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RNA 2001 7: 1743-1752

RNA (2001), *7*:1743–1752. Cambridge University Press. Printed in the USA.

DOI: 10.1017.S1355838201011487

Poly(A)-binding protein interaction with eIF4G stimulates picornavirus IRES-dependent translation

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ABSTRACT

The eukaryotic mRNA 3′ poly(A) tail and the 5′ cap cooperate to synergistically enhance translation. This interaction is mediated, at least in part, by eIF4G, which bridges the mRNA termini by simultaneous binding the poly(A)-binding protein (PABP) and the cap-binding protein, eIF4E. The poly(A) tail also stimulates translation from the internal ribosome binding sites (IRES) of a number of picornaviruses. eIF4G is likely to mediate this translational stimulation through its direct interaction with the IRES. Here, we support this hypothesis by cleaving eIF4G to separate the PABP-binding site from the portion that promotes internal initiation. eIF4G cleavage abrogates the stimulatory effect of poly(A) tail on translation. In addition, translation in extracts in which eIF4G is cleaved is resistant to inhibition by the PABP-binding protein 2 (Paip2). The eIF4G cleavage-induced loss of the stimulatory effect of poly(A) on translation was mimicked by the addition of the C-terminal portion of eIF4G. Thus, PABP stimulates picornavirus translation through its interaction with eIF4G.

Keywords: cap-poly A synergy; eIF4E; encephalomyocarditis virus; poliovirus; poly A tail

INTRODUCTION

Eukaryotic translation is controlled at the initiation step by the mRNA's terminal structures, the 5′ cap (m^7GpppN, where N is any nucleotide) and the 3′ poly(A) tail (Sachs, 2000). Although both structures stimulate translation on their own, when present together, they exert a synergistic effect. This synergy was demonstrated in yeast, plant, and mammalian cells in vivo (Gallie, 1991; Sachs, 2000). It was also recapitulated in vitro (Iizuka et al., 1994; Tarun & Sachs, 1997; Preiss & Hentze, 1998; Gebauer et al., 1999; Michel et al., 2000), and currently represents an attractive paradigm for control of translational initiation by 3′ end sequences.

The mechanism by which the mRNA 3′ poly(A) functionally interacts with the 5′ end has been first elucidated in yeast (Tarun & Sachs, 1996). This interaction is mediated by the association of the poly(A)-binding protein (PABP) with the eIF4G subunit of the cap-binding complex eIF4F (Tarun & Sachs, 1996; Gray et al., 2000). PABP is a ~70 kDa protein, which contains four RNA recognition motifs (RRMs) and a proline-rich C-terminal region (Adam et al., 1986; Sachs et al., 1986). When bound to poly(A), PABP organizes the ribonucleoprotein in a repeating structure with a periodicity of about 27 nt (Baer & Kornberg, 1983). eIF4F is composed of the cap-binding subunit, eIF4E, an RNA-dependent ATPase/ATP-dependent RNA helicase, eIF4A, and eIF4G. The latter protein serves as a scaffold for binding of several proteins, including eIF4E (Mader et al., 1995), eIF4A (Morino et al., 2000), and eIF3 (Morino et al., 2000), a 40S ribosome-associated initiation factor (Hershey & Merrick, 2000). Importantly, the N-terminal region of eIF4G harbors a binding site for PABP (Tarun & Sachs, 1996; Imataka et al., 1998), which brings about the circularization of the mRNA (Wells et al., 1998). It is thought that mRNA circularization leads to the enhancement of translation (Jacobson, 1996; Sachs, 2000). Notwithstanding the

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Abbreviations: CV: coxsackievirus; eIF: eukaryotic initiation factor; eIF4G-C_t: eIF4G C-terminal fragment; eIF4G-N_t: N-terminal fragment; EMCV: encephalomyocarditis virus; IRES: internal ribosome entry site(s); HCV: hepatitis C virus; PV: poliovirus.

demonstrated interaction between PABP and eIF4G, it was suggested that the poly(A) ribonucleoprotein stimulates the 60S subunit joining step (Sachs & Davis, 1989; Munroe & Jacobson, 1990; Searfoss et al., 2001) in addition to the recruitment of the 40S ribosomal subunit to the mRNA (Tarun & Sachs, 1995). An eIF4G-independent pathway of poly(A)-dependent regulation of translation has recently been proposed for yeast (Searfoss et al., 2001). This model posits that the PABP–poly(A) complex exerts its function by inhibiting the activities of two nonessential putative RNA helicases (*SKI2* and *SLH1*; Searfoss & Wickner, 2000) that, in turn, repress the activity of the factors required for 60S ribosomal subunit joining, that is, eIF5 and eIF5B (Pestova et al., 2000).

Poly(A)-mediated translation is a target for regulation by the PABP-interacting proteins, Paip1 and Paip2. Paip1 stimulates translation, following overexpression in COS-7 cells, apparently by establishing an additional route by which the stimulatory signal is relayed from the poly(A) tail to the translation machinery (Craig et al., 1998). In contrast, Paip2 inhibits the stimulatory effect of poly(A) on translation by dissociating PABP from poly(A), and competing with Paip1 for binding to PABP (Khaleghpour et al., 2001a, 2001b).

