Picornavirus IRESes and the poly(A) tail jointly promote cap-independent translation in a mammalian cell-free system

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Picornavirus IRESes and the poly(A) tail jointly promote cap-independent translation in a mammalian cell-free system

GIOVANNA BERGAMINI, THOMAS PREISS, and MATTHIAS W. HENTZE
Gene Expression Programme, European Molecular Biology Laboratory, D-69117 Heidelberg, Germany

ABSTRACT

In eukaryotic cells, efficient translation of most cellular mRNAs requires the synergistic interplay between the m^7GpppN cap structure and the poly(A) tail during initiation. We have developed and characterized a cell-free system from human HeLa cells that recapitulates this important feature, displaying more than one order of magnitude of translational synergism between the cap structure and the poly(A) tail. The stimulation of cap-dependent translation by the poly(A) tail is length-dependent, but not mediated by changes in mRNA stability. Using this system, we investigated the effect of the poly(A) tail on the translation of picornaviral RNAs, which are naturally polyadenylated but initiate translation via internal ribosome entry sites (IRESs). We show that translation driven by the IRESs of poliovirus (PV), encephalomyocarditis virus (EMCV), and hepatitis A virus is also significantly augmented by a poly(A) tail, ranging from an approximately 3-fold stimulation for the EMCV-IRES to a more than 10-fold effect for the PV IRES. These results raise interesting questions concerning the underlying molecular mechanism(s). The cell-free system described here should prove useful in studying these questions as well as providing a general biochemical tool to examine the translation initiation pathway in a more physiological setting.

Keywords: encephalomyocarditis; internal ribosome entry site; hepatitis A virus; poliovirus; poly(A) tail; virus translation

INTRODUCTION

Translation initiation of capped mRNAs is greatly stimulated by the poly(A) tail (Gallie, 1991; Tarun & Sachs, 1995; Sachs et al., 1997). In eukaryotic cells of fungal, plant, and animal origin, the cap structure and the poly(A) tail synergize to drive translation initiation (Deshpande et al., 1979; Drummond et al., 1985; Galili et al., 1988; Gallie, 1991). This synergism involves the cap-binding protein eIF4E and the poly(A)-binding protein Pab1p/PABP. A simultaneous interaction of the translation initiation factor eIF4G with eIF4E and Pab1p/PABP has first been found in yeast (Tarun & Sachs, 1996), and has subsequently been reported for plant and mammalian cells (Le et al., 1997; Imataka et al., 1998). These interactions appear to account, at least in part, for the synergism between the 5′ cap and the poly(A) tail, although the exact mechanism(s) by which this synergy is achieved remains to be defined (Tarun & Sachs, 1995; Sachs et al., 1997; Preiss & Hentze, 1998; Otero et al., 1999).

Picornaviral mRNAs also have a poly(A) tail at their 3′ end. Picornaviridae are single-stranded positive sense RNA viruses that are among the most diverse and oldest "known" viruses, consisting of more than 200 viruses that include human and agricultural pathogens (Stanway, 1990). The viral genome is modified at the 5′ end by a covalently attached protein, Vpg, instead of the cap structure (Nomoto et al., 1977). Upon entry into the cell, the viral genome efficiently competes with cellular mRNAs for the translational machinery. Expression of the viral genome results in a large single polyprotein that is cleaved cotranslationally by virally encoded proteases. The efficiency of picornavirus translation and RNA synthesis is astounding, resulting in the amplification of a single viral particle into thousands of infectious viruses within a few hours of infection (Andino et al., 1999).

To drive translation initiation, the picornaviral 5′ UTRs contain a *cis*-acting element known as an internal ribosome entry site (IRES) (Pelletier & Sonenberg, 1988; Jang & Wimmer, 1990; Belsham & Sonenberg, 1996).

Reprint requests to: Matthias W. Hentze, Gene Expression Programme, European Molecular Biology Laboratory, Meyerhofstraße 1, D-69117 Heidelberg, Germany; e-mail: Hentze@EMBL-Heidelberg.de.

On the basis of primary sequence, predicted secondary structure, and biological properties, the picornavirus IRESs have been divided into three classes (Hellen & Wimmer, 1995; Borman et al., 1995, 1997a): the enterovirus and rhinovirus IRESs, including the prototypic poliovirus (PV) IRES; the cardiovirus and aphtovirus IRESs, to which the encephalomyocarditis virus (EMCV) IRES belongs, and the hepatitis A virus (HAV) IRES (Jackson et al., 1994). Several proteins that appear to participate in IRES-mediated translation initiation have been reported. These proteins differ between classes of IRESs and include the canonical initiation factors eIF4G, eIF4A, eIF-2, and eIF-3 (Scheper et al., 1992; Pause et al., 1994; Pestova et al., 1996a, 1996b), and other cellular proteins like the poly(rC)-binding proteins PCBP1 and PCBP2 (Blyn et al., 1997; Parsley et al., 1997; Walter et al., 1999), the La autoantigen (Meerovitch et al., 1993; Svitkin et al., 1994), the polypyrimidine tract-binding protein PTB (Hunt & Jackson, 1999), and the RNA-binding protein UNR (Hunt et al., 1999). The identification of factors involved in IRES-mediated translation initiation and a description of the functional roles of those proteins already known to interact with the IRES is not yet complete. In general, mammalian cells possess the machinery required for IRES-directed translation, although some IRESs are not active in all cell types, and tissue-specific activity profiles have been described for some IRESs (Borman et al., 1997b; Creancier et al., 2000).

