational machinery, although the hydrolisis of PABP did not correlate with the shutoff of cellular translation in PV-infected cells (10). Therefore, the individual contribution of eIF4GI, eIF4GII, and PABP proteinase to the shutoff of cellular protein synthesis needs further investigation.

We describe an effective system based on PV 2Apro that induces the differential cleavage of eIF4GI and eIF4GII without affecting PABP. Hydrolysis of eIF4GI by 2Apro occurs before proteinase of eIF4GII in HeLa cells, whereas the opposite is true for BHK cells. The effect of differential degradation of each eIF4G isoform in the translation of cellular mRNAs in culture cells has been examined.

MATERIALS AND METHODS

Cell Cultures—HeLa and BHK-21 (baby hamster kidney) cells were grown at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum and nonessential amino acids. HeLa clone X1/5 cells express luciferase (Luc) in a tetracycline-dependent manner (19, 17). Repression of Luc gene transcription was obtained with 20 ng/ml tetracycline in the culture medium (17).

Plasmids—The plasmids pKS-Luc, pTM1-Luc, pTM1–2C, and pTM1–2A and the plasmids pTM1–2AG60R, pTM1–2AG121E, pTM1–2AD135N, and pTM1–2AV119M, which encodes a PV 2Apro mutant, have been described previously (20, 21).

RNA Transcription—The in vitro transcription was carried out with T7 polymerase (Promega) according to the indications of the manufacturer and the corresponding plasmid as a template. In vitro polyadenylation was performed with poly(A) polymerase (Invitrogen). The mRNA was purified using the chroma spin columns kit (BD Biosciences). The amount of mRNA was analyzed with the NanoDrop ND-1000 spectrophotometer.

Transfection of HeLa and BHK-21 Cells—HeLa cells were electroportorated with in vitro synthesized mRNAs. Subconfluent cells were harvested, washed with ice-cold phosphate-buffered saline (PBS), and resuspended in PBS at a density of about 2.5 × 10⁸ cells/ml. Fifty μl of transcription mixture (Promega) with the amounts of RNA indicated in each figure legend were added to 0.8 ml of cell suspension, and the mixtures were transferred to 4-mm electroportoration cuvettes (Bio-Rad). Electroporation was performed at room temperature by one 350-V, 975-microfarad pulse using a Gene Pulser apparatus (Bio-Rad). BHK-21 cells were electroportorated as previously described (22). Coupled infection/transfection by vaccinia virus T7/pTM1 system has been described previously (23). Protein synthesis was analyzed by metabolic labeling with 50 μCi of [³⁵S]Met–[³⁵S]Cys/ml (Promix; Amersham Biosciences) for 1 h followed by SDS-PAGE, fluorography, and autoradiography. The integrity of translation initiation factors was analyzed by Western blotting with anti-eIF4GI antisera raised against peptides derived from the N- and C-terminal regions of human eIF4GI at a 1:1000 dilution, rabbit antiseras raised against the N-terminal and C-terminal region of eIF4GII (a gift from N. Sonenberg, McGill University, Montreal, Canada) at a 1:500 dilution, and mouse monoclonal anti-PABP antibody (Abcam) at a 1:250 dilution or anti-eIF4A at a 1:50 dilution (a gift from Dr. H. Trachsel, Institute for Biochemistry and Molecular Biology, University of Berne, Switzerland). Heat shock protein 70 (Hsp70) was detected using rabbit anti-Hsp70 antiseras at a 1:200 dilution (Santa Cruz). Anti-rabbit and anti-mouse immunoglobulin G antibodies coupled to peroxidase (Pierce) were used at 1:10,000 dilution. Percentage of protein synthesis and percentage of intact eIF4G were determined by densitometric scanning of the corresponding protein band.

Analysis of mRNA by Real-time RT-PCR—β-Actin, Luc, and Hsp70 mRNA levels in transfected HeLa cells were determined by real-time quantitative reverse transcription RT-PCR. Total RNA was extracted from 2 × 10⁶ cells at the times indicated in each figure using the RNeasy commercial kit (Qiagen) according to the manufacturer’s recommendations (22). Analysis of the actin mRNA level was performed using the Hs99999903-m1 assay, whereas the Hsp70 mRNA level was estimated with the Hs00359163-s1 assay. As a control, 18 S rRNA was measured using the Hs9999901-m1 assay (Applied Biosystems). In the case of Luc mRNA, primers and probe were designed and provided by Applied Biosystems. The amount of the different mRNAs was determined by taking into consideration the 18 S rRNA levels. RT-PCR was carried out in 20 μl of reaction mixture containing 0.9 μM concentrations of each primer and 0.25 μM TaqMan probe. Reverse transcription was performed at 25 °C for 10 min and 37 °C for 2 h. Afterward, PCR amplification was started by incubation at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min using the ABI PRISM 7000 (Applied Biosystems). Data analysis was carried out using the SDS-7000 software (Version 1.1). The corresponding figures were plotted with the 95% confidence intervals from three independent experiments as error bars.