In contrast to most cellular mRNAs, which are translated by a cap- and 5′ end-dependent mechanism, picornavirus mRNAs utilize an internal ribosome entry site (IRES; Jang et al., 1988; Pelletier & Sonenberg, 1988). Two major types of IRES structure organization were described for picornaviruses (Jackson, 2000). Enteroviruses [such as poliovirus (PV)] and rhinoviruses constitute one class, whereas cardioviruses [such as encephalomyocarditis (EMCV)] and aphthoviruses [such as foot-and-mouth disease virus (FMDV)] constitute another. 48S initiation complex formation on EMCV IRES requires the canonical set of initiation factors, including eIF4G, which binds to the IRES directly, and eIF4A, which stimulates this binding, but does not require eIF4E (Pestova et al., 1996a; Lomakin et al., 2000; Lópes de Quinto & Martínez-Salas, 2000; Marcotrigiano et al., 2001). Strikingly, IRES activity is maintained, or even enhanced, following the cleavage of eIF4G by PV or human rhinovirus (HRV) 2A protease ($2A^{pro}$), which leads to the inhibition of cap-dependent translation because of the physical separation of eIF4E- and eIF4A/eIF3-interacting domains of eIF4G (Belsham & Jackson, 2000).

IRES-mediated picornavirus mRNA translation is stimulated by the poly(A) tail (Hruby & Roberts, 1977; Bergamini et al., 2000; Michel et al., 2000; Khaleghpour et al., 2001b). However, the role of the PABP-eIF4G in this system has not been addressed. Here, we address this question. We show that either cleavage of eIF4G by HRV $2A^{pro}$ or Paip2-mediated disruption of the PABP–poly(A) complex renders IRES activity independent of the poly(A). Thus, the poly(A) tail stimulates initiation on IRES via PABP–eIF4G interaction. These results further support the importance of PABP–eIF4G interaction for efficient translation.

RESULTS

Cap-poly(A) tail synergy in a Krebs-2 cell extract

Previous studies demonstrated translational synergy between the mRNA 5′ terminal cap structure and the 3′ poly(A) tail in vitro under conditions of mRNA competition or after partial depletion of ribosomes and initiation factors (Gebauer et al., 1999; Bergamini et al., 2000; Michel et al., 2000). However, a significant stimulation of translation (approximately eightfold) by the poly(A) tail was also observed in a micrococcal nuclease-treated Krebs-2 cell extract (Khaleghpour et al., 2001b). To further characterize the synergism in the latter system, cap-poly(A) synergy was studied under different conditions by translating capped or uncapped luciferase mRNAs, each either containing or lacking a poly(A) tail (Fig. 1). The ratio of translational enhancement by the combination of cap and poly(A) to the sum of enhancement by the cap and poly(A) individually was greater than one (e.g., 5.1 at 2 μg/mL mRNA concentration), indicating a synergistic rather than additive effect on translation (Fig. 1A). High mRNA concentrations decreased both the cap- and poly(A) tail-dependence of translation and synergy (Fig. 1B,C). The reason for this is not immediately clear, but may reflect titration of general RNA-binding proteins (Svitkin et al., 1996) by mRNA excess.

Poly(A) tail-mediated stimulation of IRES activity

The effect of the poly(A) tail on IRES activity was studied using well-documented IRES, such as PV, EMCV, and coxsakievirus B1 (CV) IRES (Jackson, 2000). The poly(A) tail stimulated IRES-dependent translation of PV (Fig. 2A, left panel) and CV (Khaleghpour et al., 2001b). Stimulation of PV IRES was more pronounced at low mRNA concentrations, similar to what was seen for cap-dependent translation (Fig. 2B). At 2 μg/mL mRNA, the stimulation of PV IRES activity was ~5-fold (Fig. 2B). In agreement with the results reported previously in the HeLa cell-free system (Bergamini et al., 2000), EMCV IRES was 20–30-fold more active in translation than PV IRES and responded modestly to the poly(A) tail (~2-fold stimulation at 2 μg/mL mRNA; Fig. 2A, right panel, and Fig. 2B). Also, stimulation of translation from EMCV IRES by the poly(A) tail was not influenced by the mRNA concentration (Fig. 2B).