As a starting point for biochemical examinations of the function of the poly(A) tail in the translation of mammalian mRNAs and picornaviral RNAs, we aimed to establish an in vitro system from mammalian cells in which translation is dependent on the presence of the poly(A) tail at the 3′ end of the messenger. Previously, poly(A) tail-dependent translation extracts have been reported for *Saccharomyces cerevisiae* (Iizuka et al., 1994) and *Drosophila melanogaster* (Gebauer et al., 1999; Castagnetti et al., 2000; Lie & Macdonald, 2000), but no such system was available for mammalian cells. We describe and characterize here a cell-free translation system from HeLa cells that is simple to prepare and displays strong (greater than 10-fold) translational synergy between the cap structure and the poly(A) tail. Using this system, we show that the translation of picornaviral RNAs from the three different classes of IRESs is strongly stimulated by their poly(A) tails.

RESULTS

A HeLa cell-derived translation extract that displays strong synergy between the cap structure and the poly(A) tail

Based on our earlier work with *Drosophila* embryos and ovary translation extracts (Gebauer et al., 1999; Castagnetti et al., 2000), we reasoned that a gentle hypotonic lysis of cells might most likely yield a mammalian translation extract that preserves the synergism between the cap structure and the poly(A) tail as seen in vivo. We therefore prepared a translation extract from a suspension culture of HeLa cells as outlined in Figure 1 and described in detail in Materials and Methods. Table 1 shows the optimization of various parameters for the translation assay in the HeLa extracts. In vitro-translation assays were subsequently carried out with four types of reporter mRNAs containing a 5′ m^7GpppG cap (Cap) or an ApppG cap analog (Acap), that does not bind eIF4E, and with (pA) or without a poly(A) tail of 98 residues. A time-course experiment with these four types of otherwise identical luciferase mRNAs is shown in Figure 2A (note: the right and the left panel depict the same data, but the scales on the Y-axis differ). Luciferase expression from Cap-luc-pA mRNA is approximately 10–20 times higher than the luciferase activity yielded by either Acap-luc-pA or Cap-luc mRNAs, that is, those mRNAs that lack either a functional cap structure or a poly(A) tail. The synergy (translational

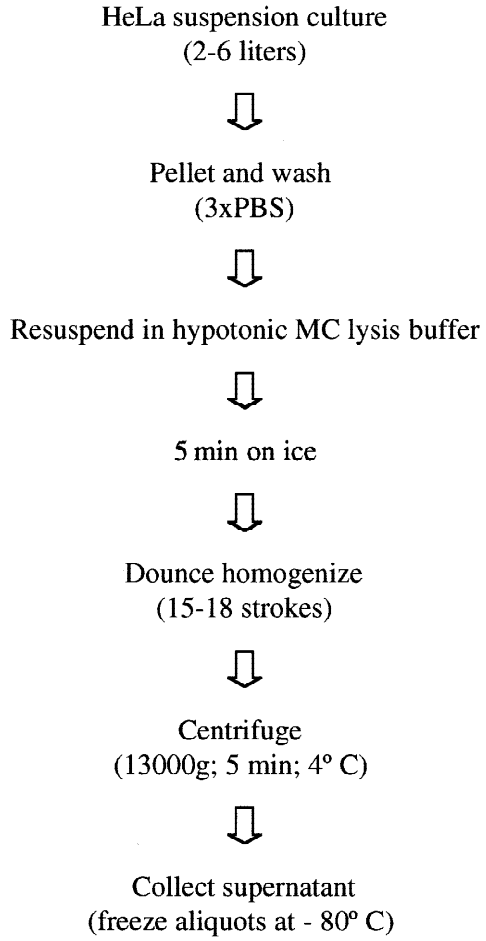

FIGURE 1. Outline of the preparation of translation extracts from HeLa cells. For more details, see Materials and Methods.

TABLE 1. Optimization of translation in HeLa cell extracts.

Component	Range tested	Optimal[a]
Creatine phosphate	5–30 μM	20 μM
Creatine kinase	0.08–0.12 μg/μL	0.1 μg/μL
K-acetate[b]	0–125 mM	25–50 mM
Mg-acetate[b]	0–5 mM	2.5 mM
Spermidine	0–0.3 mM	0.1 mM
Amino acids	15–120 μM	100 μM
HEPES buffer, pH 7.6	14–20 mM	16 mM
mRNA template[b]	1–150 ng/μL	1–4 ng/μL
Temperature	30–37 °C	37 °C
Incubation time[b]	0–300 min	60–90 min

[a]Optimal is defined as maximal luc activity using a Cap-luc-p(A) mRNA when all other parameters are kept constant.
[b]Should be optimized for each mRNA template.

output from Cap-luc-pA mRNA divided by the sum of the luciferase activities from Acap-luc-pA and Cap-luc mRNAs) is therefore around 10–20-fold. By contrast, the luciferase activity from Acap-luc mRNA that lacks both a functional cap structure and a poly(A) tail is barely detectable above background.

To assess the stability of the differently end-modified mRNAs, samples were taken at different time points of the translation reactions. After addition of an RNA recovery control, RNA was extracted and analyzed by Northern blotting (Fig. 2B). This analysis shows that the stabilities of the mRNAs bearing a cap structure or the ApppG cap analog are sufficiently similar to conclude that the synergy found in the luciferase assays reflects bona fide translational synergy. It is also apparent that the lack of any cap structure (−luc-pA and −luc mRNAs) renders the RNAs very unstable. The poly(A) tail exerts a minor but reproducible stabilizing effect on capped mRNAs (compare Cap-luc with Cap-luc-pA, and Acap-luc with Acap-luc-pA RNAs). We conclude that translation in the HeLa cell extracts appears to faithfully recapitulate the in vivo synergism between the cap and the poly(A) tail. Similar results were obtained with translation extracts prepared by the same procedure from the human astrocytoma cell line MG373 (data not shown), suggesting that the extract preparation procedure can be applied to different mammalian cell types.

