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A novel protein–RNA binding assay: Functional interactions of the foot-and-mouth disease virus internal ribosome entry site with cellular proteins

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ABSTRACT

Translation initiation on foot-and-mouth disease virus (FMDV) RNA occurs by a cap-independent mechanism directed by a highly structured element (~435 nt) termed an internal ribosome entry site (IRES). A functional assay to identify proteins that bind to the FMDV IRES and are necessary for FMDV IRES-mediated translation initiation has been developed. In vitro-transcribed polyadenylated RNAs corresponding to the whole or part of the FMDV IRES were immobilized on oligo-dT Dynabeads and used to deplete rabbit reticulocyte lysate (RRL) of IRES-binding proteins. Translation initiation factors eIF4G, eIF4A, and eIF4B bound to the 3′ domain of the FMDV IRES. Depletion of eIF4G from RRL by this region of the FMDV IRES correlated with the loss of translational capacity of the RRL for capped, uncapped, and FMDV IRES-dependent mRNAs. However, this depleted RRL still supported hepatitis C virus IRES-directed translation. Poly (rC) binding protein-2 bound to the central domain of the FMDV IRES, but depletion of RRL with this IRES domain had no effect on FMDV IRES-directed translation initiation.

Keywords: eukaryotic translation initiation factors; IRES; picornavirus; poly (rC) binding protein; protein synthesis

INTRODUCTION

Foot-and-mouth disease virus (FMDV) belongs to the aphthovirus genus within the picornavirus family that also includes poliovirus (PV), encephalomyocarditis virus (EMCV) and human rhinovirus (HRV). All picornaviruses have a single copy of a positive sense RNA genome that is translated to produce the viral polyprotein but also serves as the template for viral RNA replication. In contrast to cellular mRNAs, the picornaviral RNA lacks a 5′ terminal cap structure (m^7G pppN, where N is any nucleotide). The viral RNA contains, within the long (600–1,300 nt) 5′ untranslated region (UTR), a highly structured RNA element known as an internal ribosome entry site (IRES) that directs the ribosome to the initiation codon. Internal initiation of protein synthesis was initially demonstrated using the 5′ UTR of PV RNA (Pelletier & Sonenberg, 1988). Identification of the functionally equivalent IRES elements from EMCV (Jang et al., 1988), FMDV (Belsham & Brangwyn, 1990; Kuhn et al., 1990), and hepatitis A virus (Brown et al., 1991) soon followed. Certain cellular mRNAs (e.g., encoding BiP and c-myc; see Johannes et al., 1999) and other viral RNAs (e.g., from hepatitis C virus, HCV; Tsukiyama-Kohara et al., 1992; Reynolds et al., 1995) have also been reported to contain IRES elements.

Picornavirus IRES elements are about 450 nt in length and can be divided into at least two classes (see reviews by Belsham & Sonenberg, 1996; Belsham & Jackson, 2000). The cardiovirus (e.g., EMCV) and aphthovirus (e.g., FMDV) IRES elements represent one class that functions efficiently within the rabbit reticulocyte lysate (RRL) in vitro translation system. The enterovirus (e.g., PV) and HRV IRES elements represent the second major class of IRES and have a very different secondary structure. The PV and HRV IRES elements function poorly within the RRL system unless it is supplemented with HeLa cell proteins (Dorner et al., 1984; Hunt et al., 1999) indicating that proteins in addition to the canonical translation initiation factors may play a role in IRES-directed translation.

In an in vitro assay for 48S preinitiation complex formation on the EMCV IRES, it was shown that this ele-

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ment required each of the canonical translation initiation factors for maximal activity (Pestova et al., 1996a, 1996b). However, the role of eIF4F (consisting of eIF4E, eIF4A, and eIF4G) could be fulfilled by eIF4A with the central domain of eIF4G alone. The HCV IRES is shorter than the picornavirus IRES elements and it has been shown that, unlike the EMCV IRES, it does not need eIF4G or eIF4A (Pestova et al., 1998), indicating that the HCV IRES functions by a significantly different mechanism from the picornavirus IRESs.

