Cleavage of p220 by Purified Poliovirus 2A^{pro} in Cell-Free Systems: Effects on Translation of Capped and Uncapped mRNAs†

Isabel Novoa,‡ Francisco Martínez-Abarca,‡ Purificación Fortes,§,|| Juan Ortín,|| and Luis Carrasco*,‡

Centro de Biología Molecular, UAM-CSIC, y Centro Nacional de Biotecnología, CSIC, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

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ABSTRACT: Poliovirus protease 2A^{pro} has been obtained in soluble form as a fusion protein with maltose binding protein (MBP). Addition of MBP-2A^{pro} to rabbit reticulocyte cell-free systems gives rise to efficient cleavage of the initiation factor of translation p220 (eIF-4G). Translation of capped mRNA encoding the influenza virus NP protein is severely impaired in lysates in which p220 has been proteolytically cleaved. This inhibition is dependent on the concentration of mRNA added to the lysate. Thus, increasing the concentrations of mRNA substantially overcomes the blockade of NP synthesis after p220 cleavage. Notably, translation of uncapped NP mRNA is also compromised in p220-deficient rabbit reticulocyte lysates, suggesting that p220 participates in the translation of both capped and uncapped NP mRNAs. The effects of p220 proteolysis by poliovirus 2A^{pro} have also been assayed on luciferase mRNA translation. Three types of mRNAs encoding for luciferase have been examined: capped, uncapped, and mRNA bearing the poliovirus 5′ leader region (leader luc mRNA). Synthesis of luciferase directed by any of these mRNAs was inhibited after cleavage of p220 in rabbit reticulocyte lysates. Interestingly, supplementation of the lysate with HeLa cell extracts stimulates leader luc mRNA translation by poliovirus 2A^{pro}. These results indicate that activation of translation of mRNAs bearing the poliovirus leader region promoted by this poliovirus protease requires a factor present in HeLa cell extracts. These findings agree well with recent experiments implicating p220 not only in protein synthesis directed by capped mRNAs but also in the translation of naturally uncapped mRNAs.

The finding that poliovirus 2A^{pro} (Etchison et al., 1984), as well as other picornavirus proteases, efficiently cleaves p220 in infected cells (Krausslich & Wimmer, 1988) led to the proposal that the shutoff of host translation that occurs after poliovirus infection was mediated by the proteolytic cleavage of p220 (Sonenberg, 1990; Carrasco, 1994). This suggestion has not been supported by more recent evidence illustrating that there is no correlation between p220 cleavage and the kinetics or extent of the inhibition of cellular translation (Bonneau & Sonenberg, 1987; Perez & Carrasco, 1992; Lloyd & Bovee, 1993; Irurzun et al., 1995; Novoa et al., 1996). Nevertheless, the fact that p220 is cleaved by several picornavirus proteases (Toyoda et al., 1986) raised the question of the role that p220 plays in cellular gene expression. Few studies have been carried out on the effects of the individual expression of poliovirus 2A^{pro} on cellular translation in cultured cells (Sun & Baltimore, 1989; Davies et al., 1991). To our knowledge, only one study has addressed the question of which step in gene expression is blocked by poliovirus 2A^{pro}, leading to the conclusion that transcription was more affected than translation in the case of chloramphenicol acetyltransferase reporter gene (Davies et al., 1991). We have used a different approach to analyze the role of p220 in gene expression that involves the transient expression of poliovirus 2A triggered by recombinant vaccinia virus infection (Aldabe et al., 1995). Cleavage of p220 in this system leads to a great reduction in vaccinia virus protein synthesis. No inhibition of virus transcription was found nor of the *in vitro* translatability of the mRNAs synthesized, suggesting that p220 cleavage impairs the translation of newly synthesized vaccinia virus mRNA (Feduchi et al., 1995). But still, no studies have been carried out on the impact of p220 cleavage on ongoing cellular mRNA translation. These experiments should mimic the shutoff of protein synthesis by poliovirus.

Despite this paucity of data on the action of p220 cleavage in cellular gene expression, a number of recent publications have investigated the effects of several picornavirus proteases, such as coxsackie 2A^{pro}, rhinovirus 2A^{pro}, and FMDV L proteases on the translation of exogenous mRNAs in cell lysates (Liebig et al., 1991; Borman et al., 1995; Ohlmann et al., 1995; Ziegler et al., 1995). Poliovirus 2A^{pro} has not been analyzed as yet in this type of study, perhaps due to the fact that this protease is synthesized in bacteria in a rather insoluble and unstable form (Konig & Rosenwirth, 1988; Alvey et al., 1991; Martínez-Abarca et al., 1993). Attempts to solubilize poliovirus 2A^{pro} produced a protease with low capacity to cleave p220 in cell-free systems (Martínez-Abarca et al., 1993). The initial results by Liebig et al. (1993) suggested that depletion of p220 after incubation with rhinovirus 2A led to the inhibition of translation of capped globin mRNA in HeLa cell extracts. Moreover, translation driven by the rhinovirus 5′ untranslated region (UTR) was

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* Corresponding author. Fax (34-1) 397-4799.

‡ Centro de Biología Molecular.

§ Present address: EMBL, Meyerhofstr. 1, 6900 Heidelberg, Germany.

|| Centro Nacional de Biotecnología.

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enhanced in the presence of rhinovirus 2A and consequently with p220 degraded (Liebig et al., 1991). These findings were in good agreement with the pioneering finding that poliovirus 2A stimulated translation of reporter mRNAs containing the poliovirus 5′-UTR (Hambidge & Sarnow, 1992). Subsequent analyses of the translation of several capped mRNAs in rabbit reticulocyte lysates incubated with foot-and-mouth disease virus (FMDV) L protease showed moderate inhibition of translation of capped mRNAs, ranging from 30% to 60% inhibition, depending on the mRNA tested (Ohlmann et al., 1995). Protein synthesis directed by several artificially uncapped mRNAs was stimulated by addition of L protease, but curiously this stimulation did not occur when translation was driven by Theiler's murine encephalomyelitis virus internal ribosome entry site (Ohlmann et al., 1995). Even more puzzling are the recent findings of Ziegler et al. (1995), who found no inhibition of translation of capped mRNAs after p220 cleavage by coxsackie $2A^{pro}$ or FMDV L proteases (Ziegler et al., 1995). Additional studies are necessary to assess the effects of picornavirus proteases on translation of capped and uncapped mRNAs not only in the cell-free system but also in the intact cells.