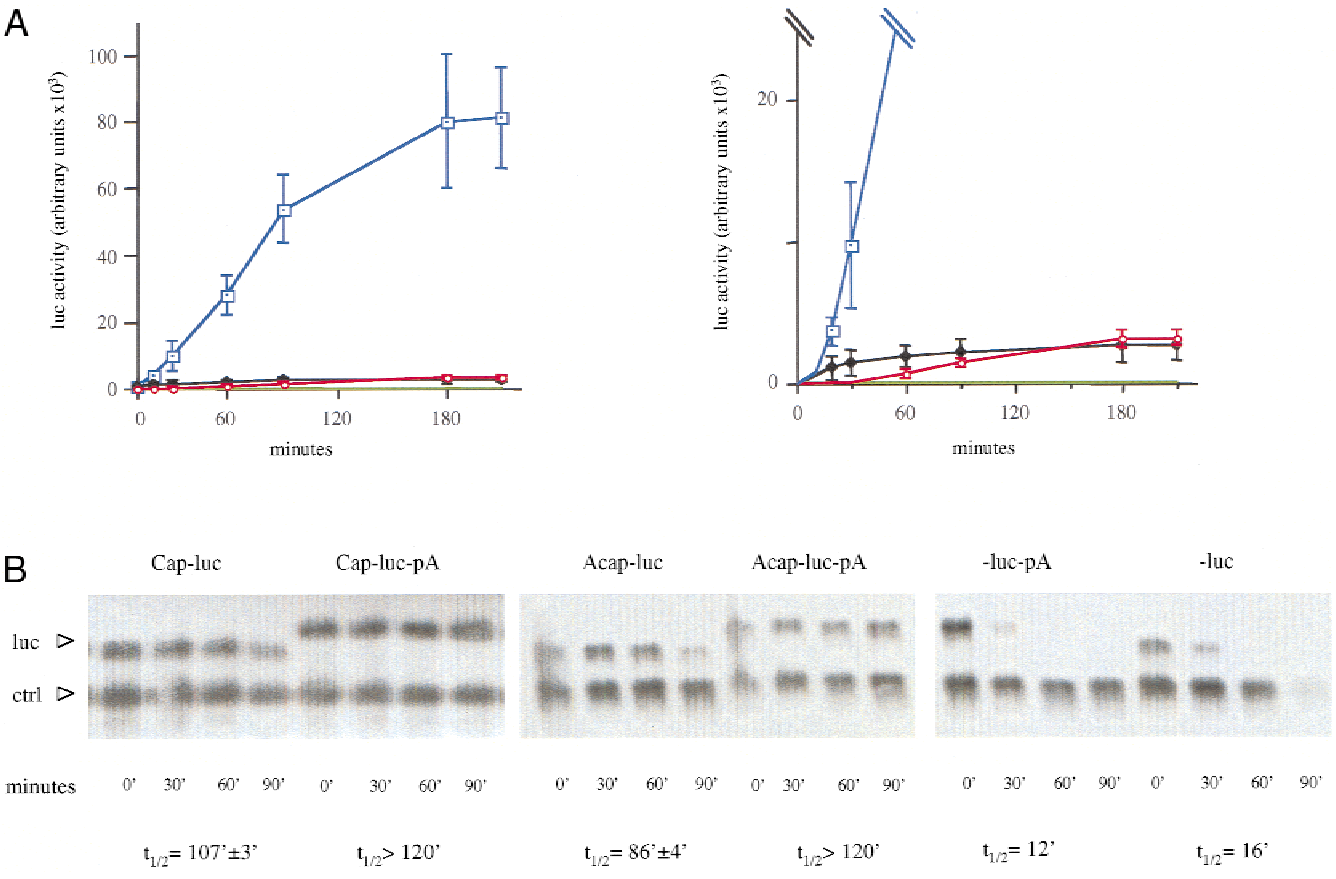

FIGURE 2. Translational synergism between the cap structure and the poly(A) tail in the HeLa cell-free system. **A**: Time course of translation of luciferase mRNAs containing the following end modifications: Cap, m^7GpppG (black line with full diamond); Cap-pA, m^7GpppG and poly(A) tail (blue line with dotted square); Acap, ApppG, (green dotted line); Acap-pA (red line with empty circle). Aliquots of the translation reactions were taken at 0, 10, 20, 30, 60, 90, 180, and 210 min to measure luciferase activity (light units). **B**: Same data as in **A**, with different scale of Y axis. **C**: Physical stabilities of the RNAs used in **A** analyzed by Northern blotting. As a control for the extraction procedure, a CAT RNA was added to each sample.

To further characterize the properties of these extracts, we tested the influence of mRNA concentration, incubation temperature and incubation time, buffer conditions, cation concentrations, and so forth on the translation of Cap-luc-pA mRNA in the HeLa cell extract. The results are summarized in Table 1. To evaluate whether features that may be particular to the luciferase mRNAs influence the experimental outcome, we also tested a series of CAT reporter mRNAs. These mRNAs are totally unrelated to the luciferase mRNAs and bear 5′ and 3′ untranslated regions of different lengths and nucleotide sequences. Comparison of Cap-CAT mRNA with Cap-CAT-pA mRNA bearing a tail of 98 A-residues shows that the latter yields an approximately 10-fold higher CAT activity than the former (Fig. 3), confirming the data obtained with the luciferase reporter mRNAs. Consistent with findings in yeast and *Drosophila* embryo translation systems (Iizuka et al., 1994; Gebauer et al., 1999), an A_{15} tail does not suffice to stimulate translation. Increasing the length of the poly(A) tail between 31 and 98 A-residues causes increasingly stronger translational enhancement (Fig. 3). This shows that the HeLa translation system not only recapitulates cap structure/poly(A) tail synergism, but reflects differences in poly(A) tail length.

The poly(A) tail promotes translation from the PV IRES

To address the question of whether the poly(A) tail affects translation initiation from the PV IRES, the 5′ region of PV (Mahoney strain) containing the IRES plus the region encoding the first 37 amino acids of the viral nucleocapsid was cloned upstream of the luciferase reporter RNA bearing an A_{98} tail or lacking a poly(A) tail (Fig. 4).