Measurement of Luc Activity—HeLa X1/5 and HeLa cells electroporated with the in vitro synthesized Luc mRNAs were lysed in a buffer containing 0.5% Triton X-100, 25 mM glycylglycine (pH 7.8), and 1 mM dithiothreitol at different post-electroporation times. Luc activity was determined using a Monolight 2010 apparatus (Analytical Luminescence Laboratory) as described previously (23). S.D. determined from three independent experiments are indicated as error bars in each figure.

RESULTS

Transfection of HeLa Cells with mRNAs Containing the PV 2A Sequence—We initially constructed a plasmid that encoded for the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) internal ribosome entry site (IRES) followed by the PV 2Apro sequence (pTM1–2A) (21). In vitro transcription from this plasmid leads to the synthesis of EMC-2A mRNA. Electroporation of 9 μg of this mRNA was sufficient to drastically inhibit translation in HeLa cells accompanied by hydrolysis of eIF4G (Fig. 1, A and B). The cleavage products were similar to those found after transfection of cells with pTM1–2A plasmid and infection with recombinant vaccinia virus T7 but differed from the eIF4G peptides produced by caspase-3 activity in apoptotic cells (data not shown). Notably, PABP remained intact (Fig. 1C), whereas potent hydrolysis was found using this antibody in cells trans-
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FIGURE 1. Expression of PV 2A<sup>pro</sup> in HeLa cells on transfection of EMC-2A mRNA. HeLa cells were electroporated with 9 μg of EMC-2A, EMC-2C, EMC-2AM2, EMC-2AM15 mRNAs or transcription buffer. Proteins were labeled from 7 to 8 hpe and processed as described under "Materials and Methods." A, analysis of protein synthesis. B, Western blotting against eIF4GI (upper panel) and eIF4GII (lower panel). C, Western blotting against eIF4A (upper panel) and PABP (lower panel). Ac, actin; N-t, N-terminal fragments of eIF4GI or eIF4GII; C-t, C-terminal fragments of eIF4GI or eIF4GII. Mr, molecular weight markers.

Differential Cleavage of eIF4GI and eIF4GII remained intact at 8 h post-electroporation (hpe) (Fig. 1, A and B). These data suggest that the inhibition of host mRNA translation and the modifications of eIF4G are, indeed, due to the synthesis of PV 2A<sup>pro</sup> and not to competition from the transfected mRNAs.

Transfection of HeLa X1/5 Cells with EMC-2A; Effect on Translation of Preexisting and de Novo-synthesized mRNAs—Previous observations have shown that eIF4GI is preferentially hydrolyzed as compared with eIF4GII in PV-infected HeLa cells (14). Our aim was to establish conditions under which these two isoforms were differentially cleaved in the absence of PABP hydrolysis in culture cells. We wanted to analyze the effect of differential eIF4GI and eIF4GII hydrolysis on the initiation of translation of de novo synthesized mRNAs as compared with the translation of mRNAs already engaged in the protein synthesis machinery. For this, we used Luc inducible HeLa X1/5 cells (19). These cells were electroporated with different amounts of in vitro transcribed EMC-2A mRNA: 1, 2, 4, or 9 μg. As controls, cells were electroporated with transcription buffer or with 9 μg of EMC-2C mRNA. During electroporation, tetracycline was removed from the culture medium to trigger Luc mRNA synthesis. Host protein synthesis, Luc expression, integrity of initiation factors, and mRNA levels were analyzed at 4, 6, and 8 hpe (Fig. 2 and supplemental Fig. 10). The 2A<sup>pro</sup> expression induced a time- and dose-dependent inhibition of host protein synthesis (Fig. 2A). To ensure that similar amounts of proteins were loaded in each lane of the gel, the level of eIF4A was also estimated (Fig. 2B). eIF4GI and eIF4GII were proteolized in a time- and dose-dependent manner. As expected, eIF4GII was cleaved by 2A<sup>pro</sup> with delayed kinetics as compared with eIF4GII hydrolysis (Fig. 2B and see Fig. 6C) (14). Interestingly, PABP remained intact during this assay (Fig. 2C). Significant hydrolysis of eIF4GI accompanied by partial cleavage of eIF4GII had only a limited effect on host translation of preexisting mRNAs (Fig. 2, A and B, second and eighth lanes). However, extensive inhibition of ongoing protein synthesis was observed when both eIF4G isoforms were hydrolyzed by 2A<sup>pro</sup> (Fig. 2, A and B). Protein synthesis was not shut off by a reduction in the total amount of preexisting mRNAs since significant differences in the level of actin mRNA were not observed in these cells (supplemental Fig. 10). These findings indicate that a strong abrogation of translation of preexisting mRNAs takes place when 2A<sup>pro</sup> extensively cleaves both eIF4GI and eIF4GII. Moreover, PABP degradation is not essential for this inhibition to occur.