Picornavirus proteases have served not only for a better understanding of p220 in translation but also to map different functional regions within this initiation factor. A single cleavage by poliovirus $2A^{pro}$ in p220 takes place between amino acids Arg486 and Gly487, while FMDV L protease cleaves p220 at multiple positions (Lamphear et al., 1993; Kirchweger et al., 1994). The cleavage of p220 at position 486 releases an N-terminal moiety that interacts with eIF-4E, while the C-terminal moiety of p220 interacts with eIF-4A and eIF-3 (Mader et al., 1995; Lamphear et al., 1995). A model has been advanced where the C-terminal cleavage product of p220, together with eIF-4A, participates in the translation of some picornavirus mRNAs (Lamphear et al., 1995). If this model is correct, neither the N-terminal portion nor eIF-4E would be involved in the initiation of picornavirus protein synthesis. It remains to be established if intact p220 may function in the translation of mRNAs bearing the poliovirus leader region or if p220 cleavage is mandatory for this event to take place. The fact that cardioviruses do not cleave p220 during infection (Krausslich & Wimmer, 1988) and the finding that inhibition of eIF-4F functioning blocks both cardiovirus and poliovirus mRNA translation (Pause et al., 1994) suggest that uncleaved p220 can participate in translation initiation of these mRNAs.

MATERIALS AND METHODS

Construction of Plasmids. Plasmid pKS.2A was constructed using standard polymerase chain reaction (PCR) techniques. Oligonucleotides were designed to hybridize with regions 3386−3403 (italic) (primer 5′-2A.B1/E1) and 3815−3832 (italic) (primer 3′-2A.B1/E1) of poliovirus type 1 cDNA from vector pT7XLD, generously provided by Dr. E. Wimmer (Stony Brook, NY). Primer 5′-2A.B1/E1 has the sequence 5′CCCGGGGATATCATG*GGATTCGGACAC-CAAAAC*; primer 3′-2A.B1/E1 has the sequence 5′GGGC-CCGTCGACTATTA*TTGTTCCATGGCTT CTTC*. The amplified product of 446 bp containing the sequences of poliovirus $2A^{pro}$ was purified using the Gene Clean kit (Bio 101, Inc.), doubly digested by *Eco*RV and *Sal*I endonucleases and finally ligated to pBluescript KS vector (Stratagene) previously incubated with the same enzymes. The region amplified by PCR was sequenced by the dideoxy method (Sequenase, U.S. Biochemical Corp.). The resulting plasmid pKS.2A was then digested with *Eco*RV and *Sal*I and the 446 bp fragment corresponding to poliovirus 2A was ligated to the pMal-c vector (New England Biolabs) doubly digested with *Stu*I and *Sal*I to generate the construct pMal-c.2A.

The luciferase gene from plasmid pDO432 was ligated into the *Bam*HI site of pBluescript KS to obtain plasmid pKS.Luc (Wet et al., 1987). Sense orientation was determined by restriction digestion analyses. Plasmid pKS.L15′ containing the poliovirus 5′ untranslated leader sequence was constructed using standard PCR techniques. Oligonucleotides were designed to hybridize with regions 1−23 (italic) (primer 5′-5′L.E1) and 722−743 (italic) (primer 3′-5′L.E1) of poliovirus type 1 cDNA from vector pT7XLD. Primer 5′-5′L.E1 has the sequence 5′GAATT*CTTAAAA-CAGCTCTGGGGTTGTA*; primer 3′-5′L.E1 has the sequence 5′GAATT*CTATGATACAATTGTCTGATTG*. The amplified product of 746 bp was digested with *Eco*RI and ligated to pBluescript KS vector previously incubated with *Eco*RI. Sense orientation was determined by restriction digestion analyses. Plasmid pKS.Luc was digested with *Spe*I, blunt-ended-and subsequently digested with *Sal*I. The 1.8 kb fragment containing the luciferase gene was finally ligated to pKS.L15′ doubly digested with *Eco*RV and *Sal*I to obtain plasmid pKS.L1.Luc.

The plasmid pGNPpoly-A was obtained by ligation of the *Hpa*I−*Stu*I fragment of plasmid pSVa963 (encoding influenza virus NP gene with a poly-A tail) (Portela et al., 1985) into the *Sma*I site of pGEM3 (Promega). Orientation was determined by restriction digestion analyses.

Purification of the Fusion Proteins (MBP-2A and MBP-β-gal-α). *Escherichia coli* (DH5 strain) cells transformed with either pMal-c2 (New England Biolabs) or pMal-c.2A were grown in LB medium containing 0.2% glucose and 100 μg/mL ampicillin to an optical density (660 nm) of 0.5. IPTG (isopropyl 1-thio-β-D-galactopyranoside) was added to a final concentration of 1 mM. After 2 h at 37 °C the cells were harvested by centrifugation, and the pellet was stored at −20 °C. Cell lysis was carried out by resuspending the bacteria in 10 mM sodium phosphate buffer (pH 7.0), 0.5 M NaCl, 0.25% Tween 20, 1 mM dithiothreitol (DTT), 10 mM EDTA, and 10 mM EGTA (lysis buffer). Lysozyme (Boehringer) was added at a final concentration of 0.5 mg/mL and the samples were incubated on ice for 30 min. The resuspended cells were sonicated at 4 °C in a Soniprep (MSE 7100) (three bursts at 14 μW) and then centrifuged at 9000*g* for 30 min. The supernatant was diluted (1:5) in 10 mM sodium phosphate buffer (pH 7.0), 1 mM DTT, 3 μg/mL DNase I, and 10 mM $MgCl_2$. The diluted supernatant was loaded onto a 10 mL column of amylose resin (New England Biolabs) equilibrated in 10 mM sodium phosphate, 100 mM NaCl, 1 mM EGTA, and 1 mM DTT (column buffer). The column was developed at a flow rate of 1 mL/min, washed with 30 mL of column buffer containing 500 mM NaCl and 0.25% Tween 20, and subsequently washed with 50 mL of column buffer containing 500 mM NaCl, and finally with 50 mL of PBS. Elution was carried out with PBS supplemented with 10 mM maltose, fractions were collected, and protein content was determined (Bio-Rad protein assay). Cleavage with factor Xa (New England Biolabs) was carried out at a 1% (w/w) ratio of factor Xa compared to the amount of MBP-2A purified protein and in the presence of 1 mM $CaCl_2$. The