Many picornaviruses induce eIF4G cleavage early in virus infection of cells (see Belsham & Sonenberg, 1996). The cleavage of eIF4G releases the N-terminal domain that contains the binding sites for eIF4E (Mader et al., 1995) and the poly(A) binding protein (PABP; Imataka et al., 1998). The residual portion of eIF4G, which is sufficient for IRES-directed translation, retains two binding sites for eIF4A (Imataka & Sonenberg, 1997) and binding sites for eIF3 (Lamphear et al., 1995) and Mnk-1 (Pyronnet et al., 1999).

eIF4A is an ATP-dependent RNA helicase; it is believed to unwind RNA secondary structure and is the prototypic member of the DEAD box family of helicases (Pause & Sonenberg, 1993). Multiple forms of eIF4A have also been described; eIF4AI and eIF4AII seem functionally homologous (Yoder-Hill et al., 1993) but eIF4AIII has different properties (Li et al., 1999). eIF4A is the most abundant of the eukaryotic translation initiation factors and is present both within the eIF4F complex (bound to eIF4G) and also free in the cytoplasm. Dominant negative mutants of eIF4AI block cap-dependent and picornavirus IRES-directed translation (Pause et al., 1994) but not HCV IRES-directed translation (Pestova et al., 1998). The helicase activity of eIF4A is strongly stimulated by eIF4B (which has RNA-binding activity; Rozen et al., 1990; Methot et al., 1996) but there is no evidence for stable physical interaction between these two proteins. ATP-dependent UV cross-linking of eIF4B to the FMDV IRES has been reported (Meyer et al., 1995) and eIF4B has also been shown to enhance 48S preinitiation complex formation on the EMCV IRES (Pestova et al., 1996a).

There is considerable interest in the role of cellular proteins, in addition to the translation initiation factors, in determining the activity of the picornavirus IRES elements. Four cellular proteins have been shown to bind to certain picornavirus IRES elements and stimulate their activity. These are La (Meerovitch et al. 1993; Svitkin et al., 1994), the polypyrimidine tract binding protein (PTB; Borman et al., 1993; Hellen et al., 1993; Niepmann et al., 1997; Hunt & Jackson, 1999), the poly (rC) binding protein 2 (PCBP-2; Blyn et al., 1996, 1997; Walter et al., 1999) and unr (for upstream of N-ras; Hunt et al., 1999). These proteins may function as RNA chaperones to maintain the secondary and tertiary structures of the IRES elements (see Belsham & Sonenberg, 2000).

In this study, a novel assay has been developed in which RNAs corresponding to the whole or part of the FMDV IRES were immobilized and used to deplete RRL of IRES-interacting proteins. The effect of protein depletion by the different RNA transcripts was assessed using a functional assay with the depleted RRL and the cellular proteins bound to the immobilized RNA transcripts were analyzed.

RESULTS

Development of an IRES-binding protein depletion assay

Fragments of FMDV cDNA encoding regions of the 5′ UTR were inserted into the pSP64Poly(A) transcription vector. Following linearization of these plasmids, RNA transcripts corresponding to the whole or part of the FMDV IRES linked at their 3′ termini to poly(A) tails of 30 nt were produced in vitro (see Fig. 1). Such transcripts bound efficiently to oligo dT magnetic beads and thus were readily immobilized to produce bead–RNA complexes. This procedure selected from the pop-