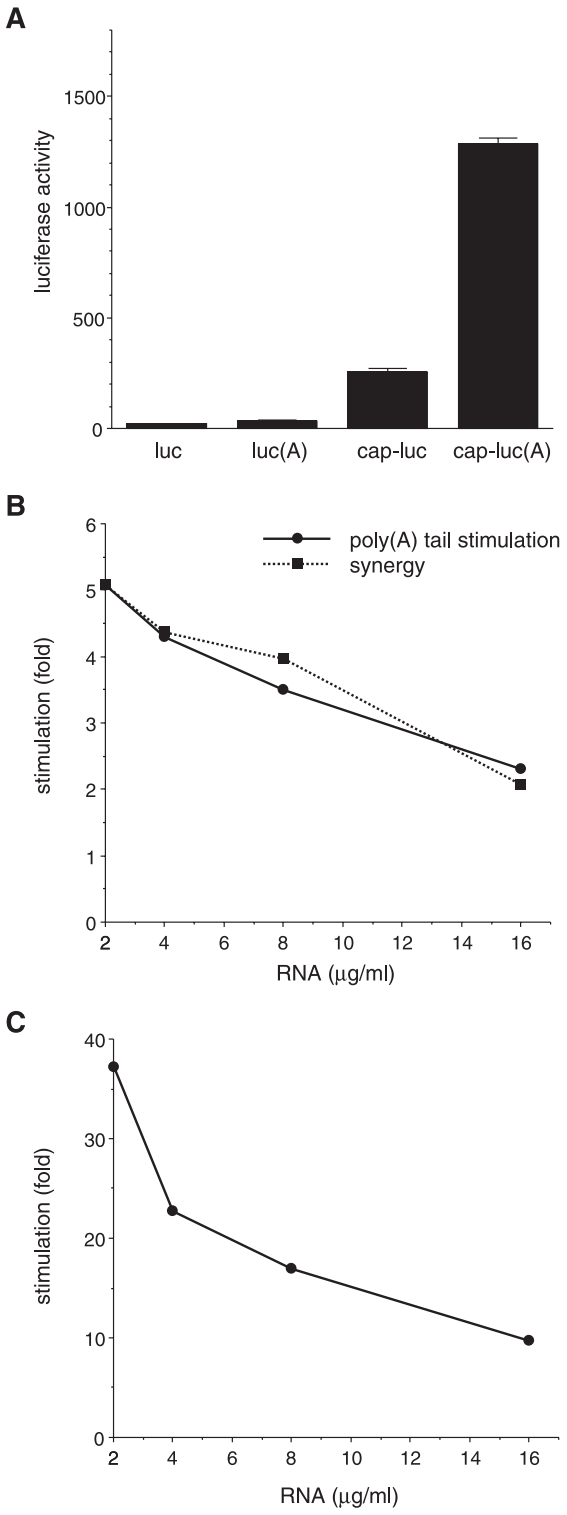

FIGURE 1. Synergistic enhancement of translation by the mRNA 5′ cap and the 3′ poly(A) tail in a Krebs-2 cell extract. **A:** Translation of luciferase mRNA (2 μg/mL) was carried out as described in Materials and Methods. The data represent an average of three independent determinations with the standard deviation from the mean. **B:** Fold-stimulation of cap-luc mRNA translation by the poly(A) tail (circles) and cap-poly(A) tail synergy (squares) as a function of mRNA concentration. Synergy is defined as stimulation of luc mRNA translation by combination of the cap and the poly(A) tail divided by the sum of stimulations by each of these structures alone (Michel et al., 2000). **C:** Fold-stimulation of luc(A) mRNA translation by the cap structure.

Poly(A)-IRES functional interaction is abrogated by eIF4G cleavage

We next addressed the role of eIF4G in poly(A) stimulated IRES-driven translation. eIF4G binds directly to the EMCV or FMDV IRES (Lomakin et al., 2000; Lópes de Quinto & Martínez-Salas, 2000; Marcotrigiano et al., 2001), and is essential for ribosome binding in a reconstituted in vitro ribosome binding assay (Pestova et al., 1996a). However, the eIF4G requirement for the activity of PV-HRV group IRES has not been demonstrated directly, but is inferred from the ability of dominant-negative eIF4A mutants to inhibit translation from these IRES (Pause et al., 1994b; Svitkin et al., 2001). We reasoned that if eIF4G mediates the poly(A)-PV IRES functional interaction, then its cleavage by picornavirus $2A^{pro}$ [which results in the separation of the eIF4G PABP-interacting N-terminal portion (Imataka et al., 1998) from the eIF4A and eIF3 binding portion (Imataka & Sonenberg, 1997; Morino et al., 2000)] would render translation from the PV IRES refractory to stimulation by poly(A). To cleave eIF4G, we used HRV2 $2A^{pro}$, which was obtained in a highly purified form (Liebig et al., 1993), and cleaved both eIF4GI and eIF4GII isoforms within the eIF4F complex (Haghighat et al., 1996; Gradi et al., 1998; Glaser & Skern, 2000). $2A^{pro}$ treatment of the Krebs-2 extract resulted in stimulation of PV IRES activity (Fig. 2A), consistent with previous in vitro and in vivo studies (Hambidge & Sarnow, 1992; Liebig et al., 1993; Macadam et al., 1994; Ziegler et al., 1995a; Borman et al., 1997; Roberts et al., 1998). Strikingly, at all mRNA concentrations, translation of poly(A)− mRNA was more strongly stimulated by $2A^{pro}$ treatment than translation of poly(A)+ mRNA. Consequently, the difference in translation of poly(A)− and poly(A)+ mRNAs disappeared in the $2A^{pro}$-treated extract (Fig. 2A, left panel, and Fig. 2B). EMCV (Fig. 2A, right panel) or CV (see below, Fig. 3C) IRES also directed translation with higher efficiency and in a poly(A)-independent manner after $2A^{pro}$ treatment. The reason for the stimulation of translation after eIF4G cleavage will be addressed in the Discussion. Because PABP might also be cleaved by picornavirus $2A^{pro}$ (Joachims et al., 1999; Kerekatte et al., 1999), it was pertinent to analyze its integrity. No cleavage of PABP was observed under the conditions where eIF4GI was completely cleaved (Fig. 2C) and cap-dependent translation of both poly(A)+ and poly(A)− mRNAs was abolished (Fig. 2D). Also, Paip1, which might also be involved in bridging the mRNA termini (Craig et al., 1998), remained intact (Fig. 2C). Finally, $2A^{pro}$ treatment of Krebs-2 cell extracts did not affect stability of poly(A)+ and poly(A)− PV or EMCV IRES containing luciferase mRNAs (Fig. 2E and data not shown). Taken together, these results indicate that eIF4G integrity is critical for poly(A)–IRES cooperation in translation.