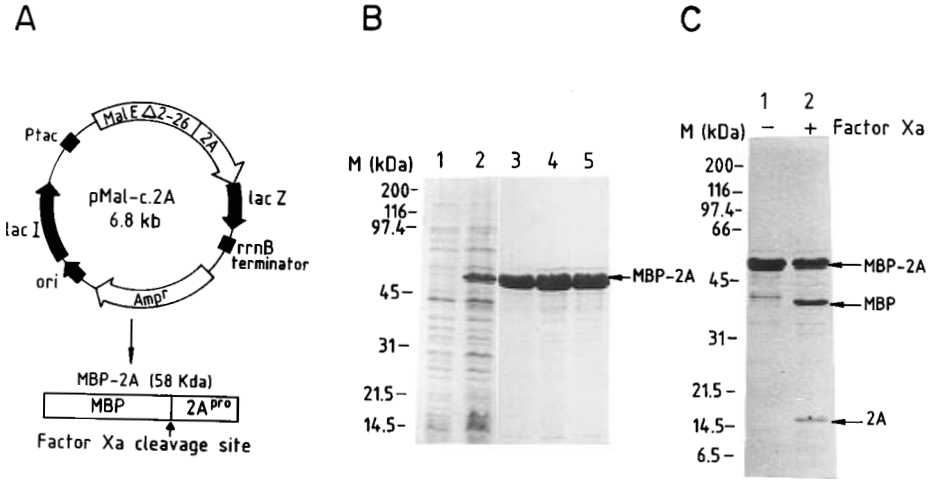

FIGURE 1: Cloning, synthesis, and purification of poliovirus $2A^{pro}$. (Panel A) Schematic representation of the plasmid pMal-c.2A and the fusion protein encoded: maltose binding protein (MBP) fused to poliovirus $2A^{pro}$. (Panel B) Expression and purification of MBP-2A. Lane 1, proteins from uninduced *E. coli* (DH5) cells; lane 2, proteins from cells induced with 1 mM IPTG; lanes 3−5, protein fractions eluted from the amylose resin column. (Panel C) Factor Xa cleavage assay of MBP-2A. Purified protein was incubated in the absence (lane 1) or in the presence (lane 2) of 1% factor Xa for 2 days at room temperature. Samples were separated by SDS−15% PAGE and stained with Coomassie blue. The positions of MBP-2A, MBP, 2A, and molecular size markers are indicated.

reaction mixture was incubated for 2 days at room temperature.

In Vitro Transcription and Translation. Purified plasmids pKS.Luc and pKS.L1.Luc linearized with *Sal*I were incubated with T7 RNA polymerase, in the presence or absence of m7G(5′)ppp(5′)G to yield capped or uncapped mRNA transcripts, respectively (Promega). Purified plasmid pGNP-poly-A was linearized with *Sal*I and transcription was carried out with the Megascript kit (Ambion) in the presence or absence of m7G(5′)ppp(5′)G to yield capped or uncapped NP mRNA transcripts, respectively. These NP transcripts contain a poly(A) tail of about 100 residues. For translation assays, rabbit reticulocyte lysates (Promega) were preincubated either with purified proteins MBP-2A to cleave p220 or with MBP-β-gal-α used as control. Then, translation was carried out in the presence of *in vitro* synthesized transcripts (capped NP, uncapped NP, capped luc, uncapped luc, or leader luc mRNAs) at 30 °C for 1 h 30 min using the conditions described by the supplier (Promega). Where indicated, HeLa cell extracts (Molla et al., 1991) were added to the rabbit reticulocyte assay. The reaction was stopped by adding 2 volumes of 2× sample buffer (Martínez-Abarca et al., 1993). [^{35}S]Methionine-labeled translation products were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). The integrity of the p220 component of eIF-4F in the translation assays was analyzed by Western blotting (Novoa et al., 1994; Aldabe et al., 1995). Aliquots of lysates containing equivalent amounts of protein were separated by SDS−7.5% PAGE gels. Proteins were transferred overnight to a nitrocellulose membrane (Transblot transfer medium, Bio-Rad) at 200 mA in a transfer buffer [25 mM Tris-HCl (pH 8.3), 90 mM glycine, 20% methanol, and 0.1% SDS). After the nitrocellulose membrane was blocked with 5% nonfat dry milk in PBS buffer and incubated with anti-human p220 rabbit polyclonal antibodies, the specific bands were visualized with peroxidase-coupled secondary antibodies (Pierce) and enhanced chemiluminiscence (ECL, Amersham) (Aldabe et al., 1995). Where indicated, aliquots of 4 μL of translation assays were removed and luciferase activity was assayed as described (Wet et al., 1987; Ventoso & Carrasco, 1995).

RESULTS

Purification and in Vitro Activity of Fusion Protein MBP-$2A^{pro}$. Initial attempts from our laboratory to obtain poliovirus $2A^{pro}$ in large quantities led to the purification of this protease from *E. coli* cells in an insoluble form that could only be solubilized in the presence of 6 M urea (Martínez-Abarca et al., 1993). In addition, the urea-treated protease cleaved p220 in lysates very inefficiently (Martínez-Abarca et al., 1993). To obtain soluble poliovirus $2A^{pro}$, a fusion protein between maltose binding protein (MBP) and $2A^{pro}$ was engineered. To this end, *E. coli* clones bearing pMal-c.2A plasmid were isolated (Figure 1A). These clones express a major 58 kDa polypeptide band upon induction with IPTG (Figure 1B). Purification of total cell extracts with amylose columns renders the purified MBP-$2A^{pro}$ fusion protein, which was soluble without the further use of detergents (Figure 1B). This protein can be cleaved to MBP and $2A^{pro}$ by incubation with factor Xa, which recognizes and cleaves just after the sequence IEGR located at the C-terminus of MBP (Rodríguez & Carrasco, 1995). Cleavage of MBP-$2A^{pro}$ generates two proteins, MBP and poliovirus $2A^{pro}$ that migrates similarly to the poliovirus protease from infected HeLa cells (Figure 1C).