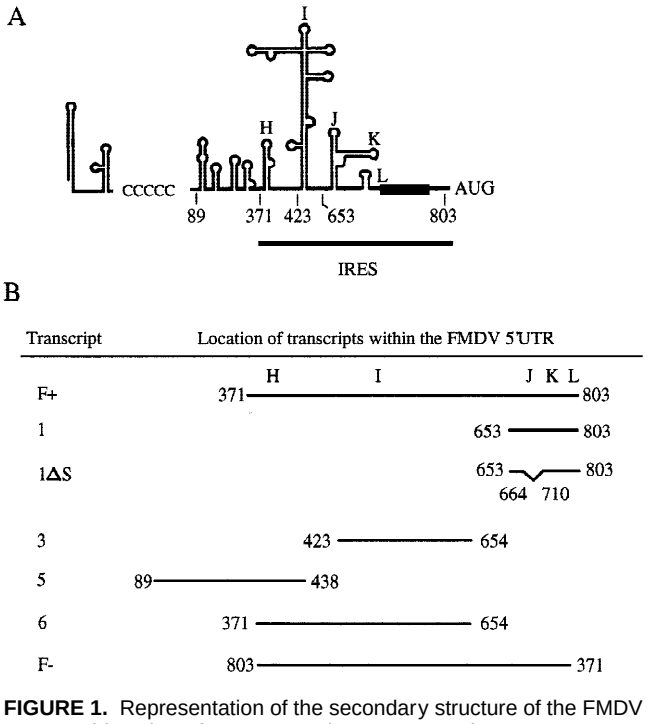

FIGURE 1. Representation of the secondary structure of the FMDV IRES and location of RNA transcripts. **A**: Secondary structure prediction of the FMDV 5′ UTR, based on the model of Pilipenko et al. (1989). The individual domains within the IRES are annotated as described by Duke et al. (1992). The polypyrimidine tract near the 3′ terminus of the IRES is indicated as a solid bar and the poly (C) tract within the FMDV 5′ UTR is shown. **B**: The location of RNA transcripts used in this study are indicated. cDNA fragments corresponding to these transcripts were prepared by PCR as described in Materials and Methods and inserted into pSP64Poly(A). RNA transcripts were prepared from *Eco*RI-linearized plasmids and contained a 30 nt poly(A) 3′ terminal tail.

ulation of RNAs synthesized by the RNA polymerase those full-length transcripts that included the poly(A) tail, resulting in an essentially homogeneous collection of molecules associated with the oligo dT beads. In preliminary experiments, the effect of depletion of RRL proteins using the full-length IRES (F+) transcript (containing domains H, I, J, K, and L; see Fig. 1) bound to the oligo-dT beads was determined. The production of chloramphenicol acetyl transferase (CAT) and luciferase (LUC) from the dicistronic mRNA transcribed from pGEM-CAT/FMD/LUC was assayed. Dose-dependent inhibition of both CAT and LUC expression was observed following depletion of the RRL in the TNT reaction mixture with increasing amounts of the beads–IRES complex (data not shown).

To monitor the IRES-dependent depletion of proteins from the RRL in more detail, beads–RNA complexes containing the positive sense IRES transcript (F+) or the antisense IRES transcript (F−) were prepared. The F− transcript acts as a control RNA of the same size and sequence complexity as the F+ (IRES) transcript. The complexes were incubated with 20 μL or 100 μL of RRL and depleted lysates were generated. These lysates were analyzed in the translation assay using a CAT/FMD/LUC transcript (Fig. 2, panels a and b) and for the presence of the translation initiation factors eIF4A and eIF4G by immunoblotting (Fig. 2, panels d and f). Furthermore, the proteins bound to the immobilized RNA transcripts were also analyzed for the presence of these factors (Fig. 2, panels c and e). A constant amount of beads–RNA complex was used in each depletion assay. Hence, it was expected that reactions using 20 μL of RRL should be more efficiently depleted of IRES-binding proteins, but the amounts of these factors retained on the beads–RNA could be greatest when 100 μL of RRL was used. As noted above, depletion of the RRL with the F+ transcript severely reduced the ability of the RRL to produce both LUC and CAT within the functional assay (Fig. 2, panels a and b). In contrast, incubation of the RRL with the F− transcript–beads complex had only a modest effect on the translational capacity of the RRL. The F+ transcript greatly depleted the 20-μL aliquot of RRL of eIF4G (Fig. 2, panel f) and also to a lesser extent of the more abundant eIF4A. Significant depletion of the eIF4G by the F+ transcript from the 100-μL aliquot of RRL was also apparent. In accord with these results, eIF4A and eIF4G were efficiently bound to the F+ transcript and thus retained on the beads–RNA complex (Fig. 2, panels c and e) but only much lower levels of these proteins were associated with the F− transcript. As expected, greater amounts of eIF4A and eIF4G were bound to the beads when incubated with 100 μL rather than 20 μL of RRL. Taken together, these results indicated that depletion of eIF4G from the RRL by the full-length FMDV IRES correlated with the loss of translational capacity.