The monocistronic IRES RNAs were capped with the analog ApppG to avoid potential cap-mediated ribosome entry and to stabilize the RNAs. Concentration of salts, in particular Mg^{2+}, showed the most influence on translation efficiency of these mRNAs. As shown in the time-course experiment in Figure 4A and the corresponding RNA stability analysis in Figure 4B, the translation of PV.IRES-pA RNA is about 10-fold more efficient than the translation of the nonpolyadenylated counterpart PV.IRES. Because about three times more RNA

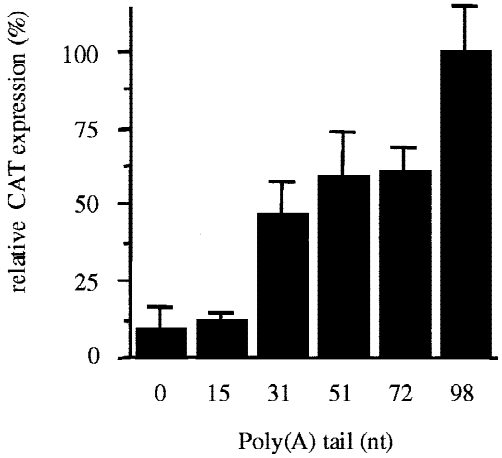

FIGURE 3. The length of the poly(A) affects the translational efficiency of capped mRNAs in HeLa extracts. Capped CAT mRNAs with poly(A) tails ranging from 0 to 98 adenosines were added to translational reactions. CAT protein levels were measured by ELISA and are expressed relative to the value obtained for the Cap-CAT-pA_{98} mRNA.

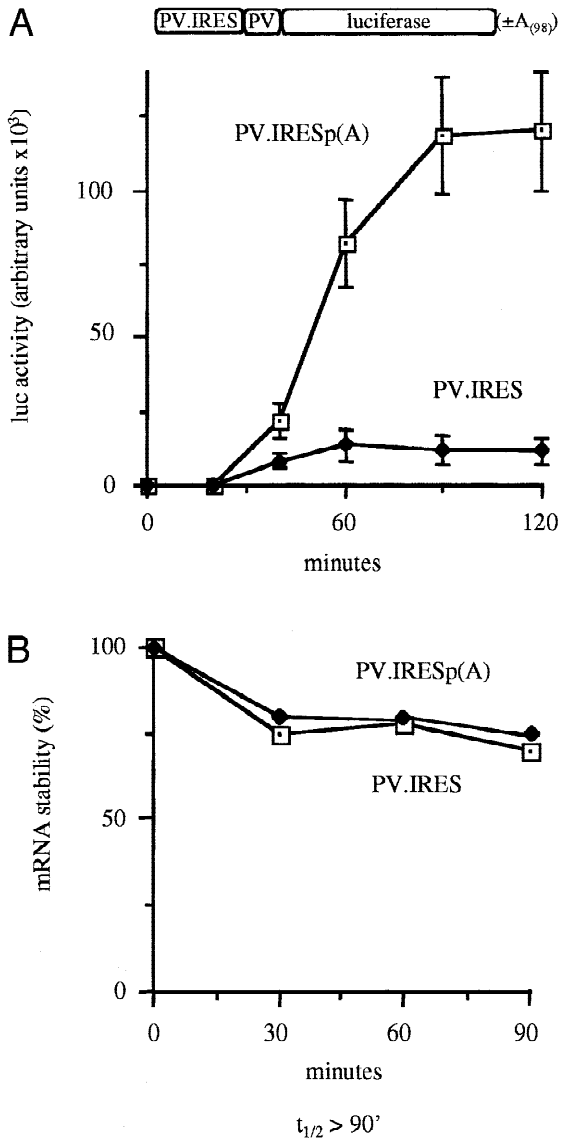

FIGURE 4. Translation driven from PV IRES is poly(A) dependent. **A**: Translational time course of trace-labeled mRNAs PV.IRES-luc (full diamonds) and PV.IRES-luc-pA (dotted squares). Aliquots were taken at 20, 40, 60, and 90 min and used to measure the luc activity. **B**: Physical stabilities of the mRNAs used in **A** have been compared by Northern blot as in Figure 2.

was used than in the experiments with capped luciferase mRNAs, the results also indicate that, like cap-mediated translation, the PV IRES-mediated translation is efficient.

We next performed competition experiments titrating the cap analog m^7GpppG (or ApppG as a specificity control), and setting the luciferase activity obtained in the absence of any added analog at 100%. As shown in Figure 5, the translation of both PV.IRES and PV.IRES-pA RNAs is unaffected by increasing amounts of m^7GpppG competitor. By contrast, this competitor inhibits the cap-dependent translation of Cap-luc and Cap-luc-pA mRNAs (Fig. 5, bottom panels). Addition of ApppG does not affect cap-dependent translation, but reproducibly stimulates the translation from the PV IRES; the reason for this is currently unknown.

We conclude that the presence of a poly(A) tail strongly stimulates translation from the PV IRES.

Poly(A) tail stimulation of translation is a general feature of picornavirus IRESs

The PV IRES is the prototype of class I picornavirus IRESs. We next examined whether translation mediated by IRESs from the other two classes was also augmented by the poly(A) tail. The 5′ region of HAV containing the IRES element plus the region encoding the first 49 amino acids of the nucleocapsid was cloned upstream of and in frame with the luciferase open reading frame (ORF), with or without an A_{98} tail at the 3′ end (Fig. 6). The time-course experiment comparing HAV.IRES and HAV.IRES-pA RNAs shows the approximately 10-fold higher translation of the polyadenylated RNA, without corresponding differences in RNA stability (data not shown). As in the case of the PV IRES, translation from the HAV IRES was very efficient (comparable amounts of mRNA were used in both cases).