To assess the activity of the fusion protein on p220 cleavage, MBP-$2A^{pro}$ was incubated with a rabbit reticulocyte lysate and the proteins were separated by SDS−PAGE and immunoblotted with an antibody against human p220 (Aldabe et al., 1995). Extensive cleavage of p220 was shown by the disappearance of intact p220 and the generation of a 110 kDa protein band (Figure 2). This protein corresponds to the C-terminal region of rabbit reticulocyte p220, since the N-terminal moiety of rabbit p220 (but not human p220) is not recognized by our antibodies (Aldabe et al., 1995). Incubation of the lysate with as little as 5 μg of MBP-$2A^{pro}$ for 180 min leads to extensive degradation of p220 that is virtually complete when 10 μg of MBP-$2A^{pro}$ is employed, as judged by the disappearance of intact p220 (Figure 2A). The kinetics of p220 cleavage by 4 μg of MBP-$2A^{pro}$ shows about 50% cleavage after 60 min of incubation and extensive disappearance of intact p220 after 150 min (Figure 2B). To assure an extensive cleavage of p220 before addition of the

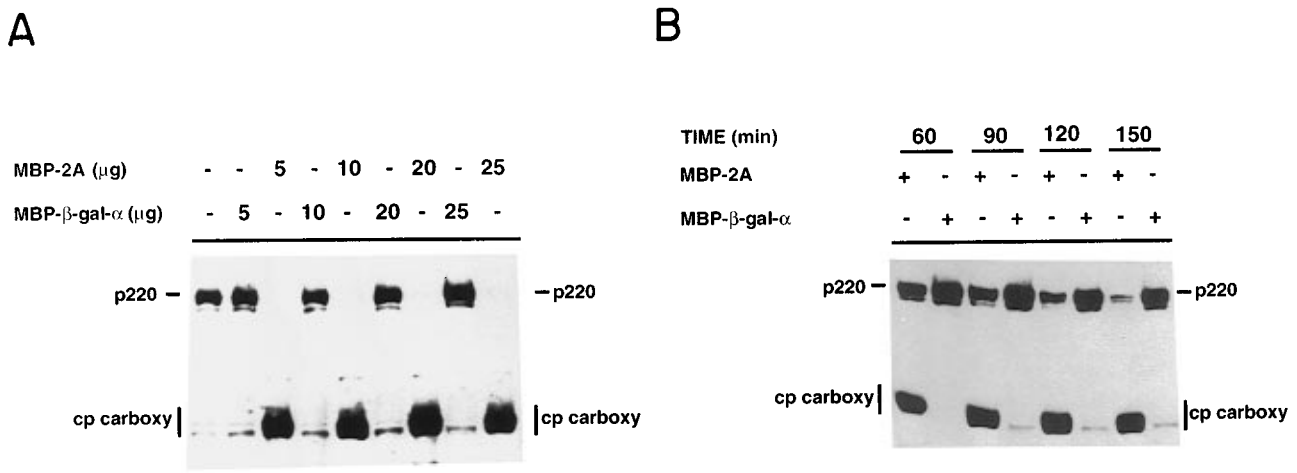

FIGURE 2: Cleavage of eIF-4G (p220) by MBP-2A in rabbit reticulocyte lysates. (Panel A) Rabbit reticulocyte lysates were incubated at 30 °C for 180 min in the presence of 5, 10, 20, or 25 μg of MBP-2A or MBP-β-gal-α, as indicated. (Panel B) Rabbit reticulocyte lysates were incubated at 30 °C in the presence of 4 μg of MBP-2A or MBP-β-gal-α and cleavage of p220 was analyzed at the indicated times. Proteins were separated by SDS−7.5% PAGE and analyzed by Western blot using anti-human p220 polyclonal antibodies. Intact p220 and C-terminal fragment of p220 are shown (cp carboxy).

mRNA, the lysate was preincubated for 150 min with 5 μg of the fusion protein in subsequent experiments. The specificity of the reaction is also observed in Figure 2, since addition of purified MBP-β-gal-α had no effect on the integrity of p220, while addition of MBP-2A^{pro} leads to the cleavage of this initiation factor. These results indicate that poliovirus 2A^{pro} is active when extra sequences are placed at the amino terminus of this protein. Cleavage of p220 by MBP plus 2A^{pro} generated by the incubation with factor Xa is similar to that found with the fusion protein (results not shown). Therefore we decided to use MBP-2A^{pro} in our experiments to avoid the presence of another protease (factor Xa) in the cell-free system.

Effect of p220 Cleavage on in Vitro Translation of Capped and Uncapped Influenza Virus NP mRNA. Once the conditions for efficient cleavage of p220 in the lysate were established, the effect of poliovirus 2A^{pro} on the translation of an exogenous capped mRNA in rabbit reticulocyte lysates deficient in p220 was analyzed. To this end, the influenza virus mRNA coding for NP (nucleoprotein) was chosen. This RNA was synthesized by T7 RNA polymerase and artificially capped with an efficiency of about 50−60%. In addition this mRNA also contains a poly(A) tail of about 100 residues.

The effect of p220 cleavage on the translation of capped NP mRNA was assayed under different concentrations of mRNA. A clear inhibition of NP translation was observed when p220 had been proteolytically cleaved, particularly when limiting amounts of mRNA were present (40−80 ng) (Figure 3A). Over 95% inhibition of NP synthesis occurred with 80 ng of mRNA, as estimated by densitometric scan analyses using a computing densitometer, Model 300A (Molecular Dynamics). The inhibition diminished when the amount of capped NP mRNA added to the cell-free system was increased (200−400 ng). Thus, about 40% of NP synthesis occurs after p220 cleavage when 400 ng of mRNA is present. Clearly, NP synthesis directed by capped mRNAs is inhibited at the different time points assayed in lysates containing p220 cleaved (Figure 4A). The dependence of the inhibition of translation by p220 cleavage on the concentration of capped mRNA may help to explain previous results from other groups, who obtained variable degrees of inhibition of capped mRNA translation after p220 cleavage (Ohlmann et al., 1995; Ziegler et al., 1995).