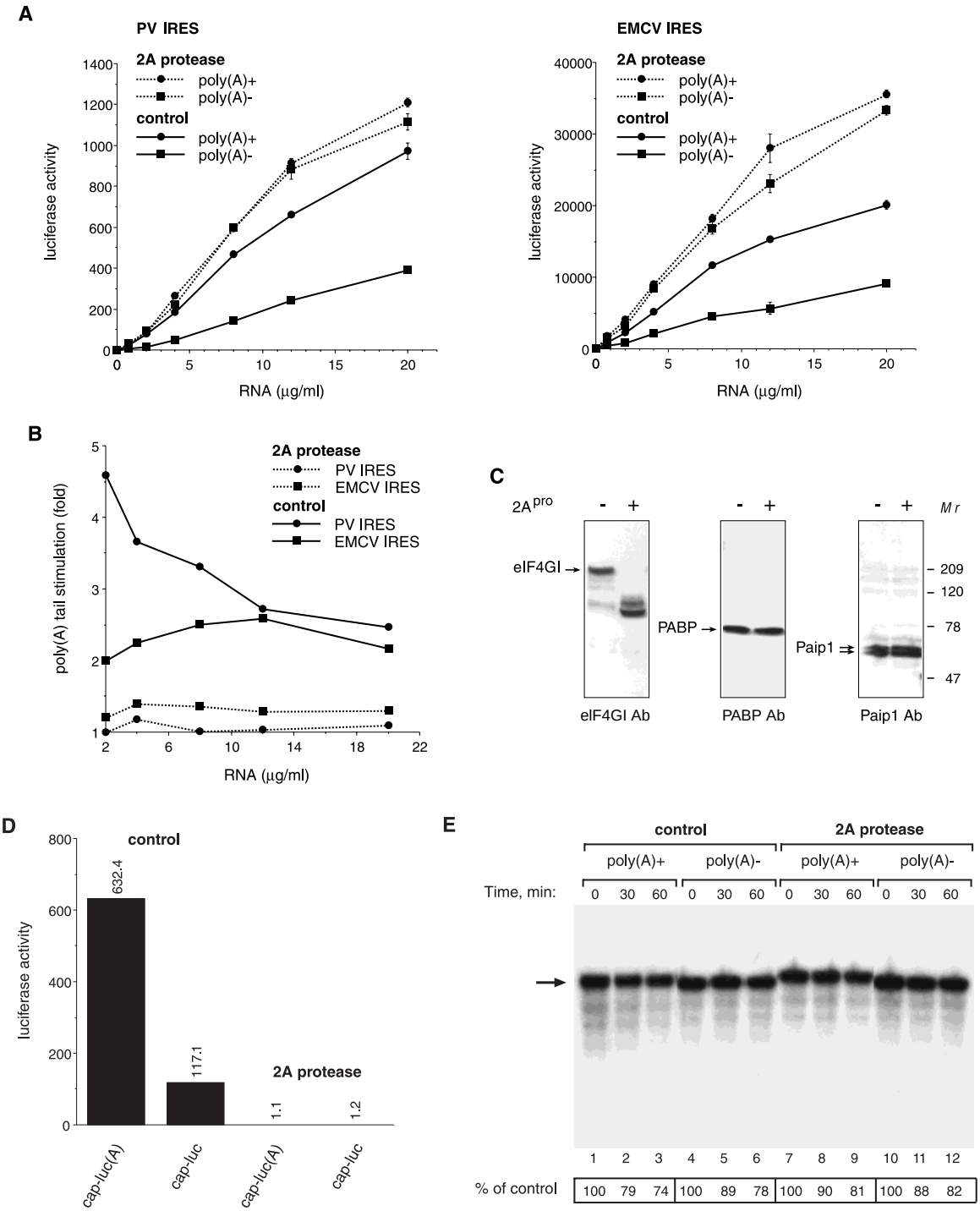

FIGURE 2. Poly(A) tail stimulates translation from PV and EMCV IRES. **A:** Effects of mRNA concentration and 2A^{pro} treatment. Krebs-2 ascites cell extracts were preincubated at 30 °C for 5 min with HRV 2A^{pro} (25 μg/mL) or control buffer. Following preincubation, the extracts were supplemented with poly(A)+ or poly(A)− mRNA that contained either PV (left panel) or EMCV (right panel) IRES. Conditions for incubation and luciferase activity assay were the same as those in Figure 1. Error bars denote the standard deviation from the mean from four different experiments. **B:** Fold-stimulation of PV and EMCV IRES by the poly(A) tail as affected by mRNA concentration and HRV 2A^{pro}-treatment (calculations are based on data shown in **A** and **B**). **C:** Western blot analysis of eIF4GI (left panel), PABP (middle panel), and Paip1 (right panel) in the control and 2A^{pro}-treated Krebs-2 translation extract. Rabbit antibodies raised against the indicated proteins were used in 1:1,000 dilution and protein signals were detected using enhanced chemiluminescent (ECL) procedure. **D:** Cap-dependent translation is abolished in the 2A^{pro}-treated Krebs-2 extract. Capped poly(A)+ or poly(A)− were translated at 2 μg/mL in the extracts preincubated either with control buffer (control) or 2A protease as specified above. The average result of two independent determinations of luciferase activity is shown. **E:** 2A^{pro} treatment does not affect stability of PV IRES containing luciferase mRNAs. Poly(A)+ or poly(A)− mRNAs (4 μg/mL) were incubated in the extracts that were pretreated with either control buffer or 2A protease as above. Total RNA was isolated at the indicated times and the integrity of luciferase mRNA was analyzed by formaldehyde-agarose gel electrophoresis and northern blotting using a ^{32}P-labeled luciferase probe (Khaleghpour et al., 2001b). Values obtained for time 0 were set as 100%. The position of the intact luciferase mRNA is indicated by an arrow.