Luc mRNA translation was measured to analyze the effect on translation of de novo synthesized mRNAs when eIF4GI and eIF4GII are differentially hydrolyzed. Synthesis of Luc mRNAs was not significantly affected by the expression of PV 2A<sup>pro</sup> (supplemental Fig. 10) (17). Notably, 1 μg of EMC-2A mRNA sufficed to abrogate Luc mRNA translation (Fig. 2D). This amount of EMC-2A mRNA induced efficient cleavage of eIF4GI but only partially hydrolyzed eIF4GII (Fig. 2B, second, seventh, and fourteenth lanes). These results suggest that translation of de novo synthesized mRNAs is more affected by eIF4GI proteolysis than mRNAs engaged in the translation machinery. Because the amounts of actin and Luc mRNAs were similar in transfected cells regardless of the EMC-2A dose.
employed (supplemental Fig. 10), the inhibition of actin or Luc synthesis could not be ascribed to a decrease in the level of these mRNAs.

Translation of de novo synthesized Luc mRNA was more dependent on eIF4GI integrity than preexisting actin mRNAs. To determine whether eIF4G is required to translate preexisting Luc mRNA, tetracycline was removed from the culture medium 4 h before electroporation. Thus, Luc mRNA started to be synthesized before transfection of EMC-2A mRNA. Cells were then electroporated with 0.1, 0.5, 1, 3, and 9 μg of EMC-2A, and actinomycin D (ActD), an inhibitor of transcription (26), was immediately added at a final concentration of 0.5 μg/ml. Luciferase activity and eIF4G integrity were measured at 0 and 4 hpe in ActD-treated as well as untreated cells (Fig. 3A). eIF4GI was significantly hydrolyzed at 0.1 μg of EMC-2A mRNA. However, eIF4GI was only proteolyzed in cells electroporated with 9 μg of EMC-2A mRNA. Partial hydrolysis of eIF4GI with partial cleavage of eIF4GII was induced by electroporation of HeLa cells with 1 μg of EMC-2A mRNA, whereas total proteolysis of both isoforms of eIF4G was achieved in cells electroporated with 9 μg of this mRNA. Cleavage of PABP did not occur in either case (data not shown).

Luc activity increased up to 6 hpe in cells electroporated with cap-Luc in the absence of EMC-2A mRNA. Partial inhibition of Luc activity was detected in cells co-electroporated with 1 μg of EMC-2A, whereas in cells co-electroporated with a higher
Differential Cleavage of eIF4GI and eIF4GII

**A**

Effect of eIF4GI hydrolysis on translation of preexisting Luc mRNA. X1/5 HeLa cells were washed twice with phosphate-buffered saline, and then culture medium was added. 4 h after Luc induction cells were electroporated with 0.1, 0.5, 1, 3, and 9 μg of EMC-2A. Before electroporation cells were treated with 0.5 μg/ml ActD or left untreated. Luciferase activity and integrity of initiation factor were analyzed at 0 and 4 hpe. A, relative representation of luciferase activity from ActD treated or untreated cells at 4 hpe compared with control cells. B, comparative representation of the level of intact eIF4GI and eIF4GII.

**B**

Translation of an IRES-containing mRNA under Conditions of eIF4G Cleavage—The mRNAs of picornavirus are uncapped and polyadenylated. The 5′-untranslated region of these mRNAs allows recruitment of the 40 S ribosomal subunit under conditions in which the eIF4F complex is disrupted by viral protein activity (2). Translation of picornavirus IRES-containing mRNAs is enhanced by the presence of a poly(A) tail at its 3′ end (27, 28). EMCV IRES has been classified as type II on the basis of its primary sequence and secondary structure conservation and its requirements for optimal internal initiation in vitro (29). We, therefore, used the IRES of EMCV to investigate the effect of differential cleavage of eIF4GI and eIF4GII in IRES-driven translation in transfected cells.