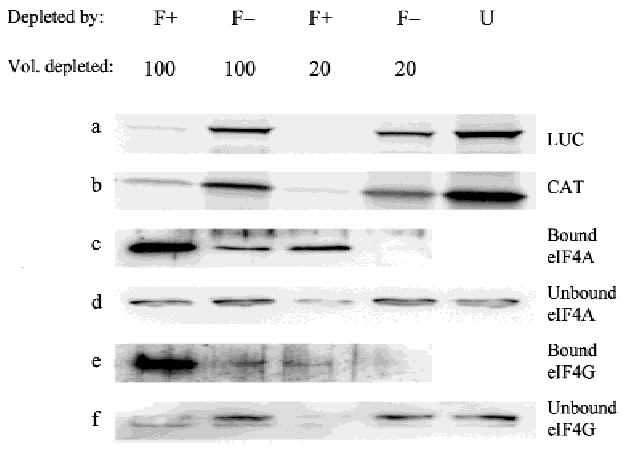

FIGURE 2. Depletion of RRL of proteins necessary for FMDV IRES function. RNA transcripts (5 μg) corresponding to the full-length FMDV IRES in the sense (F+) or antisense (F−) orientation were bound to oligo dT Dynabeads. The beads–IRES complex was incubated with 100 or 20 μL of RRL as indicated. Depleted RRL was incubated with the CAT/FMD/LUC RNA (1 μg) and [^{35}S]-methionine and samples analyzed by SDS-PAGE and autoradiography (panels a and b). Proteins bound to the beads–RNA complex (panels c and e) and the depleted lysates (panels d and f) were analyzed by SDS PAGE and immunoblotting for eIF4G (panels e and f) and eIF4A (panels c and d). Detection onto X-ray film was achieved with chemiluminescent reagents.

Delimitation of protein-binding sites on the FMDV IRES

In the studies described above, the whole FMDV IRES element (~435 nt) was used. To define the regions of the IRES that were active in binding eIF4G and eIF4A and were responsible for the loss of translational capacity by the RRL, sections of the FMDV IRES cDNA were amplified by PCR and inserted into the pSP64Poly(A) transcription vector. RNA transcripts corresponding to specific regions of the IRES (see Fig. 1) were prepared. Transcript 1 corresponds to the 3′ region of the IRES (containing domains J, K, and L) and transcript 3 corresponds to the large central region (domain I) of the element (see Fig. 1). These transcripts were used in parallel with the F+ and F− transcripts to deplete aliquots of RRL as described above. Once again, the F+ transcript severely reduced the capacity of the depleted RRL to translate the CAT/FMD/LUC transcript; only low levels of LUC and CAT protein were synthesized (Fig. 3, panels a and b). Depletion of the RRL by transcript 1 had a similar effect. In contrast, the transcripts F− and 3 each had similarly modest effects on the translation activity of the RRL. Analysis for the eIF4A and eIF4G in the depleted RRL was entirely consistent with the functional assay data (Fig. 3, panels c and d). Transcripts F+ and 1 severely depleted the RRL of eIF4G and significantly reduced the level of eIF4A. However, the transcripts F− and 3 had no significant