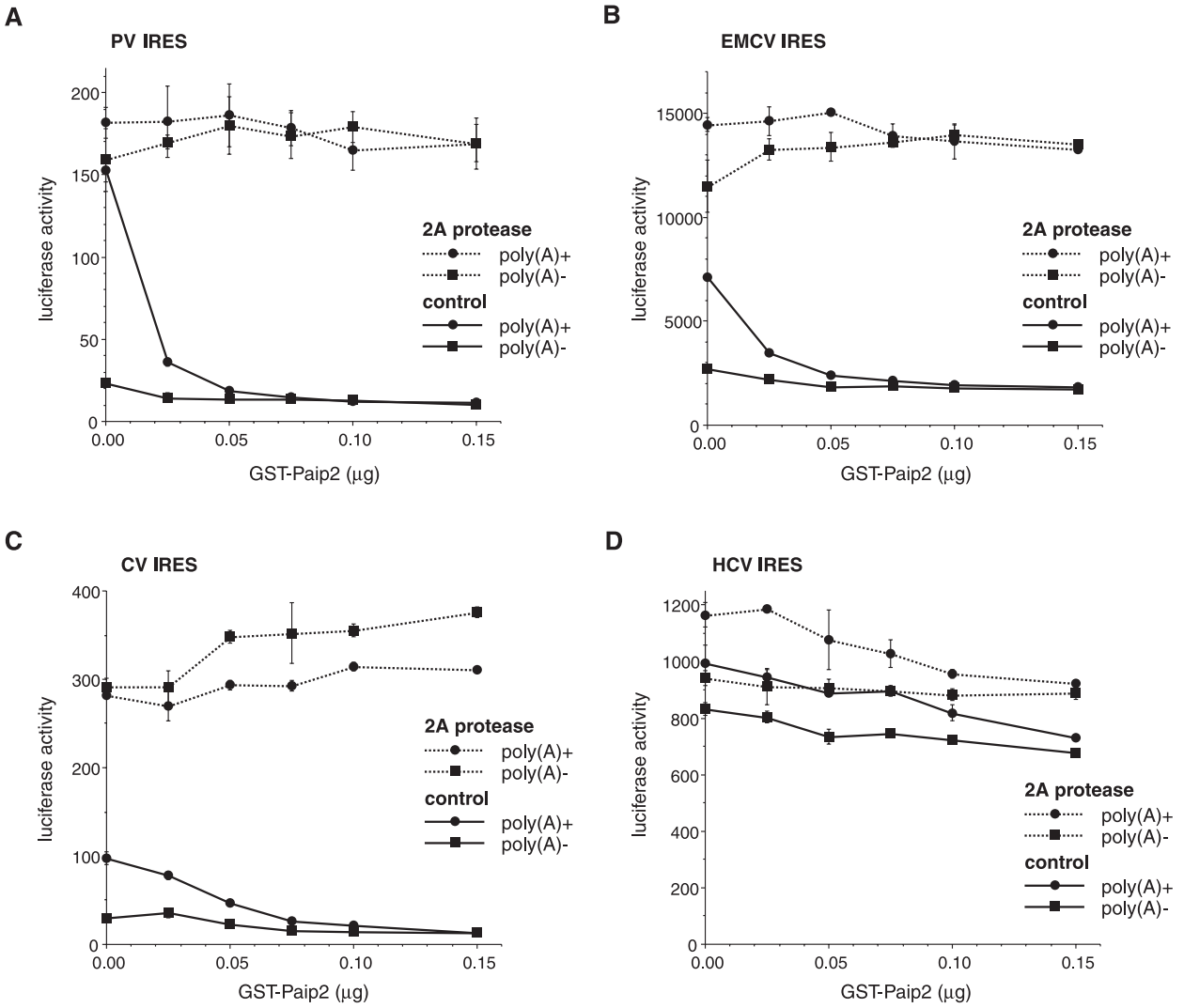

FIGURE 3. Paip2 mediated translational repression is prevented in the $2A^{pro}$-treated extract. GST-Paip2 titration of reactions that were pretreated with either control buffer or $2A^{pro}$ and programmed with PV (**A**), EMCV (**B**), CV (**C**), or HCV (**D**) IRES-containing poly(A)+ or poly(A)− luciferase mRNA (2 μg/mL). The data represent an average of three (**A**, **B**, and **D**) or four (**C**) independent determinations with the standard deviation from the mean.

eIF4G cleavage prevents translational repression by Paip2

To further substantiate the role of PABP-poly(A) interaction in poly(A) tail-mediated translational enhancement, we made use of the ability of the translational repressor Paip2 to disrupt the PABP-poly(A) complex (Khaleghpour et al., 2001a, 2001b). Titration of endogenous PABP by Paip2 preferentially inhibited translation of poly(A)+ as compared to poly(A)− mRNAs containing different types of IRES (PV, EMCV, and CV IRES; Fig. 3A–C). At maximal inhibition by Paip2, there was no difference in translation between poly(A)+ and poly(A)− mRNAs. Thus, the PABP–poly(A) tail interaction is important for efficient IRES function. Strikingly, the results obtained in extracts treated with $2A^{pro}$ were very different. eIF4G proteolysis not only eliminated the differential translation of poly(A)+ versus poly(A)− mRNA, but also abrogated the inhibitory effect of Paip2 on translation (Fig. 3A–C). Thus, the eIF4G fragments generated by the $2A^{pro}$ treatment cannot substitute for the intact eIF4G to effect the poly(A) tail-mediated stimulation of IRES activity. Importantly, translation from HCV IRES, for which eIF4G is dispensable (Pestova et al., 1998), was not affected significantly by poly(A) tail addition and Paip2 or $2A^{pro}$, either alone or in combination (Fig. 3D; see also Khaleghpour et al., 2001b).