Thus, two mRNAs were obtained by in vitro transcription from the pTM1-Luc plasmid. One mRNA contained the 5′-untranslated region of EMCV followed by the Luc gene (EMC-Luc), whereas in the other mRNA, the poly(A) tail was added by in vitro polyadenylation (EMC-Luc-poly(A)) (Fig. 5A). HeLa cells were co-electroporated with 9 μg of each EMCV IRES-containing mRNA together with 1 or 9 μg of EMC-2A or transcription buffer as a control. The Luc activity and the integrity of initiation factors were analyzed at 4, 6, and 8 hpe. The PV 2Apro expression system described above is highly reproducible. Thus, 1 μg of EMC-2A mRNA brought about significant cleavage of eIF4GI, whereas eIF4GII remained largely intact. However, electroporation of 9 μg of this mRNA led to total cleavage of both eIF4G isoforms (data not shown). Most of the Luc synthesized from EMC-Luc was produced before 4 hpe. After this point only a slight increase in Luc activity was achieved (Fig. 5B). The same effect was found with this mRNA when it was co-transfected with a low dose of EMC-2A. However, powerful stimulation of the Luc activity was observed when a high dose of EMC-2A was employed (Fig. 5B). Translation of this mRNA was enhanced at all times compared with control cells. These data suggest that significant hydrolysis of eIF4GI and eIF4GII is required to induce total transactivation of EMCV IRES-driven translation in culture cells. To our knowledge these findings provide the first evidence in culture cells that extensive cleavage of both isoforms of eIF4G is necessary for full stimulation of EMCV IRES-driven translation.

Synthesis of Luc from EMC-Luc-poly(A) was significantly stimulated compared with its counterpart lacking the poly(A) tail (~7-fold at 8 hpe) (Fig. 5B). Only slight enhancement was observed when this mRNA was co-expressed with 2Apro regardless of the EMC-2A dose employed (Fig. 5B). Therefore, EMC-Luc is more extensively transactivated by 2Apro activity than EMC-Luc-poly(A). Notably, the differences in the Luc activity obtained from EMC-Luc and EMC-Luc-poly(A) disappeared when a high dose of EMC-2A was used (Fig. 5B).

**Synthesis of Heat Shock Proteins; Effect of the Differential Cleavage of eIF4GI and eIF4GII**—Several host mRNAs can be translated by a cap-independent mechanism. This is the case...
for Hsp70 mRNA (17), which may contain an IRES element at its 5'-untranslated region (30). Alternatively, it may be translated by a "shunting mechanism" under heat shock conditions (31). We, therefore, considered it of interest to assay the action of the differential cleavage of both eIF4G isoforms in the initiation of translation of Hsp70 mRNA. HeLa cells were electroporated with 1, 2, 4, or 9 μg of EMC-2A mRNA. As controls, cells were transfected with 9 μg of EMC-2C mRNA or with transcription buffer. At 5 hpe cells were incubated at 42 °C for 3 h to trigger the heat shock response. Host protein synthesis and the integrity of the different initiation factors were analyzed at 4 and 8 hpe (3 h post-heat shock). As shown in Fig. 2, the repression of host protein synthesis and proteolysis of both eIF4G isoforms took place in a dose- and time-dependent manner (Fig. 6, A and B). Once again, significant hydrolysis of eIF4GI without eIF4GII cleavage had only a slight effect on cellular protein synthesis. Notably, a strong shut off of host translation was observed that correlated with eIF4GII hydrolysis (Fig. 6, A and B). At 3 h post-heat shock, Hsp70 synthesis was detected (Fig. 6A). De novo synthesized Hsp70 mRNA can be translated at a level similar to that observed in control cells (Fig. 6, A and B). However, a decrease in Hsp70 synthesis was detected coinciding with an increase in the dose of EMC-2A (Fig. 6, A and C). When eIF4GI and eIF4GII were significantly cleaved by PV 2Apro, Hsp70 synthesis was about 35–25%, whereas endogenous mRNAs were strongly inhibited (about 10% of actin synthesis) (Fig. 6, A and B). The amount of Hsp70 mRNA was analyzed by RT-PCR. At 3 h post-heat shock, the amount of this mRNA decreased in a dose-dependent manner in transfected cells (supplemental Fig. 12). A decrease in the amount of Hsp70 mRNA had been observed previously when hybrid proteins that contain PV 2Apro were introduced in HeLa cells (17). Nevertheless, the electroporation of 9 μg of EMC-2A induced a 30–40% decrease in the amount of Hsp70 mRNA, whereas the synthesis of the heat shock protein was greatly inhibited (65–75%). Therefore, blockade of the initiation of Hsp70 mRNA translation was not entirely due to decreased mRNA concentrations. These findings reveal that the first initiation event in some host mRNAs might be more susceptible to eIF4GII proteolysis than to eIF4GI degradation. These results taken together with the findings reported earlier suggest that eIF4GI and eIF4GII may have differential roles in the translation of different cellular mRNAs.