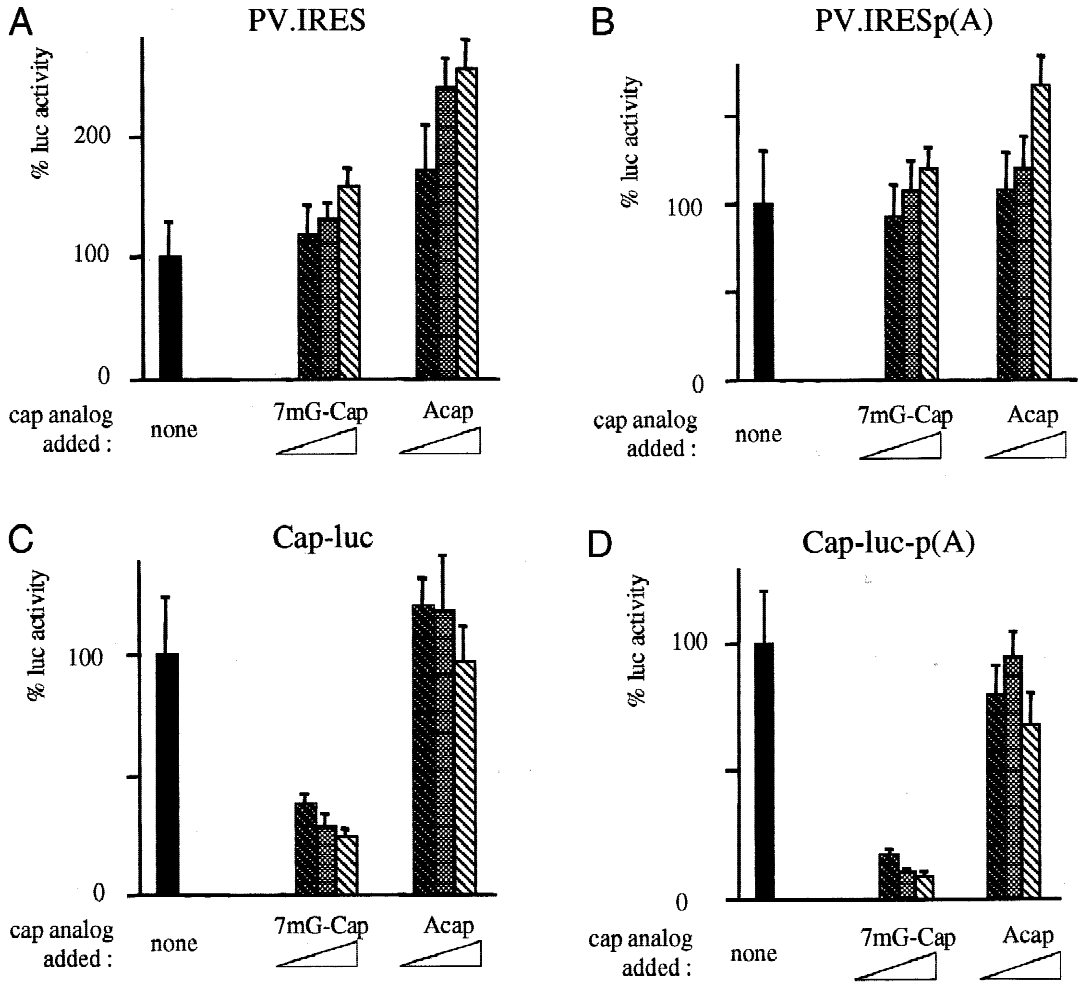

FIGURE 5. The cap analogs m^7GpppG (indicated as +Cap) and ApppG (+Acap) were added in increasing concentrations at the beginning of translational reactions containing each of the mRNAs PV.IRES-luc (**A**), PV.IRES-luc-pA (**B**), Cap-luc (**C**), and Cap-luc-pA (**D**). Black bar: 0 mM; stripes on black background: 0.25 mM; gray bar: 0.5 mM; stripes on white background, 1 mM.

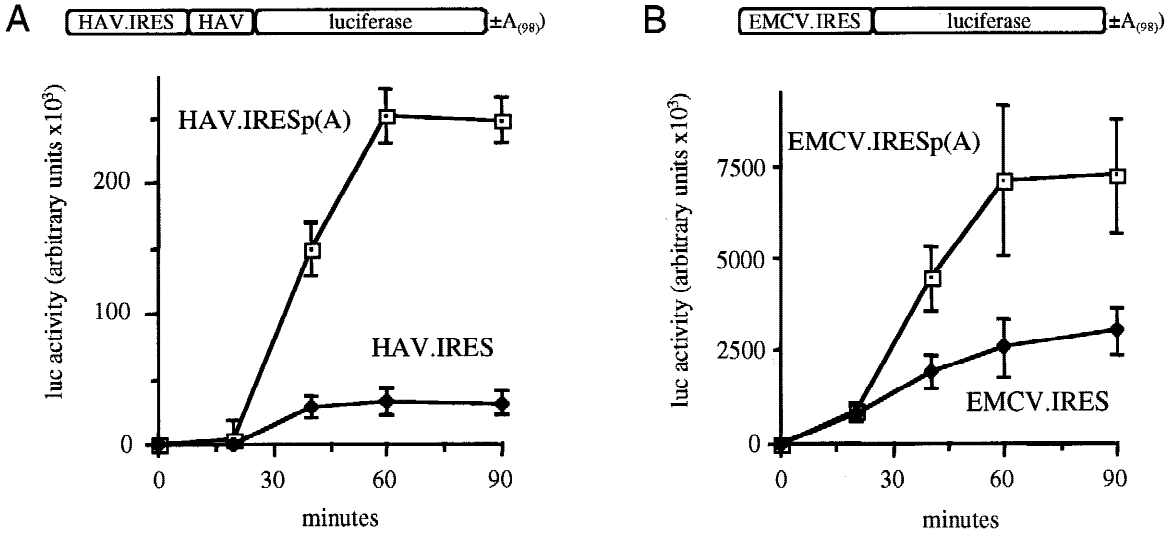

FIGURE 6. Translation driven from HAV IRES and EMCV IRES is stimulated by the poly(A) tail. Translational time course of HAV.IRES (full diamonds) and HAV.IRES-pA (dotted squares) RNAs (**A**) and, EMCV.IRES (full diamonds) and EMCV.IRES-pA (dotted squares) RNAs (**B**). Aliquots were taken at 20, 40, 60, 90, 120, and 180 min and used to measure luciferase activity.