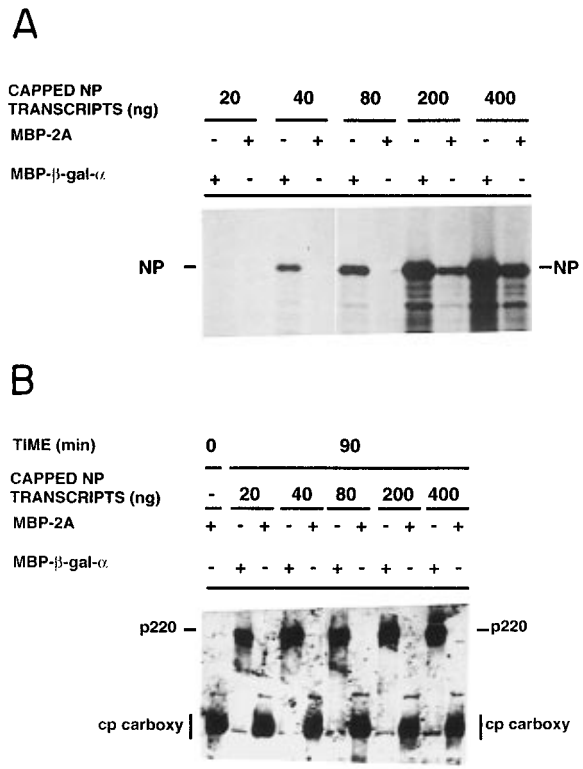

FIGURE 3: Effect of p220 cleavage by MBP-2A on the translation of different amounts of capped NP transcripts in rabbit reticulocyte lysates. (Panel A) Rabbit reticulocyte lysates were preincubated at 30 °C for 150 min in the presence of 5 μg of MBP-2A or MBP-β-gal-α. Subsequently, translation was carried out at 30 °C for 90 min in the presence of different amounts of *in vitro* synthesized capped NP transcripts. [^{35}S]Methionine-labeled proteins were analyzed by SDS−15% PAGE. (Panel B) The integrity of p220 was analyzed by Western blotting at time 0 and at the end of translation as described in Materials and Methods. NP protein, intact p220, and C-terminal fragment of p220 (cp carboxy) are indicated.

The integrity of p220 was tested in each assay shown in Figure 3A. To this end, aliquots of the lysate were taken after the preincubation step with MBP-2A^{pro}, before mRNA addition to the lysate (time 0) and at the end of the incubation period. These aliquots were immunoblotted with the polyclonal antibody against p220 (Figure 3B). No intact p220 was detected either at the beginning or at the end of the

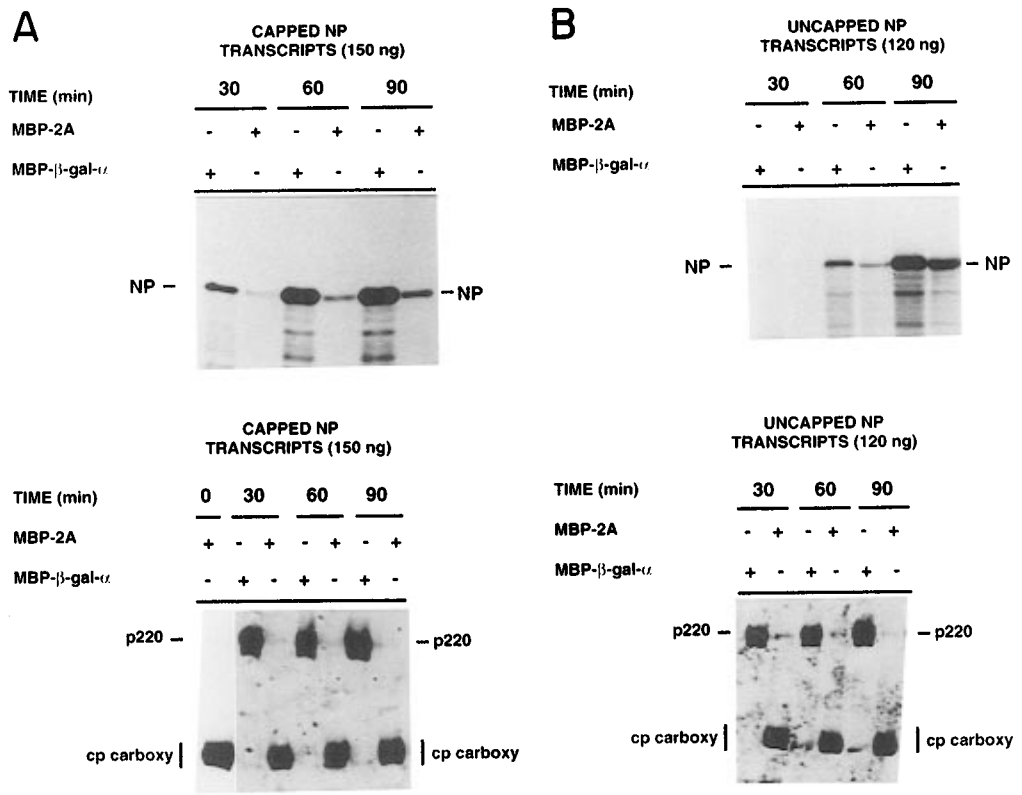

FIGURE 4: Effect of p220 cleavage on the translation of capped or uncapped NP transcripts. (Panel A) Rabbit reticulocyte lysates were preincubated at 30 °C for 150 min in the presence of 5 μg of MBP-2A or MBP-β-gal-α. Subsequently the translation reaction was carried out in the presence of 150 ng of *in vitro* synthesized capped NP transcripts at 30 °C and the reaction was stopped at the times indicated in the figure. Labeled proteins were analyzed by SDS−15% PAGE (upper panels). The integrity of p220 was analyzed by Western blotting at time 0 of the translation assay and at the times indicated in the figure as described in Materials and Methods (lower panels). (Panel B) Rabbit reticulocyte lysates were preincubated at 30 °C for 1 h in the presence of 20 μg of MBP-2A or MBP-β-gal-α. Afterwards, translation was carried out in the presence of 120 ng of *in vitro* synthesized uncapped NP transcripts at 30 °C and incubated for the indicated time periods. NP protein, intact p220, and C-terminal fragment of p220 (cp carboxy) are indicated.

incubation period in the samples containing 5 μg of MBP-2A^{pro} (Figure 3B).

Translation of artificially uncapped mRNAs does take place in lysates, albeit with a reduced efficiency as compared to the capped mRNA counterpart (Rose & Lodish, 1976; Lodish & Rose, 1977; Munroe & Jacobson, 1990; Gallie & Tanguay, 1994; Russell et al., 1991). About 30−50% inhibition of translation as estimated by densitometric scan analyses is found when uncapped poly(A)-containing mRNAs are compared to their capped counterparts (Munroe & Jacobson, 1990; Gallie & Tanguay, 1994). In principle, the initiation factor complex eIF-4F only recognizes capped mRNAs and does not participate in the translation of uncapped mRNAs (Grifo et al., 1983; Sonenberg, 1994; Rhoads, 1993). To assess the effect of poliovirus 2A^{pro} on the translation of uncapped influenza virus NP mRNA, the experiment shown in Figure 4 was conducted. Notably, synthesis of NP directed by uncapped mRNA was also dependent on the integrity of p220. This inhibition of uncaped NP mRNA translation occured at different time points during the translation reaction (75% inhibition after 60 min and 50% inhibition after 90 min) (Figure 4B). Once again, the degree of inhibition of NP synthesis is partially reversed by increasing the amount of uncapped mRNA present in the lysate. Densitometric quantitation of the gels indicated that 80% inhibition occurred when 80 ng of uncapped mRNAs was translated in lysates deficient in intact p220, while only 50% inhibition was observed when 150 ng of uncapped NP mRNA was used (results not shown). Therefore, we conclude that the degree of translation of the NP mRNA after p220 cleavage depends on the amount of mRNA present in rabbit reticulocyte lysates, irrespective of whether the mRNA bears a cap structure or not.