Two PV 2Apro variants, D135N (M6) and V119M (M7), that failed to repress host transcription but maintained their capacity to proteolyze eIF4GI, have been described (21). Because
Hsp70 mRNA transcription was partially inhibited by 2A<sup>pro</sup> expression, mRNAs that encode these two 2A<sup>pro</sup> mutants were transfected to analyze their effect on Hsp70 synthesis. Transfection of high doses of these mRNAs largely hydrolyzes both eIF4G isofoms leading to blockade of actin as well as Hsp70 synthesis. Analysis of the Hsp70 mRNA by real-time RT-PCR revealed that 2A<sup>pro</sup> M6 and M7 failed to repress host transcription (supplemental Fig. 13). Thus, the inhibition of Hsp70 synthesis in 2A<sup>pro</sup> M6- and M7-expressing cells is not only due to the blockade of transcription but also to their direct effect on translation.

Ongoing mRNA Translation and Hsp70 Synthesis in BHK Cells Transfected with EMC-2A—To analyze the differential cleavage of the two isoforms of eIF4G by 2A<sup>pro</sup> in another cell line, BHK cells were electroporated with 1, 2, 4, 6, 9, or 18 μg of EMC-2A. At 5 hpe, cells were incubated at 42 °C for 3 h to trigger the heat shock response. Host protein synthesis and integrity of translation initiation factors were analyzed at 8 hpe in cells incubated at 37 °C and in cells subjected to heat shock for 3 h (8 hpe and 3 h post-heat shock). In both cases a gradual dose-dependent inhibition of protein synthesis was achieved (Fig. 6A). eIF4GI and eIF4GII were also proteolyzed in a dose-dependent manner, whereas PABP remained intact in each case (Fig. 7, B and C). Previous analyses of eIF4G using specific antibodies have revealed the existence of two proteins of ∼220 and ∼150 kDa, respectively, in BHK cells (22). As described earlier, eIF4G exhibits different mobility patterns in SDS-PAGE of mammalian cells, possibly due to post-translational modifications (32). Alternatively, one of the proteins could be a breakdown product of eIF4G. Both 220- and 150-kDa polypeptides disappeared in 2A<sup>pro</sup>-expressing cells. Only the C-terminal proteolytic fragment could be detected with anti-eIF4G antibodies (22). Surprisingly, proteolysis kinetics of eIF4GII was delayed with respect to eIF4GII in BHK cells (Fig. 7B).

Hsp70 synthesis was also analyzed in BHK cells. The heat shock treatment was carried out at 5 hpe. Thus, Hsp70 mRNA synthesis started when eIF4GI and eIF4GII were already engaged in translation.

Cytoplasmic Initiation of Translation in EMC-2A-transfected HeLa and BHK Cells—To determine the involvement of eIF4GII in the first translation event of preexisting mRNAs, polysome run-off was induced both in HeLa and BHK cells (Fig. 8). Cells were incubated with hypertonic medium to inhibit the initiation of translation, although elongation synthesis in 2A<sup>pro</sup> M6- and M7-expressing cells is not only due to the blockade of transcription but also to their direct effect on translation.