Examination of mRNAs with the EMCV shows that these RNAs appear to be extraordinarily well translated, and that the stimulatory effect of the poly(A) tail is significant, but quantitatively less impressive than that seen with the PV and the HAV IRESs (Fig. 6B). We conclude that the stimulation of translation from picornavirus IRESs is a general feature that includes all three classes. This stimulation is moderate (~3-fold) for the EMCV IRES to strong (>10-fold) for the PV IRES. Unexpectedly, the stimulatory effect of the poly(A) tail on translation driven by the PV and the HAV IRES appears to approach that of the cap-dependent translation of Cap-luc and Cap-CAT mRNAs.

In contrast to the translation from the PV (Fig. 5) and the EMCV (Fig. 7B) IRES, we noticed that the translation of both the polyadenylated and the nonpolyadenylated versions of HAV-luciferase reporter RNAs were sensitive to the addition of m^7GpppG, but not ApppG competitor (Fig. 7A). Because the reporter RNAs bear an ApppG cap, this result cannot be explained by an inadvertent 5′ end-mediated translation of the HAV RNAs. This surprising result rather appears to indicate that HAV IRES-mediated translation involves a cofactor that cannot function in m^7GpppG-treated extracts.

DISCUSSION

A novel mammalian cell-free system to examine translation

We describe the preparation of translation extracts from HeLa cells that display critical and characteristic features of translation in vivo: (a) Translation is stimulated by the m^7GpppG cap structure; (b) translation is stimulated by the poly(A) tail; (c) the cap structure and the poly(A) tail act synergistically; (d) translation is affected by the length of the poly(A) tail; and (e) the three classes of picornavirus IRESs mediate efficient translation of RNAs that lack a m^7GpppG cap structure. Therefore, it appears as though this novel translation system should be suitable to study the translation of mammalian and viral mRNAs in a more "physiological setting" than cell-free translation systems that have traditionally been used. Because we have obtained similar results with extracts prepared from an astrocytoma cell line growing in monolayer cultures (data not shown), we believe that the simple and gentle method described here should also prove useful for the preparation of translation extracts from other sources.

Comparing our preparation protocol with that for other translation extracts, it is not obvious why the procedure described here yields an extract with the described properties. The indicator mRNAs used to assay for synergy between the cap structure and the poly(A) tail had previously been found to be suitable for this purpose in translation extracts from yeast (Iizuka et al., 1994; Preiss & Hentze, 1998), *Drosophila* embryos (Gebauer et al., 1999), and *Drosophila* ovaries (Castagnetti et al., 2000). However, because the luciferase and the CAT reporter mRNAs share no sequence or apparent structural similarities, we regard it as unlikely that an unrecognized denominator that is common between these two mRNAs but might be lacking from others would solely explain our results. In fact, the same reporter mRNAs fail to reflect translational synergism between the cap structure and the poly(A) tail in commercially available extracts from rabbit reticulocytes or wheat germ (data not shown). At present, we do not know