Action of p220 Cleavage on Translation of Luciferase mRNAs. Since influenza NP mRNA encodes a viral protein and contains a poly(A) tail, it may display special requirements for some factors involved in translation (Iizuka et al., 1994; Munroe & Jacobson, 1990; Tarun & Sachs, 1995). Therefore, synthesis of the protein luciferase directed by capped or uncapped mRNAs without a poly(A) tail was examined. Three types of luciferase mRNAs were obtained by *in vitro* transcription of the corresponding plasmids with the T7 RNA polymerase: capped luc mRNA, uncapped luc mRNA, and leader luc mRNA containing the poliovirus untranslated 5′ leader sequence that contains the IRES region upstream of the luciferase gene. In agreement with the results found with influenza NP mRNA, the inhibition of translation of all three types of luciferase mRNA after p220 cleavage was dependent on the concentration of mRNA present (Figure 5). Translation of capped luc mRNAs was strongly inhibited at low mRNA concentrations (50 ng) when p220 was cleaved, whereas the inhibition was diminished by increasing the mRNA concentration (100 ng) (Figure 5A). Only 25% inhibition of uncapped luciferase mRNA translation was obtained after p220 cleavage when 250 ng of this transcript was used, while the inhibition was much stronger when lower mRNA concentrations were present (100% inhibition with 75 ng of mRNA) (Figure 5B). Therefore, we propose that a mRNA concentration curve should be tested when the inhibition of translation by picornavirus

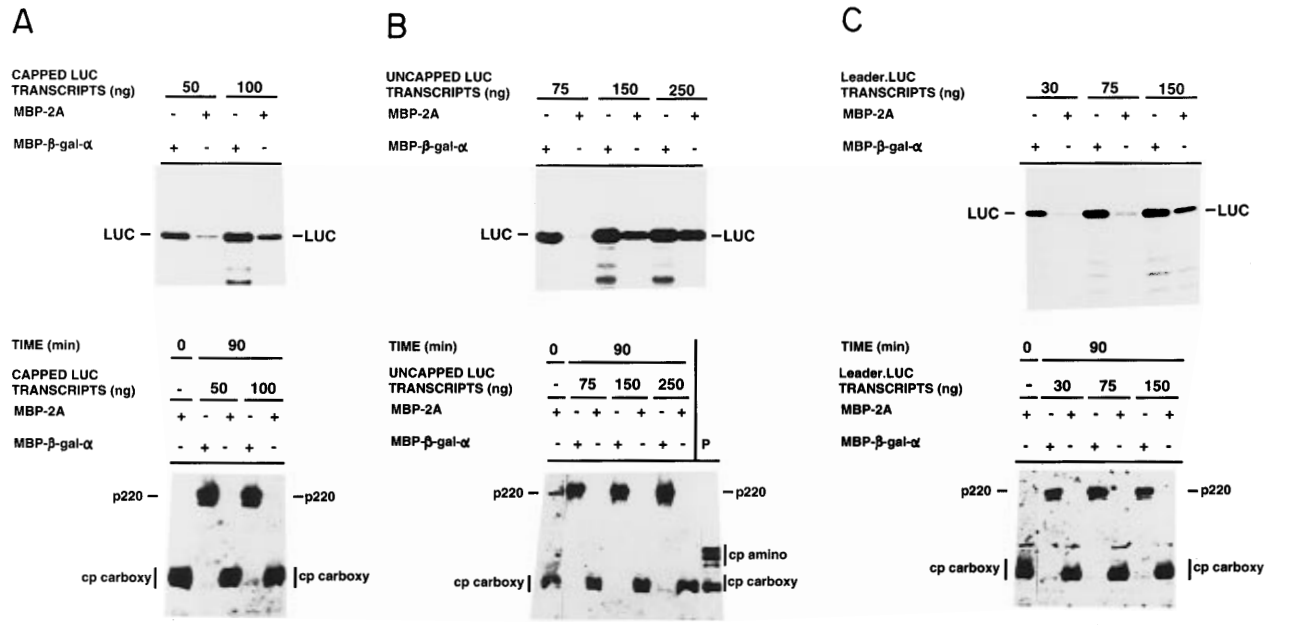

FIGURE 5: Effect of p220 cleavage on the translation of different amounts of capped, uncapped, or leader luc transcripts. Rabbit reticulocyte lysates were preincubated at 30 °C for 150 min in the presence of 5 μg of MBP-2A or MBP-β-gal-α. Subsequently, translation was carried out in the presence of different amounts of *in vitro* synthesized capped (panel A), uncapped (panel B), or leader luc (panel C) transcripts at 30 °C for 90 min. Upper panels correspond to [^{35}S]-labeled proteins analyzed by SDS−15% PAGE. Lower panels correspond to the analysis of p220 by Western blotting at time 0 and after 90 min of translation as described in Materials and Methods. Luc (luciferase), intact p220, C-terminal fragment (cp carboxy), and N-terminal fragment (cp amino) of p220 are indicated. P, poliovirus-infected HeLa cells.

proteases is examined. It is noteworthy that artificially uncapped mRNAs are translated rather efficiently in the cell-free system. These results are consistent with recent findings indicating that sequences placed at both the 5′ and the 3′ ends of mRNA participate in translation initiation (Munroe & Jacobson, 1990; Gallie & Tanguay, 1994).

Notably, not only translation of uncapped luciferase mRNA (Figure 5B) but also protein synthesis directed by luciferase mRNA containing the poliovirus leader region at the 5′ end was strongly compromised after p220 cleavage (85% inhibition with 75 ng of mRNA) (Figure 5C). These findings imply that p220 not only participates in the translation of artificially uncapped mRNAs but also is involved in the translation of uncapped mRNA containing the poliovirus leader region. These results are in good agreement with those reported by Pause et al. (1994) on the blockade of poliovirus leader CAT by inhibiting eIF-4F. The idea that p220 or one of its cleavage products (the C-terminal product) participates in poliovirus translation is very attractive (Lamphear et al., 1995), although a detailed analysis of the exact role that p220 plays in picornavirus translation is still lacking.