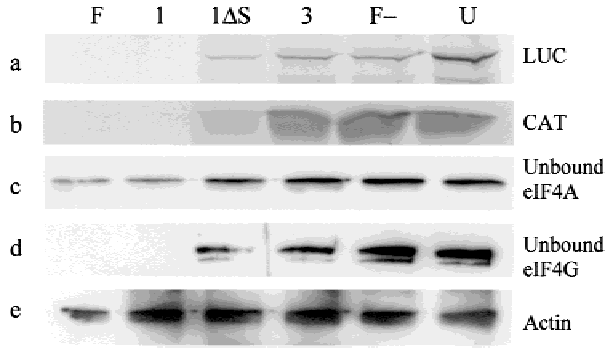

FIGURE 3. The 3′ end of the FMDV IRES depletes RRL of proteins required for protein synthesis. RNA transcripts corresponding to different regions of the FMDV IRES were bound to oligo-dT magnetic beads and used to deplete RRL prior to performing in vitro translation reactions as in Figure 2. Synthesis of LUC (panel a) and CAT (panel b) were detected by autoradiography. Western blot analysis of the depleted lysates for eIF4A (panel c), eIF4G (panel d), and actin (panel e) was performed as described in Materials and Methods.

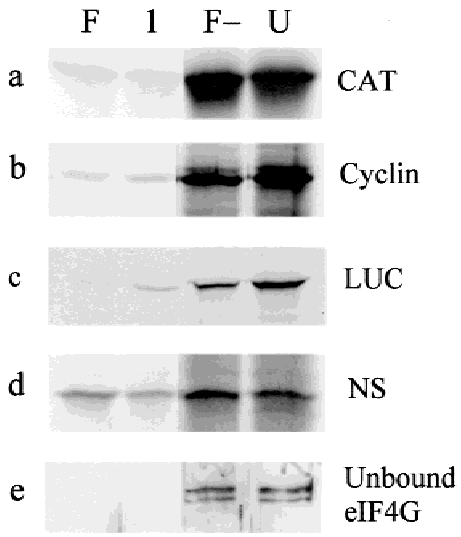

FIGURE 4. RRL depleted of FMDV IRES-interacting proteins is competent for HCV IRES-directed translation. The indicated capped RNA transcripts were translated in vitro as described in Materials and Methods using depleted (by transcripts F+, 1, or F−) or undepleted lysate as indicated. Synthesis of CAT (panel a) and LUC (panel c) from the cCAT/FMD/LUC transcript and of cyclin (panel b) and NS (panel d) from the cXL/HCV/NS transcript was detected on Kodak-imaging K phosphorimager screens. The residual eIF4G in the RRL (panel e) was determined by immunoblotting as described in Materials and Methods and visualized with chemiluminescent reagents.

effect on the level of these proteins in the RRL. To delimit the functional region of transcript 1 further, an internal deletion was made within this sequence. The deleted region of the cDNA corresponds to part of stem-loop J within the IRES (see Fig. 1); a similar deletion within the EMCV IRES rendered the element nonfunctional (Roberts & Belsham, 1997). The modified cDNA was again inserted into the pSP64Poly(A) transcription vector and the transcript, termed 1ΔS (containing a truncated J domain, with domains K and L), was assayed as before. In contrast to transcript 1, depletion of RRL with transcript 1ΔS had only a weak inhibitory effect on LUC and CAT expression within the translation assay (Fig. 3, panels a and b). Consistent with these observations, incubation of the RRL with this transcript had little effect on the levels of eIF4A and eIF4G (Fig. 3, panels c and d). None of the transcripts tested had a significant effect on the level of actin (used as a control) within the RRL (Fig. 3, panel e). These results indicated that the 3′ terminal region of the FMDV IRES, which includes the J, K, and L stem-loops plus the polypyrimidine tract (see Fig. 1A), depleted eIF4G together with eIF4A and that stem-loop J was required to achieve this.