Poly(A) tail-mediated translational stimulation is inhibited by the eIF4G C-terminal fragment

Although the results described above demonstrate that IRES activation by the poly(A) tail correlates with eIF4G

integrity, the involvement of other proteins, which are cleaved by $2A^{pro}$ could not be ruled out. To address this question, we examined whether the C-terminal portion of eIF4G could mimic the effect of $2A^{pro}$ on translation, presumably by competing with the endogenous eIF4G for binding eIF3, eIF4A, or some unknown factors. A C-terminal fragment of eIF4G (eIF4G-C_t, 940 amino acids) that is only slightly longer than the C-terminal eIF4G cleavage product (919 amino acids; Morino et al., 2000) was obtained as described in Materials and Methods. The fragment was examined for its effect on translation of poly(A)+ or poly(A)− luciferase mRNA containing viral IRES. Poly(A)+ mRNA translated more efficiently than the poly(A)− mRNA for both PV and EMCV IRES, as already shown above (Fig. 4A,B). However, the difference between translational efficiencies of poly(A)+ and poly(A)− mRNA was gradually diminished with increasing concentrations of the eIF4G-C_t (Fig. 4A,B). The minimal concentration of eIF4G-C_t that rendered translation refractory to stimulation by poly(A) was ~30 μg/mL. This roughly equals the concentration of endogenous eIF4G-C_t generated by $2A^{pro}$ treatment as determined by western blotting (data not shown). Furthermore, eIF4G-C_t addition, similar to $2A^{pro}$ treatment, completely prevented the inhibitory effect of Paip2 on translation from CV IRES (compare Fig. 4C to Fig. 3C). We conclude that cleavage of eIF4G alone and the accompanying accumulation of eIF4G-C_t is sufficient to render IRES-dependent translation initiation refractory to stimulation by poly(A).

DISCUSSION

This and previous works (Bergamini et al., 2000; Michel et al., 2000; Khaleghpour et al., 2001b) demonstrate that the poly(A) tail enhances the IRES-dependent translation of all picornaviruses. PABP is an important mediator of the poly(A)–IRES functional interaction, as its displacement from the poly(A) tail by Paip2 abolishes the stimulatory effect of the poly(A) tail on translation (Fig. 3; Khaleghpour et al., 2001b). eIF4G mediates this translational enhancement, because its cleavage by $2A^{pro}$ renders the IRES refractive to the stimulatory activity of poly(A) (Figs. 2 and 3). It is not well understood how the eIF4G–PABP complex facilitates translation. It might stabilize the association of eIF4G with the mRNA. Alternatively, translational enhancement may be effected by juxtaposition of the mRNA termini and subsequent ribosome shunting from the 3′ to 5′ end. Earlier results demonstrating the interaction of eIF4G with PABP (via the eIF4G N-terminal portion; Imataka et al., 1998) and with EMCV or FMDV IRES (via the eIF4G middle domain; Pestova et al., 1996a; Lomakin et al., 2000; Lópes de Quinto & Martínez-Salas, 2000; Marcotrigiano et al., 2001) are also consistent with the role of eIF4G in the poly(A) tail–IRES functional interaction.