In fact, according to our present knowledge, one would expect that addition of poliovirus 2A and subsequent p220 cleavage would stimulate translation of the leader luc mRNA, a result that certainly was not found in our experiments, indicating that cleavage of rabbit eIF-4G (p220) by 2A^{pro} does not transactivate poliovirus translation in the rabbit reticulocyte lysate.

Supplementation of Rabbit Reticulocyte Lysates with HeLa Cell Extracts. Effects of Poliovirus 2A^{pro} on Translation. It is well-known that translation of poliovirus mRNA is inefficient in rabbit reticulocyte lysates but can be increased by HeLa cell extracts. Consequently we decided to assay the translation of leader luc mRNA in rabbit reticulocyte lysates supplemented with HeLa cell extracts (Figure 6). Addition of the HeLa extract still resulted in a clear inhibition of translation of capped luciferase mRNA (92% inhibition), when cleavage of p220 is accomplished by poliovirus 2A^{pro} (Figure 6B). Note that the cleavage products corresponding to the N-terminal fragment of p220 are apparent when the lysate is supplemented with the human cell extract since our p220 antibodies recognize the N-terminal moiety of human, but not rabbit, p220 (Figure 6B, lower panel). Interestingly enough, the inhibitory effect on translation by 2A^{pro} is not observed when the leader luc mRNA is assayed (Figure 6A). Moreover, substantial stimulation of luciferase synthesis can be achieved by addition of poliovirus 2A^{pro} (5-fold stimulation) (Figure 6B). Therefore, poliovirus 2A has opposite effects on translation depending on the mRNA examined; this protease strongly blocks luciferase synthesis directed by capped mRNA, while it enhances translation directed by the leader luc mRNA (Figure 6B,C). This result suggests that the stimulation of translation by 2A^{pro} requires a factor which is present in the human cell extract. In principle, this stimulation may be mediated by the activity of the p220 cleavage products, or by the presence of 2A^{pro} itself in combination with these products. If so, the p220 cleavage products produced in the rabbit reticulocyte lysate would be unable to transactivate a mRNA containing the poliovirus leader region.

DISCUSSION

P220 Cleavage by Picornavirus 2A. The fact that several picornavirus proteases cleave p220 (eIF-4G) (Krausslich & Wimmer, 1988; Palmenberg, 1990) provides a unique tool to assay for the function of this factor, both in intact cells and in cell-free systems. Efficient cleavage of p220 can be achieved in lysates after incubation with these picornavirus proteases in soluble form. Different protocols have been followed to achieve this goal using coxsackie, rhinovirus, or FMDV proteases (Liebig et al., 1993; Ohlmann et al., 1995; Ziegler et al., 1995). The approach reported in the present work with poliovirus 2A^{pro} may facilitate further

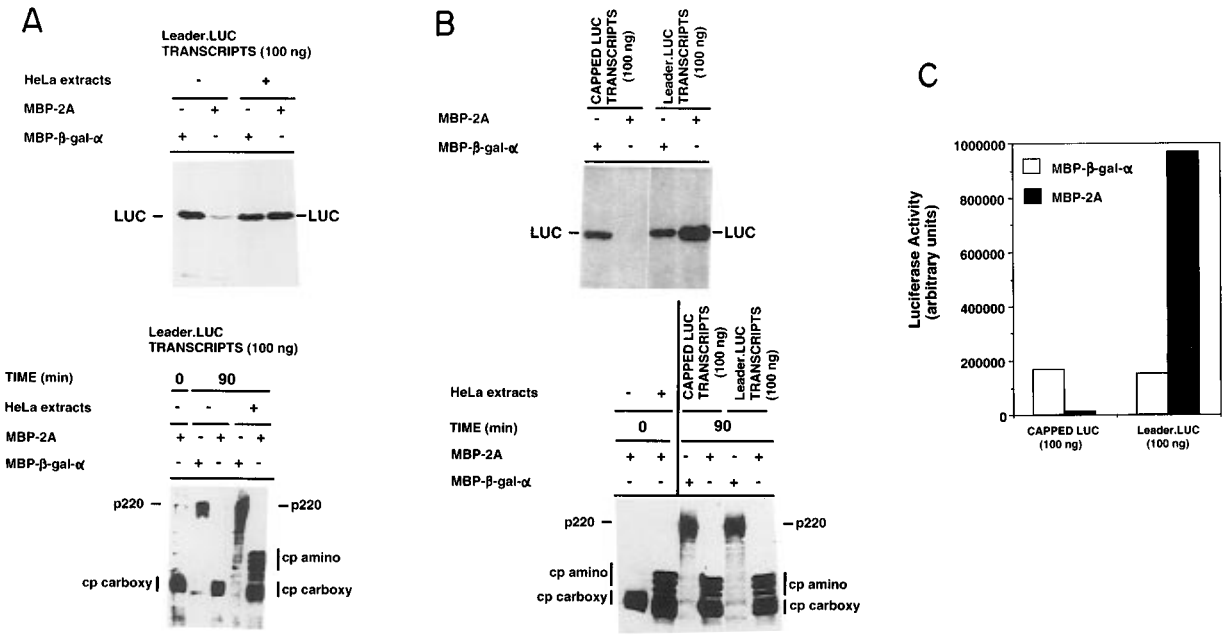

FIGURE 6: Effect of p220 cleavage on the translation of capped or leader luc transcripts, when supplemented with HeLa cell extracts. (Panel A) Rabbit reticulocyte lysates were preincubated at 30 °C for 180 min in the presence of 5 μg of MBP-2A or MBP-β-gal-α. Where indicated, HeLa cell extracts were added or not and were further incubated for 30 min. Afterward, translation was carried out in the presence of 100 ng of *in vitro* synthesized leader luc transcripts at 30 °C for 90 min. (Panel B) Rabbit reticulocyte lysates were preincubated at 30 °C for 120 min in the presence of 5 μg of MBP-2A or MBP-β-gal-α. Rabbit reticulocyte lysates were then supplemented with HeLa cell extracts and were incubated for 30 min. Then protein synthesis was carried out in the presence of 100 ng of *in vitro* synthesized capped or leader luc transcripts at 30 °C for 90 min. Upper panels correspond to SDS−15% PAGE analyses of the proteins synthesized. Lower panels correspond to the analysis of p220 by Western blotting at time 0 and after 90 min of translation as described in Materials and Methods. (Panel C) Luciferase activity of translation assay samples of panel B was determined as described in Materials and Methods. Luc (luciferase), intact p220, C-terminal fragment (cp carboxy) and N-terminal fragment (cp amino) of p220 are indicated in panels A and B.