The HCV IRES directs internal initiation in RRL depleted by the FMDV IRES

Translation initiation directed by the HCV IRES does not require eIF4G or eIF4A (Pestova et al., 1998) but ribosomes, eIF2-GTP and eIF3, are still necessary. If the inhibitory effect of RRL depletion by the FMDV IRES was due to specific depletion of eIF4G and eIF4A, then the depleted RRL should still support translation initiation directed by the HCV IRES. To investigate this, RRL was depleted using the transcripts F+, 1, and F− and used to translate the capped mRNAs cCAT/FMD/LUC and cXL/HCV/NS. As observed above, specific depletion of the RRL by the transcripts F+ and 1 severely inhibited (by ~90% compared to the control F− transcript) cap-dependent and FMDV IRES-directed translation (Fig. 4, panels a, b, and c). As anticipated, most of the eIF4G was depleted from the RRL by transcripts F+ and 1 (Fig. 4, panel e). However, this depleted RRL still supported efficient HCV IRES-directed translation of NS, (Fig. 4, panel d). Quantitation, by phosphorimager analysis, indicated that the synthesis of NS in the RRL depleted by transcript F+ or transcript 1 was about 40% of the efficiency observed in the F−-transcript-depleted RRL. The limited reduction in the ability of the RRL to mediate HCV IRES-dependent translation is likely due to limited loss of universal translation factors being depleted in association with eIF4G. Thus, it can be concluded that the complete FMDV IRES and its 3′ domain only deplete certain factors from the RRL and do not render it incompetent for all protein synthesis.

Localization of binding site for PCBP-2 on the FMDV IRES

Because the depletion assay described above can be performed on RRL that has been used in a translation assay, it was apparent that the ability of any protein to interact with the IRES could be determined by a simple

modification of the depletion procedure. Translation of cDNAs within the TNT/RRL system can produce any [^{35}S]-labeled protein of interest and then the depletion assay can be performed as above. This system permits the analysis of protein binding to the RNA transcripts in the absence of suitable antibodies. The interaction of PCBP-2 with the FMDV IRES was analyzed using this assay. PCBP-2 has been shown previously to bind to two regions of the PV 5′ UTR. One site is within the cloverleaf structure at the 5′ terminus of the genome (Gamarnik & Andino, 1997; Parsley et al., 1997) and the second site is within the IRES (Blyn et al., 1996, 1997; Gamarnik & Andino, 2000). Interaction of PCBP-2 with the FMDV 5′ UTR has also been demonstrated (Walter et al., 1999), but has not been localized. Plasmids encoding PCBP-2 and LUC (as a control) were used to program separate TNT reactions to generate the [^{35}S]-labeled proteins. When the reactions were complete, aliquots of each lysate were incubated with bead–RNA complexes containing transcripts F+, 1, 1ΔS, 3, 5, and F− as described above. The proteins bound to the bead–RNA complexes and those remaining in the depleted RRL were analyzed by SDS-PAGE and autoradiography. The [^{35}S]-labeled LUC protein (control) did not bind to any part of the FMDV IRES (Fig. 5, panel a) and all of the translated protein was detected in the depleted RRL (Fig. 5, panel b). In contrast, PCBP-2 bound specifically to the full-length IRES (F+) and the middle domain of the FMDV IRES (transcript 3) attached to the beads (Fig. 5, panel c). This result was confirmed by the significant depletion of the [^{35}S]-labeled PCBP-2 from the RRL by these transcripts (Fig. 5, panel d). It should be noted that depletion of RRL by transcript 3 had no significant effect on the ability of the RRL to support cap-dependent or FMDV IRES-directed translation (Fig. 3, panel a).