Ongoing mRNA Translation and Hsp70 Synthesis in BHK Cells Transfected with EMC-2A—To analyze the differential cleavage of the two isoforms of eIF4G by 2A<sup>pro</sup> in another cell line, BHK cells were electroporated with 1, 2, 4, 6, 9, or 18 μg of EMC-2A. At 5 hpe, cells were incubated at 42 °C for 3 h to trigger the heat shock response. Host protein synthesis and integrity of translation initiation factors were analyzed at 8 hpe in cells incubated at 37 °C and in cells subjected to heat shock for 3 h (8 hpe and 3 h post-heat shock). In both cases a gradual dose-dependent inhibition of protein synthesis was achieved (Fig. 6A). eIF4GI and eIF4GII were also proteolyzed in a dose-dependent manner, whereas PABP remained intact in each case (Fig. 7, B and C). Previous analyses of eIF4G using specific antibodies have revealed the existence of two proteins of ∼220 and ∼150 kDa, respectively, in BHK cells (22). As described earlier, eIF4G exhibits different mobility patterns in SDS-PAGE of mammalian cells, possibly due to post-translational modifications (32). Alternatively, one of the proteins could be a breakdown product of eIF4G. Both 220- and 150-kDa polypeptides disappeared in 2A<sup>pro</sup>-expressing cells. Only the C-terminal proteolytic fragment could be detected with anti-eIF4G antibodies (22). Surprisingly, proteolysis kinetics of eIF4GII was delayed with respect to eIF4GII in BHK cells (Fig. 7B). eIF4GII was extensively proteolyzed at 8 hpe using 18 μg of EMC-2A, whereas under these conditions 40–50% of the eIF4GI remained intact (Fig. 7B). Notably, the inhibition of ongoing protein synthesis correlated well with the proteolysis of eIF4GII (Fig. 7, A, B, and D). Under conditions where 75–85% of eIF4GII was cleaved by 2A<sup>pro</sup> and about 100–60% of eIF4GI remained intact, host translation dropped to nearly 35% (Fig. 7, A and B, sixth and thirteenth lanes). These results provide further evidence for the essential role that eIF4GII plays in the translation of mRNAs already engaged in translation.

Translation of Hsp70 mRNA was also analyzed in BHK cells. The heat shock treatment was carried out at 5 hpe. Thus, Hsp70 mRNA synthesis started when eIF4GI and eIF4GII were already hydrolyzed (data not shown). Synthesis of Hsp70 was detected at 3 h post-heat shock treatment. Hsp70 synthesis was also inhibited in a dose-dependent manner (Fig. 7A). Interestingly, significant inhibition of the initiation of translation of Hsp70 mRNA (about 60–65%) was observed when 6 μg of EMC-2A was used. In these cells eIF4GII was fully cleaved, although all eIF4GI remained intact (Fig. 7, A and B, twelfth lane). These results were reproduced by Western blotting with an antibody against Hsp70 (data not shown). Notably, inhibition of Hsp70 and actin synthesis correlated well with the proteolysis of eIF4GII in this cell line (Fig. 7D).
still takes place. This treatment provokes polysome run-off, whereas a return to normal medium leads to initiation of translation on ribosome-stripped mRNA (22, 17). HeLa or BHK cells were electroporated with the EMC-2A at the doses indicated (Fig. 8). At 8 hpe the culture medium was supplemented with 150 mM NaCl for 2 h, giving rise to polysome run-off. At 10 hpe, normal ionic conditions were restored to determine the first translation initiation event on preexisting cytoplasmic mRNAs. Protein synthesis, eIF4GI and eIF4GII integrity (Fig. 8), and the amount of eIF4A (data not shown) were determined at 8, 10, and 12 hpe.

Potent proteolysis of eIF4GI was achieved in HeLa cells transfected with 1 and 3 μg of EMC-2A. Under these conditions the level of protein synthesis was similar to control cells (Fig. 8A). It is noteworthy that a high dose of EMC-2A induced strong inhibition of ongoing translation concomitantly with eIF4GI and eIF4GII hydrolysis. When cells were incubated with hypertonic medium, translation was abrogated, indicating that polysome run-off took place (Fig. 8A). Notably, protein synthesis was recovered in control cells and cells electroporated with low doses of EMC-2A after restoring normal conditions, even though eIF4GII was cleaved in the latter case. Only cells transfected with high doses of EMC-2A did not recover translation (Fig. 8A). These data suggest that integrity of eIF4GII is not essential for cytoplasmic initiation of translation to occur.

The inhibition of endogenous translation in BHK-cells correlated with eIF4GII inactivation at 8 hpe (Fig. 8B). Treatment of BHK cells with hypertonic medium blocked translation in all cases irrespective of eIF4GII integrity. When normal conditions were restored, preexisting mRNAs were engaged in translation both in control cells and BHK cells transfected with a low dose of EMC-2A in which eIF4G remained intact. However, a significant inhibition of initiation of translation was observed in cells electroporated with 9 or 18 μg of EMC-2A. In cells transfected with 9 μg of mRNA, eIF4GII was hydrolyzed, with about 90% of the eIF4GI remaining intact. These results indicate that eIF4GII is essential for the first events of initiation on cytoplasmic mRNAs.