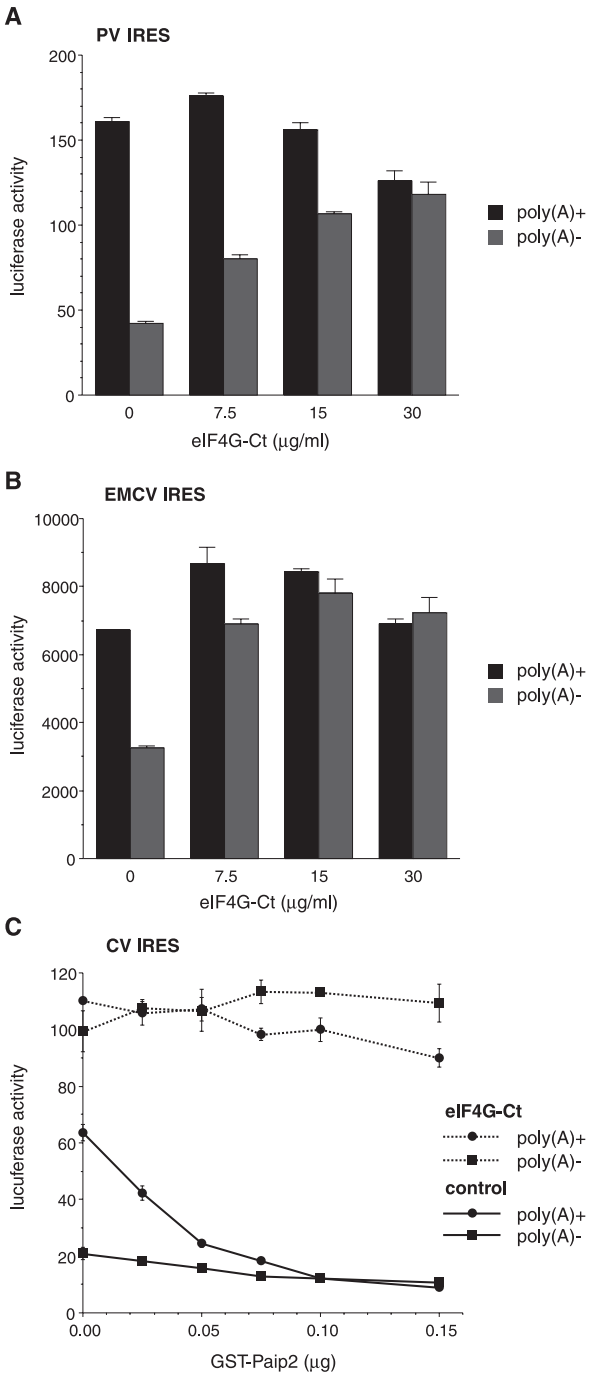

FIGURE 4. The eIF4G C-terminal fragment (eIF4G-C_t) renders IRES-directed translation poly(A) tail independent. eIF4G-C_t titration of reactions that were programmed with PV (**A**) or EMCV (**B**) IRES containing poly(A)+ or poly(A)− luciferase mRNA (2 μg/mL). **C**: GST-Paip2 titration of reactions supplemented with either control buffer or eIF4G-C_t (30 μg/mL) and programmed with CV IRES-containing poly(A)+ or poly(A)− luciferase mRNA (2 μg/mL). Average of three (**A** and **B**) or four (**C**) independent determinations with the standard deviation from the mean are shown.

As shown here (Figs. 2 and 3) and in earlier reports (Liebig et al., 1993; Ziegler et al., 1995a, 1995b; Borman et al., 1997) incubation of cell extracts with HRV $2A^{pro}$ or FMDV L^{pro}, which cleave eIF4G, stimulates

HRV/PV IRES activity. For poly(A)+ mRNA, this is the opposite of the expected inhibition of translation, as the stimulation of IRES activity by the PABP/poly(A) tail is abrogated. A plausible explanation for this effect is that an inhibitor of poly(A)− mRNA translation is inactivated by eIF4G cleavage. Candidates for such an inhibitor could be two nonessential putative RNA helicases, Ski2p and Slh1p, which were shown to inhibit translation of poly(A)− mRNA in yeast (Searfoss & Wickner, 2000). Also, the inhibitor could be a protein associated with the N-terminal portion of eIF4G (eIF4G-N_t) or eIF4G-N_t itself. For example, it is possible that eIF4G binding to the IRES is tighter upon the removal of eIF4G-N_t, and that this high affinity binding compensates for the loss of eIF4G interaction with the mRNA via the PABP-poly(A) complex. In this regard, it was suggested that the N-terminus of eIF4G inhibits translation of poliovirus RNA because it interacts with eIF4E, which induces a conformational change in eIF4G (Jackson, 2000). It is noteworthy that eIF4G cleavage should preclude the formation of a circular ribonucleoprotein complex, unless other proteins are involved. This suggests that mRNA circularization per se is not a prerequisite for efficient IRES activity with the cleaved eIF4G.

Mutations in yeast eIF5 and eIF5A, which cause a defect in 60S subunit joining, negate the stimulatory effect of poly(A) on translation (Searfoss et al., 2001). It was argued, therefore, that PABP functions to promote the 60S subunit joining step. Our results are not consistent with a model in which eIF4G is dispensable for poly(A)-mediated stimulation of translation (Searfoss et al., 2001). Also, the importance of eIF4G for poly(A)-dependent translation was demonstrated in vivo in *Xenopus* oocytes (Wakiyama et al., 2000). In this system, the expression of an eIF4GI mutant that is incapable of PABP binding inhibited translation of poly(A)+, but not poly(A)− mRNA. Finally, if the PABP/poly(A) complex were to modulate the 60S subunit joining step in our system, then translation from the HCV IRES would also be inhibited by Paip2, which is not the case (Fig. 3; Khaleghpour et al., 2001b). The discrepancy between the models, which were invoked to explain the mechanism of poly(A) enhancement of translation, could be due to the fact that when 60S ribosomal subunit joining is inhibited (as in the eIF5 or eIF5B mutants, or by diminishing 60S ribosome biogenesis), 60S ribosome joining becomes rate-limiting for initiation. Thus, under these conditions, a difference between poly(A)+ and poly(A)− mRNAs in 40S recruitment might be difficult to detect.

It is noteworthy that, in cells, the PV genome might be functionally circularized through a unique protein–protein bridge (Barton et al., 2001; Herold & Andino, 2001). This bridging is effected by the simultaneous interaction of PABP with the poly(A) tail and the cloverleaf ribonucleoprotein complex that is formed by the 5′ cloverleaf RNA structure, the viral protease–polymerase precursor (3CD) and the poly(C)-binding protein (PCBP; Barton et al., 2001; Herold & Andino, 2001). Both the cloverleaf RNA and the poly(A) tail are important *cis*-acting elements for RNA replication (Spector et al., 1975; Sarnow, 1989; Andino et al., 1993; Barton et al., 1996; Paul et al., 1998), and their proximity may be a prerequisite for both negative- and positive-strand RNA synthesis (Herold & Andino, 2001). In addition, the 3CD–cloverleaf RNA interaction down regulates translation (Gamarnik & Andino, 1998). It is thus possible that eIF4G cleavage and subsequent 3CD accumulation in the middle of the infectious cycle leads to PABP–cloverleaf RNP complex formation to yield ribosome-free replication competent RNA templates (Barton et al., 1999).