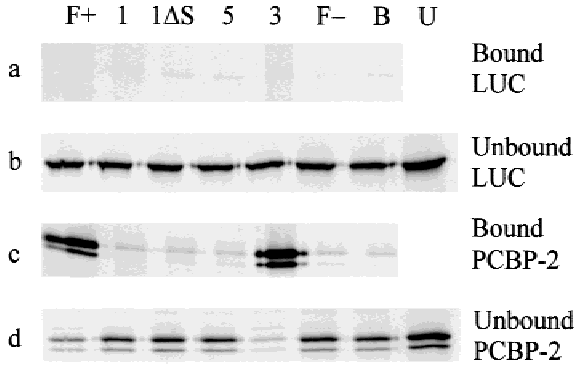

FIGURE 5. PCBP-2 binds the middle domain (I) of the FMDV IRES. [^{35}S]-labeled LUC and PCBP-2 proteins were made in vitro using the TNT system. The TNT reactions were incubated with the indicated beads–transcript complexes (or beads alone, B) as described above. LUC (panels a and b) and PCBP-2 (panels c and d) that were bound (panels a and c) to the RNAs were eluted and analyzed by SDS-PAGE (10%) and autoradiography in parallel with aliquots of the depleted and undepleted (U) lysates (panels b and d) containing the unbound [^{35}S]-labeled proteins.

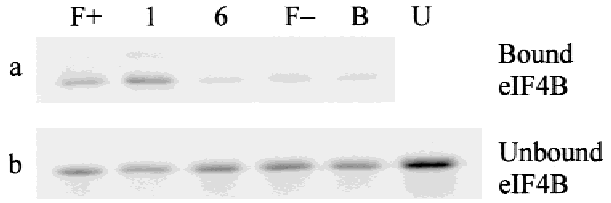

FIGURE 6. eIF4B binds to the 3′ domain of the FMDV IRES. [^{35}S]-labeled eIF4B was produced in vitro using the TNT system. Aliquots of the TNT reaction were incubated with the indicated beads–transcript complexes (or beads alone, B) as described above. eIF4B bound to the RNAs was eluted and analyzed (U) lysates.

Binding of eIF4B to the FMDV IRES

The translation initiation factor eIF4B has been shown previously to be UV-crosslinked to the FMDV IRES in an ATP-dependent manner (Meyer et al., 1995). We wished to investigate whether eIF4B could be shown to interact with the FMDV IRES within RRL using the depletion assay. Using this procedure, cDNA encoding eIF4B (kindly provided by N. Methot and N. Sonenberg) was translated within the TNT system and transcripts F+, F−, 1, and 6 were assayed for their ability to interact with the [^{35}S]-labeled eIF4B. It was found that eIF4B was efficiently bound by the full-length IRES (F+) and by transcript 1, but not by the antisense IRES (F−) or the magnetic beads alone (Fig. 6). This pattern of interaction is clearly similar to that observed above for eIF4G and eIF4A. Furthermore, additional assays of this type showed that the [^{35}S]-labeled P100 fragment of eIF4G (middle and C-terminal domains; Pestova et al., 1996b), eIF4E, and eIF4A were each depleted from the RRL by the 3′ terminal region of the FMDV IRES (data not shown).

DISCUSSION

A novel assay has been developed to identify cellular proteins that interact with the FMDV IRES. This assay has several important features: (1) the depletion from RRL of specific RNA-binding proteins does not involve any dilution or treatment with nonphysiological salt concentrations, (2) this permits a functional assay to be performed following the depletion process, (3) the depletion is rapid, (4) the RNA associated with the oligo dT magnetic beads that is used to deplete the RRL is homogeneous, and (5) it is relatively facile to analyze several different transcripts in parallel.

Using this assay, we have shown that eIF4G and eIF4A were both depleted from RRL by the 3′-terminal domain of the FMDV IRES and that the depletion of

eIF4G correlates with the loss of translational capacity. It seems probable that eIF4A binds to the FMDV IRES indirectly through its association with eIF4G. Indeed, repeating the depletion procedure on RRL that had already been extensively depleted of eIF4G in one step did not result in any of the residual eIF4A in the RRL binding to the fresh RNA–beads complex (data not shown). Thus it has not been possible to completely deplete eIF4A from the RRL. For this reason, we cannot exclude the possibility that, in our experiments, the partial eIF4A depletion has no effect on translation and that the effect of the depletion of the RRL is solely due to the loss of eIF4G. However, the ability of dominant negative mutants of eIF4A to block IRES-mediated translation (Pause et al., 1994) and the requirement for eIF4A by the EMCV IRES in the 48S preinitiation complex assay (Pestova et al., 1996a) does indicate that eIF4A has a role in picornavirus IRES-directed translation.