**DISCUSSION**

A wide variety of animal viruses such as several picornaviruses, retroviruses, and caliciviruses bring about the cleavage of initiation factors in infected cells to modulate host and viral translation. In this respect, eIF4GI, eIF4GII, and PABP are some of the most common cellular targets for viral proteases (2, 9). Nevertheless, the particular contribution of hydrolysis of each of these initiation factors to the inhibition of host protein synthesis is not yet well established. The method of PV 2Apro expression described here differentially cleaves eIF4G and eIF4GII, leaving PABP intact. Such a system can help elucidate...
HeLa cell lines that can be induced to express PV 2Apro, direct and reliable than other methods previously described, such as eIF4GII cleavage. This method is much more efficient, rapid, and reproducible and led to efficient cleavage of eIF4G very during the initiation of translation. This assay is easy to perform the exact role played by cleavage of these two isoforms of eIF4G during the initiation of translation. This assay is easy to perform and reproducible and led to efficient cleavage of eIF4G very soon after transfection of the majority of culture cells. Moreover, the proportion of the two eIF4G isoforms cleaved varied according to dose of EMC-2A mRNA transfected. Interestingly, the kinetics of eIF4G cleavage in BHK cells is delayed compared with HeLa cells. Thus, hydrolysis of eIF4GII occurs after eIF4GII cleavage. This method is much more efficient, rapid, and reliable than other methods previously described, such as HeLa cell lines that can be induced to express PV 2Apro, direct penetration of hybrid proteins that contain this protease, or coupled infection with recombinant vaccinia virus T7 and transfection with pTM1–2A (20, 17, 21).

The different kinetics of cleavage of the two isoforms of eIF4G observed in HeLa and BHK cells can be accounted for by the differences in the primary structure of the corresponding initiation factors. Comparison of the amino acid sequence of human and mouse eIF4G (since the sequence of hamster eIF4G is not available) reveals several changes in the amino acid residues around the cleavage site of 2Apro (Thr by Ser at P2, Thr by Ala at P5, and Thr by Pro at P6) (Berger and Schechter notation) (44). Variations at the P2 position of the cleavage site recognized by 2Apro are very restrictive for trans substrate proteolysis (33). On the other hand, the proteolysis site of human eIF4GII recognized by 2Apro has not yet been identified. In this regard, the rhinovirus 2Apro cleavage site (another Enterovirus) is well conserved in mouse eIF4GII (34). These data may account for the different kinetics of eIF4GII hydrolysis observed in the two cell lines and the similar susceptibility of eIF4GII to PV 2Apro expression.

Translation of Luc mRNA on induction of the HeLa cell line X1/5 is strongly inhibited by transfection of EMC-2A mRNA. This blockade correlated well with eIF4GII inactivation despite the fact that eIF4GII remained largely intact. The Luc mRNA synthesized in HeLa X1/5 cells is capped and polyadenylated by the host enzymes and contains a leader sequence typical of most cellular mRNAs (17). These data reveal that eIF4GII could participate in the recognition of newly synthesized cellular mRNAs. eIF4GII not only interacts with the cytoplasmic translation initiation complex (known as the steady-state complex) but it is also present in the pioneer translation initiation complex in the nucleus, bound to the nuclear cap binding proteins CBP80 and CBP20, and it is associated with pre-mRNAs (Fig. 9) (35–38). Hydrolysis of eIF4GII by HIV-2 PR or 2Apro blocks steady-state translation as well as the pioneer round of protein synthesis on virgin mRNAs (37). Nevertheless, HIV-2 PR also cleaves PABP, whereas eIF4GII is substrate for 2Apro (24, 14). Thus, in the present work we found that single hydrolysis of eIF4GII did not inhibit the first initiation event after polysome run-off. These data suggest that hydrolysis of eIF4GII could inhibit the pioneer translation initiation complex for translation of mRNAs transported from the nucleus, whereas this isoform is not essential for the steady-state complex (Fig. 9).

Previous findings indicated that translation of mRNAs already engaged in the protein-synthesis machinery is resistant to eIF4GII cleavage (11–13, 39). Earlier reports showed a good correlation between eIF4GII hydrolysis and the shut-off of host protein synthesis (14–16). As shown in this work, individual expression of PV 2Apro hydrolyzes eIF4GII with delayed kinetics as compared with eIF4GII in HeLa cells. In this cell line, the decrease in ongoing protein synthesis coincides with eIF4GII cleavage. Moreover, translation of a capped and polyadenylated Luc mRNA previously associated with polysomes was fully blocked when both initiation factor isoforms were proteolysed, whereas the effect was partial when only eIF4GII was cleaved in Luc mRNA transfected as well as ActD-treated HeLa cells. These observations are further reinforced in BHK cells, where eIF4GII was hydrolyzed by 2Apro more rapidly than eIF4GII. Therefore, in both HeLa and BHK cells, the inhibition of trans-