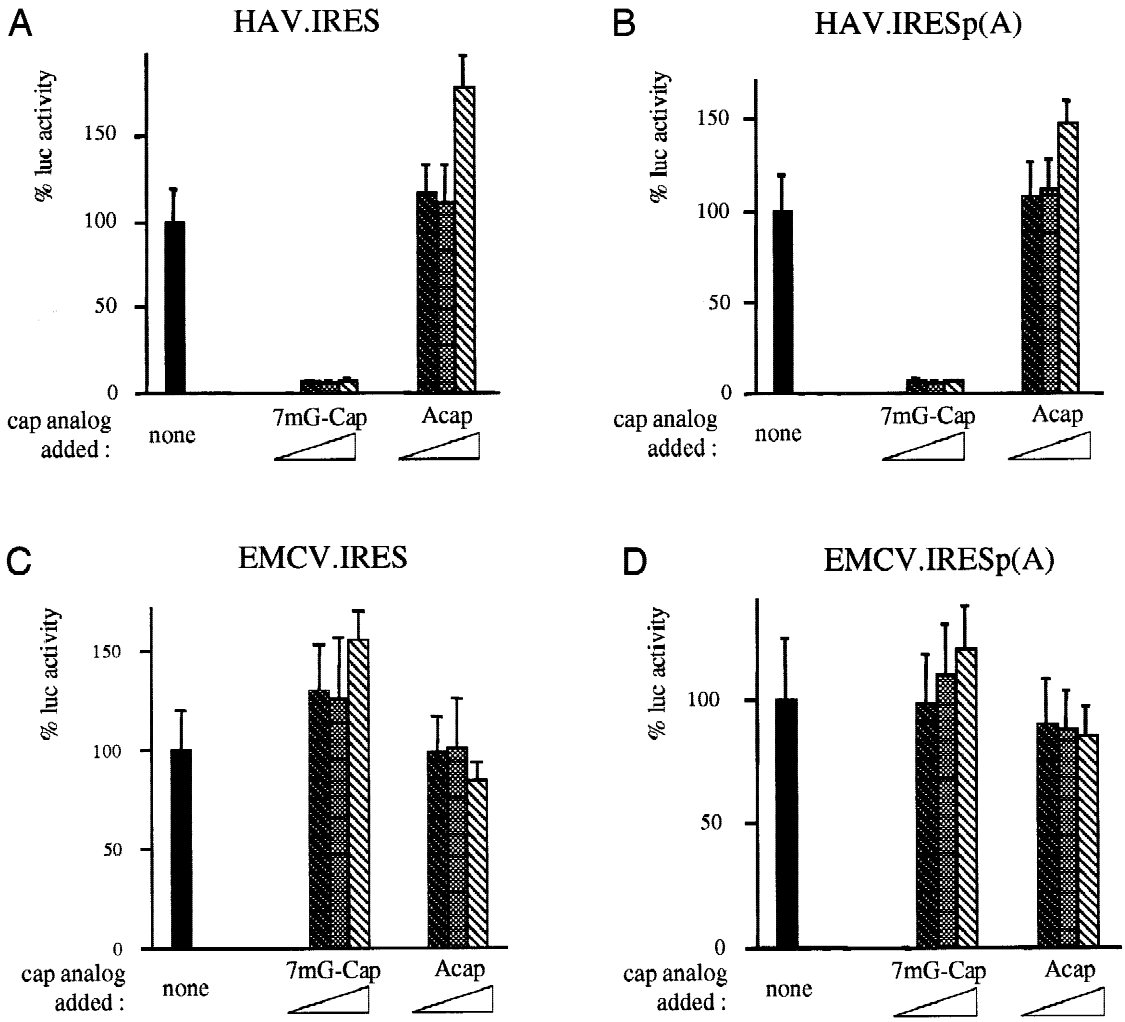

FIGURE 7. Translation from HAV IRES, but not EMVC IRES, is inhibited by addition of m^7GpppG to the HeLa extracts. The cap analogs m^7GpppG (indicated as +Cap) and ApppG (+Acap) were added at various concentrations to translational reactions containing each the mRNAs HAV.IRES (**A**), HAV.IRES-pA (**B**), EMCV.IRES (**C**), and EMCV.IRES-pA (**D**). Black bar: 0 mM; stripes on black background: 0.25 mM; gray bar: 0.5 mM; stripes on white background: 1 mM.

whether the preservation of endogenous mRNAs is important for the conservation of the "physiological features" of our translation system, because initial experiments using micrococcal nuclease treatment have led to a profound loss of translation activity. Further experiments will be necessary to answer this question.

During the final preparation of this manuscript, Michel et al. (2000) described a procedure to render rabbit reticulocyte lysate poly(A) tail dependent and to observe synergism between the cap structure and the poly(A) tail. Although the two systems appear to be similar with regard to these properties, they differ in several other aspects. First, the extract system described by Michel et al. is based on the depletion of ribosomes and associated factors by centrifugation. Therefore, the stoichiometry of components of the translation machinery is altered, and this alteration is important for the function of this system. In our system, emphasis is placed on preserving physiological conditions as much as possible. Second, our translation system displays its features at maximal levels of translational activity, whereas the depletion strategy used by Michel et al. diminishes the activity of their translation system. Third, the depleted translation system of Michel et al. displays its features even after removal of the endogenous mRNAs by micrococcal nuclease treatment, whereas the same treatment has dramatically reduced the activity of our extracts until now. Therefore, the two novel strategies reported by Michel et al. (2000) and here should serve as complementary tools to address mechanistic questions on the function of the poly(A) tail in the translation of cellular and viral mRNAs in mammalian cells.

The poly(A) tail enhances translation from picornaviral IRESs

All three classes of picornaviral IRESs mediate the translation of their viral genomes by cap-independent mech-

anisms (Jang & Wimmer, 1990; Jackson et al., 1994; Borman et al., 1995; Belsham & Sonenberg, 1996). All of these viral RNAs are polyadenylated, which has been reported to be important for infectivity (Spector & Baltimore, 1974; Hruby & Roberts, 1976), but a possible function of the poly(A) tail in translation has not yet been systematically addressed. Tobacco etch virus, a plant relative of animal picornaviruses, also has a polyadenylated RNA that is translated cap independently. Using RNA transfection, Gallie et al. (1995) reported that the poly(A) tail of this virus stimulates translation. Michel et al. (2000) tested the translation from the EMCV IRES in their cell-free system and noted a moderate stimulatory effect of the poly(A) tail on translation, consistent with our findings (Fig. 6). We observed highly efficient translation of the EMCV IRES-driven RNA lacking a poly(A) tail, which may account for the limited augmentation of translation by the addition of a poly(A) tail. The highly efficient translation mediated by the EMCV IRES is a pathophysiological characteristic observed in cells, but not reflected in rabbit reticulocyte lysates (Svitkin et al., 1978; Gingras et al., 1996). Our analysis of the IRESs of PV and HAV has yielded additional and partially surprising insights. First, it revealed that the effect of the presence of a poly(A) tail on these two other IRESs is substantially greater than on the EMCV IRES, and even approximates the poly(A) tail effect on cap-dependent translation. We have not yet tested the effect of eIF4G cleavage on the translational enhancement by the poly(A) tail. Cleavage of cellular eIF4GI and eIF4GII (Lamphear et al., 1995; Svitkin et al., 1999) as well as PABP (Joachims et al., 1999; Kerekatte et al., 1999) is a feature of polioviral infection, but does not occur following infection by EMCV or HAV. Whereas the EMCV IRES is functional when these cellular proteins are cleaved (Borman et al., 1997a), the HAV IRES requires intact eIF4G to function (Borman & Kean, 1997). In this context, it is interesting that the translation from the HAV IRES alone and from the polyadenylated HAV RNA is inhibited by the m^7GpppG analog (Fig. 7). This is a unique feature of HAV IRES translation that is not observed with the PV and EMCV IRESs (Figs. 5 and 7) (Anthony & Merrick, 1991). Because the viral RNA and our reporter RNAs do not bear a m^7GpppG cap structure, this inhibitory effect must be different from m^7GpppG-mediated inhibition of the cap-dependent translation of cellular mRNAs. Because intact eIF4G is required for HAV RNA translation, it is conceivable that eIF4E unbound to m^7GpppN is required for HAV translation. Moreover, it has recently been reported that the nuclear cap-binding complex (CBC) interacts specifically with eIF4G (Fortes et al., 2000). A possible role for CBC in HAV translation therefore also needs to be considered. An alternative interpretation of the inhibitory effect of m^7GpppG on HAV translation in our system would be that the translation of an endogenous cellular mRNA was required to generate a cofactor that is necessary for the function of the HAV IRES. The lag phase in HAV translation that we have reproducibly observed (Fig. 6A) would be consistent with such an interpretation.