studies aimed at understanding the action of picornavirus proteases in translation using cell-free systems. The use of fusion proteins containing the picornavirus protease facilitates its purification. Since $2A^{pro}$ is active as a precursor (Palmenberg, 1990; Harber & Wimmer, 1993), it is not surprising to find that the MBP-$2A^{pro}$ fusion protein is a soluble and highly active protease, capable of cleaving p220 in rabbit reticulocyte lysates even when supplemented with the human cell extract. Moreover, MBP-$2A^{pro}$ is also able to enhance translation of leader luc mRNA. The ability to mutate $2A^{pro}$ and to readily purify the different MBP-$2A^{pro}$ fusion proteins provides a rapid protocol to assay a number of $2A^{pro}$ mutants. These variant forms of $2A^{pro}$ could serve for a better understanding of the amino acid residues of $2A^{pro}$ involved in p220 cleavage. In addition, these $2A^{pro}$ variants could determine whether p220 cleavage is necessary to obtain the stimulation of translation by $2A^{pro}$ or if the presence of $2A^{pro}$ alone is sufficient to get this effect, without the need to cleave p220 (see below).

Effects of p220 Cleavage on Protein Synthesis Directed by Capped mRNAs. Two different alternatives for eIF-4F functioning have been considered. One is that it is a classical initiation factor required to initiate each round of mRNA translation by ribosomes (Grifo et al., 1983; Sonenberg, 1994; Rhoads, 1993). Another possibility, which we suggested recently, is that eIF-4F is involved in bringing the newly made mRNAs to the protein synthesizing machinery (Irurzun et al., 1995; Rose, 1996). In this regard, eIF-4F would be necessary for the first initiation event on newly made mRNAs. Once translation has started on a given mRNA, eIF-4F may not be required for subsequent initiation events (Irurzun et al., 1995). Elucidating the exact role that eIF-4F plays in cellular gene expression would benefit very much from the use of picornavirus proteases that cleave and presumably inactivate eIF-4G.

The pioneering experiments of Liebig et al. (1993) using coxsackie and rhinovirus $2A^{pro}$ to cleave p220 in HeLa cell lysates indicated that natural globin mRNA translation was depressed, while protein synthesis directed by the 5′ leader region of rhinovirus RNA was stimulated. Further experiments by other groups obtained varying inhibitory effects on mRNA translation after p220 cleavage (Ohlmann et al., 1995). These findings contrast with those recently published by Ziegler et al. (1995), who found no inhibition of cyclin synthesis from capped mRNAs after extensive p220 cleavage catalyzed by coxsackie virus 2A or FMDV L protease in rabbit reticulocyte lysates supplemented with HeLa cell extracts. Our present results point to the conditions of the cell-free system, particularly the concentration of mRNA, as an important factor that should be controlled to obtain a dependence of translation on p220 integrity. The finding that significant reversion of translation inhibition can be obtained by increasing the amount of mRNA in lysates when p220 has been proteolytically digested is intriguing. If the function of eIF-4F is to increase the affinity of mRNAs for other initiation factors in order to bring the mRNA to the 40S ribosomal subunit (Grifo et al., 1983; Sonenberg, 1994; Rhoads, 1993), it seems logical that even when intact p220 has been depleted, part of the mRNA is translated. As pointed out several years ago, the actual translation of a given mRNA depends on its affinity for limiting components of translation and on the concentration of these components, including the concentration of mRNA (Lodish, 1974). Therefore, when the amount of intact eIF-4F becomes limiting after extensive p220 cleavage, part of the inhibition of translation may be overcome by raising the concentration of mRNA. This explanation provides a rationale for understanding the variable degrees of inhibition of capped mRNA translation that have been reported to date in lysates treated with picornavirus proteases (Liebig et al., 1993;

Ohlmann et al., 1995; Borman et al., 1995; Ziegler et al., 1995).

Requirement of p220 for Translation of Uncapped mRNAs. Addition of a dominant negative mutant of eIF-4A to cell-free systems inactivates eIF-4F activity (Pause et al., 1994). Translation of EMC virus and poliovirus mRNAs under these conditions did not occur, implicating eIF-4F in the translation of picornavirus mRNA (Pause et al., 1994). Our present findings that cleavage of p220 by poliovirus $2A^{pro}$ impedes the translation of artificially uncapped mRNAs in rabbit reticulocyte lysates agree with the findings of Pause et al. (1994) but contrast with a report where enhanced translation of several uncapped mRNAs after p220 cleavage by FMDV L protease was found (Ohlmann et al., 1995). At present we have no explanation for this discrepancy. It may be that the different proteases used are responsible for this apparent inconsistency. In fact, poliovirus $2A^{pro}$ and FMDV L cleave p220 at different positions (Lamphear et al., 1993; Kirchweger et al., 1994). Certainly, uncapped mRNAs can be translated in cell-free systems (Rose & Lodish, 1976; Lodish & Rose, 1977; Munroe & Jacobson, 1990; Gallie & Tanguay, 1994; Russell et al., 1991), although the mechanism and the exact requirements for the different factors involved in the translation of artificially uncapped mRNAs have not been sufficiently explored. Our findings clearly suggest that p220 participates in the translation of both natural and artificially uncapped mRNAs. Consequently, eIF-4F may recognize not only the cap structure, if it is present on the mRNA, but also other features of the 5′ untranslated region that bind this factor to the mRNA (Rozen et al., 1990) if it is devoid of the cap structure. If so, the initiation of translation of artificially uncapped mRNAs is less efficient than the translation of capped mRNAs but is not totally abolished, at least in cell-free systems. Cleavage of p220 would impair the ability of eIF-4F to act on capped mRNAs and artificially uncapped mRNAs. The corollary of this reasoning is that the terms cap-dependent and cap-independent translation are misleading. Most types of capped or uncapped mRNAs require eIF-4F activity (Grifo et al., 1983; Pause et al., 1994), while some capped mRNAs, such as those encoding the heat-shock proteins, do not require eIF-4F (Sierra & Zapata, 1994). The term cap-independent translation implying a lack of recognition of the cap structure by eIF-4F should be used with caution. It is possible that eIF-4F actually participates in the translation of all types of mRNAs, but some mRNAs (i.e., heat-shock mRNAs) could be translated even if eIF-4F is inactivated, independently of whether the mRNA is capped or uncapped.

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