**FIGURE 8.** Effect of eIF4GII and eIF4GII cleavage on the initiation of translation after exposure to hypertonic medium. HeLa and BHK cells were electroporated with 0.5, 1, 3, and 9 or 1, 9, and 18 μg of EMC-2A, respectively. In both cases transcription buffer and high dose of EMC-2C were used as controls. At 8 hpe, 150 mM NaCl was added, giving rise to a final concentration of 300 mM in the culture medium. These conditions were maintained for 2 h. At 10 hpe, hypertonic medium was removed, and the cells were washed twice with phosphate-buffered saline. Normal medium was then restored. Protein synthesis and the integrity of initiation factors were analyzed at 8, 10, and 12 hpe. A, hypertonic treatment in HeLa cells. Shown is an analysis of protein synthesis by SDS-PAGE followed by fluorography and autoradiography (upper panel) as well as Western blotting against eIF4GII (middle panel) and eIF4GII (lower panel). Nt, N-terminal fragments of eIF4GII or eIF4GII; Ct, C-terminal fragments of eIF4GII or eIF4GII. B, hypertonic treatment in BHK cells. Shown is an analysis of protein synthesis by SDS-PAGE followed by fluorography and autoradiography (upper panel) as well as Western blotting against eIF4GII (middle panel) and eIF4GII (lower panel).
PV 2Apro has two opposite effects on gene expression. On the one hand, this protease abrogates host protein synthesis, but it can also stimulate PV translation (20, 2, 9). The findings described in the present work certainly support these opposing effects, since there is a correlation between eIF4G hydrolysis and the inhibition of cellular protein synthesis, whereas translation driven by EMCV IRES element is stimulated (high EMC-2A dose) or at least is not affected (low EMC-2A dose) under these conditions.

As observed, a mRNA containing EMCV IRES and poly(A) tail is more competitive for the translational machinery than a mRNA containing just EMCV IRES. Cleavage of eIF4G by PV 2Apro separates its PABP binding domain, abolishing the stimulation provided by poly(A) tail (41, 27). This behavior was also observed for polyadenylated picornavirus IRES-containing mRNAs (27, 28). When eIF4G is hydrolyzed, the EMC-Luc and EMC-Luc-Poly(A) mRNAs exhibit a similar translatability since poly(A) tail does not contribute to mRNA translation. Therefore, translation of EMC-Luc-poly(A) mRNA is enhanced in control cells by EMCV IRES and poly(A) tail cooperation. However, when eIF4G and eIF4GII are hydrolyzed by PV 2Apro, EMC-Luc is strongly benefited from the reduction in competition of host mRNAs for the translation machinery equalizing its translatability to EMC-Luc-Poly(A). In this regard, Paip-2 and rotavirus NSP3, two inhibitors of poly(A) tail-dependent stimulation (42, 43), strongly blocked the translation of polyadenylated EMCV IRES-containing mRNA in vitro, whereas translation was not affected in the presence of rhinovirus 2Apro (27, 28). These data suggest that poly(A) tail does not contribute to translation initiation when eIF4G is proteolyzed.

Another mRNA classified as IRES-containing is Hsp70 mRNA (30). The dependence of translation of this mRNA on some initiation factors is low, and translation was significant when eIF4G was cleaved by 2Apro (17). In good agreement with these data, our present findings suggest that the initiation of translation of de novo synthesized Hsp70 mRNA is not dependent on eIF4GII integrity. However, an increase in the dose of EMC-2A induced a partial reduction in the Hsp70 mRNA level, although this decrease did not fully account for inhibition of the Hsp70 synthesis. Strikingly, a gradual block of the translation of

![Diagram of the differential mechanism of action of eIF4G and PABP](image-url)
Differential Cleavage of eIF4GI and eIF4GII

Hsp70 mRNA occurs at the same time as eIF4GII cleavage. Moreover, high doses of EMC-2AM6 and EMC-2AM7 also induced the cleavage of the two isoforms of eIF4G and strongly inhibited Hsp70 and actin synthesis, although host transcription was only slightly affected. These findings could support the view that eIF4GII has a differential participation in the initiation of translation of different cellular mRNAs.

Therefore, the effect of eIF4GI and/or eIF4GII hydrolysis on translation varies according to the mRNA analyzed. The use of different viral proteases that cleave the two isoforms of eIF4G and PABP in a differential manner may help to elucidate the exact role that each of these factors plays in the translation of cellular and viral mRNAs.

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