Answers to these questions and a more detailed understanding of how the poly(A) tail stimulates cap-dependent and IRES-mediated translation should be forthcoming from biochemical analyses with poly(A) tail-dependent cell-free translation systems.

MATERIALS AND METHODS

Plasmids

The constructs IRE.CATa(0–98) used for the tail length series were described by Preiss et al. (1998). Plasmids with a poly(A) tail of more than 98 A-residues proved too unstable for isolation in pure form due to frequent spontaneous deletions in the A-T segment. The plasmids T3LUC and T3LUC(pA) were described by Iizuka et al. (1994). The 5′ region of the PV genome, corresponding to nt 78–851 of PV type 1, Mahoney strain, was amplified by PCR from the plasmid pT7PV1 (Haller & Semler, 1992), creating *Xho*I sites at both ends; this fragment, containing the PV IRES and 37 amino acids of the capsid protein, was inserted in frame with the luc ORF into the *Xho*I site of the plasmids pT3LUCp(A) and pT3LUC, and the resulting constructs were pPV.IRES-luc-p(A) and PV.IRES-luc, respectively. A fragment of 828 nt, corresponding to the 5′ region of the HAV genome from nt 48–886, was amplified by PCR from the plasmid pT7-HAV1 (Cohen et al., 1987), which contains the cell culture-adapted HAV cDNA (HM175p35) under control of the T7 promoter, creating *Xho*I sites at both ends. This fragment, containing the HAV IRES and 49 amino acids of the N-terminus of the HAV capsid protein, was inserted in frame with the luc ORF into the *Xho*I site of both plasmids pT3LUCp(A) and pT3LUC, resulting in the constructs pHAV.IRES-luc-p(A) and pHAV.IRES-luc, respectively. The 612-nt EMCV IRES, obtained by digestion of pIRES2-EGFP (Clontech, Palo Alto, California) with *Pst*I-*Bst*XI, was inserted blunt-ended into the filled-in *Xho*I site of both plasmids pT3LUCp(A) and pT3LUC; the resulting plasmids are referred to as pEMCV.IRES-luc-p(A) and pEMCV.IRES-luc, respectively. The nucleotide sequences of any PCR-generated inserts were verified by DNA sequencing.

In vitro transcription

Following linearization of the plasmids, mRNAs were synthesized with bacteriophage RNA polymerases in the presence of either m^7GpppG (Stripecke & Hentze, 1992) or ApppG. mRNAs were trace labeled by ^{32}P incorporation to assess their concentration and integrity. Synthesis of mRNAs used in parallel experiments was performed at the same time.

Preparation of translation extracts

HeLa cells were maintained in suspension cultures at 37 °C in Joklik's Medium, supplemented with 5% newborn bovine serum at a concentration of $3–6 \times 10^5$ cells/mL. Two to six

liters of suspension culture were collected by centrifugation at 700 × *g* for 15 min and washed three times in phosphate buffered saline (PBS) at 4 °C. Pelleted cells were resuspended in 1 vol of ice-cold hypotonic MC buffer containing 10 mM HEPES, pH 7.6, 10 mM K-acetate, 0.5 mM Mg-acetate, 5 mM dithiothreitol, and proteinase inhibitors (Complete™ EDTA-free, Roche, Germany). After 5 min on ice, cells were broken with 15–18 strokes of a tight-fitting Dounce homogenizer (pestle type B). Following centrifugation of the homogenate at 13,000 × *g* for 5 min at 4 °C, supernatant was collected and divided into aliquots frozen in liquid nitrogen and stored at −80 °C.

In vitro translation

In vitro translation assays were typically performed in a final volume of 10 μL containing 4 μL of cell lysates, 100 μM amino acids, 20 μM creatine phosphate, 80 ng/μL creatine kinase, 16 mM HEPES buffer, pH 7.6, 0.8 mM ATP, 0.1 mM GTP, 100 ng/μL calf liver tRNA, 0.1 mM spermidine, and, depending on the transcript used as template for the translational reaction, K-acetate in a range of 25–75 mM and Mg-acetate in a range of 2.5–7 mM. The amount of exogenous mRNA corresponding to a linear range of translation has to be determined for each transcript. This range was 1–4 ng/μL (corresponding to about 2–7 fmol/μL) for the luc mRNAs and 3–10 ng/μL (corresponding to about 4–12 fmol/μL) for the IRES luc mRNAs. The cell extracts were not treated with micrococcal nuclease. Incubations were normally carried out at 37 °C. Incubation time was determined on the basis of physical stability of the different species of mRNAs to be compared. The amount of luciferase protein was determined by measuring luciferase activity by using the Luciferase Assay System from Promega. The CAT protein product was measured by a colorimetric enzyme immunoassay (CAT ELISA, Roche, Mannheim, Germany).

Northern blot analysis

Total RNA was extracted from 5 μL of translational reactions following addition of 10 ng of control RNA as a recovery control for the extraction procedure. Samples were separated in a 1% denaturing gel and transferred to a nylon membrane (Schleicher & Schuell, Dassel, Germany). Membranes were hybridized with ^{32}P-labeled DNA probes corresponding to the luciferase and CAT ORFs, and then subjected to phosphorimager analysis to quantify band intensities.

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