In conclusion, we have shown that eIF4G cleavage by HRV $2A^{pro}$ abolishes the preferential translation of poly(A)+ picornavirus mRNAs. Significantly, because eIF4G cleavage is an early event in the cycle of the infection of entero- and rhinoviruses (Belsham & Jackson, 2000), the bulk of viral protein translation is PABP/poly(A) independent. This suggests that the partial $2A^{pro}$/$3C^{pro}$-mediated cleavage of PABP, which lags behind eIF4G cleavage in PV- and CV-infected cells (Joachims et al., 1999; Kerekatte et al., 1999), does not play a role in virus-specific translation. In cardiovirus-infected cells, where eIF4G is not cleaved (Mosenkis et al., 1985), IRES activity apparently remains responsive to stimulation by the PABP/poly(A) tail throughout the infectious cycle.

Cleavage of eIF4G also occurs in apoptosis by caspase-3 (Marissen & Lloyd, 1998; Bushell et al., 1999, 2000) and could explain the reduction in the rate of protein synthesis during apoptosis (Clemens et al., 2000). It remains to be seen whether the caspase-3 generated cleavage eIF4G fragments can support cap-independent translation, such as that from cellular IRES, in a poly(A) tail-independent fashion.

While this article was in preparation, Michel et al. (2001) reported that treatment of a partially depleted rabbit reticulocyte lysate with HRV $2A^{pro}$ abolished the stimulation of PV IRES activity by the poly(A) tail. Our data are in agreement with these results. An important extension of our study is the demonstration that the activity of a cellular inhibitor of PABP function, Paip2, is negated by eIF4G cleavage, providing further evidence for the importance of eIF4G–PABP interaction for efficient translation. In addition, we also demonstrate that eIF4G-C_t addition is sufficient to negate the stimulatory effect of the poly(A) tail on translation (Fig. 4). It is important to note that our ascites cell translation system exhibits cap-poly(A) tail synergy even after treatment with micrococcal nuclease. Thus, this system can be used in conjunction with the depleted reticulocyte lysate and HeLa cell extract to study the mechanism of cap-poly(A) tail synergy.

MATERIALS AND METHODS

Plasmids

Plasmids encoding luciferase, T3luc, T3luc(A)$^+$, T3CVluc, and T3CVluc(A)$^+$ were described previously (Iizuka et al., 1994). T7PV (poliovirus IRES) luc, T7PVluc(A)$^+$, T7EMCV (encephalomyocarditis virus IRES) luc, T7EMCVluc(A)$^+$, T7HCV (hepatitis C virus IRES) luc, and T7HCVluc(A)$^+$ were constructed as follows. The IRES of PV type 2 Lansing (nt 71–732), EMCV (nt 281–848), and HCV (nt 40–372) were amplified by polymerase chain reaction with the templates pGemCAT-Polio-Luc (Pause et al., 1994a), pGemCAT-EMCV-Luc (Pause et al., 1994a), and pXL40-372.NS′ (Reynolds et al., 1995), respectively. The authenticity of the amplified DNA fragments was confirmed by sequencing. The fragments were cloned between the T7 promoter and the luciferase coding sequence of pSP72-LUC and pSP72-LUC-A (Imataka et al., 1998).

Proteins

Expression and purification of HRV2 2A^{pro} was described previously (Liebig et al., 1993). Paip2, was expressed in *Escherichia coli* as a fusion protein with GST and purified on a glutathione-Sepharose 4B column (APB) according to the manufacturer's recommendations (Khaleghpour et al., 2001b). Expression and purification of the recombinant protein representing the C-terminal two-thirds of eIF4GI with the N-terminal His-tag sequence, eIF4G-C$_t$ (previously termed as eIF4G$_{457–1396}$) was described (Pestova et al., 1996b).

In vitro transcription and translation

The luciferase encoding plasmids of the T3 and T7 series were linearized with *Bam*HI and transcribed with T3 or T7 RNA polymerase. Synthesis of uncapped or capped RNA transcripts (Promega) was performed with the RiboMAX system. RNA integrity was verified by electrophoresis on a denaturing agarose gel. In vitro translation reactions (12.5 μL) contained mRNA, at the concentrations indicated in the figure legends, Krebs-2 cell extract, unlabeled amino acids, and other components as specified previously (Svitkin et al., 1984). Following incubation at 30 °C for 60 min, 3-μL aliquots of the translation mixtures were assayed for luciferase activity using the luciferase assay kit (Boehringer Mannheim) in a Lumat LB 9507 bioluminometer (EG&G Bertold). Data for luciferase activity are given in arbitrary units (one arbitrary unit corresponds to 1,000 light units).

ACKNOWLEDGMENTS

We thank Francine Nault for excellent technical assistance and Reed Wickner for communicating data before publication and for critical comments. This research was supported by a grant from the Canadian Institute of Health Research to N.S. N.S. is the recipient of a Canadian Institute of Health Research Distinguished Scientist Award and a Howard Hughes Medical Institute International Scholarship.

Received July 24, 2001; returned for revision August 20, 2001; revised manuscript received September 27, 2001

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