The central role of eIF4G in the initiation of protein synthesis has only fairly recently been recognized, although it has been clear for some time that cleavage of eIF4G within picornavirus-infected cells is an important contributor towards the virus-induced inhibition of cap-dependent protein synthesis. Studies have shown that eIF4G binds near the 3′ terminus of the EMCV IRES (Pestova et al., 1996a, 1996b) and RNAse protection studies have mapped protected residues within the J/K domain (Kolupaeva et al., 1998). Recently it has also been shown that the central domain of eIF4G, in association with eIF4A, protected from RNAse digestion residues within the FMDV J/K domain (Pilipenko et al., 2000). Furthermore, mutations at the base of the FMDV J/K domain that abrogated interaction with eIF4G in UV-crosslinking studies also inhibited IRES activity (Lopez de Quinto & Martinez-Salas, 2000). The studies presented here are fully consistent with these data. Taken together, it seems clear that eIF4G binds, in association with eIF4A, to the J/K domain of the FMDV IRES and that this interaction is important for the function of this IRES.

The interaction of eIF4B with the FMDV IRES has been documented previously using UV-crosslinking assays (Meyer et al., 1995) and the depletion analysis used here confirms this interaction. It was demonstrated previously that this process required ATP (Meyer et al., 1995), which suggested the possibility that this interaction was mediated through eIF4A. Because the same region of the FMDV IRES binds to eIF4G, eIF4A, and eIF4B, it is possible that these three proteins are bound as a complex on the IRES. However, stable association of eIF4B with eIF4F/eIF4A has not been observed previously, but the mild conditions and rapidity of the depletion assay may help to maintain this complex. Alternatively, eIF4B could be bound as a result of its interaction with eIF3 (Methot et al., 1996), which also binds to eIF4G (Lamphear et al., 1995).

PCBP-2 has been shown to bind and stimulate the activity of the PV IRES (Blyn et al., 1996, 1997). Recently this protein was also shown to bind to the 5′ UTRs of FMDV and EMCV (Walter et al., 1999); however no effect of PCBP-2 depletion on the activity of these IRES elements was observed. The 5′ UTR transcripts of FMDV and EMCV used by Walter et al. (1999) both included poly(C) tracts; thus the binding of PCBP-2 to these elements may have resulted from binding to sequences outside of the IRES. In this study (Fig. 5), it was demonstrated that PCBP-2 binds to the central domain of the FMDV IRES. It is interesting to note that, within this domain, there is a C-rich loop that was highlighted by Jackson et al. (1994) as a conserved motif, but confirmation of this loop as the binding site for PCBP-2 requires further study. In any case, the functional significance of this interaction is unclear, as, in accord with the data of Walter et al. (1999), we found that depletion of RRL using this domain of the IRES (which depletes the RRL of PCBP-2) had no significant effect on FMDV IRES-directed translation.

The basic procedure used to identify protein–RNA interactions of immobilizing polyadenylated RNA transcripts on oligo dT magnetic beads provides a rapid and simple assay and should be widely applicable.

MATERIALS AND METHODS

Plasmid constructions

Plasmids encoding the FMDV IRES in the sense (F+) or antisense (F−) orientation were constructed by insertion of a blunt-ended (with Klenow enzyme plus dNTPs) *Eco*RI-*Cla*I fragment (435 bp) from pKSRCla (Drew & Belsham, 1994) into *Hin*dIII-*Sac*I-digested pSP64Poly(A) (Promega) that had been blunt ended with T4 DNA polymerase. The two orientations of the insert within the vector were identified by restriction enzyme digestion. Specific fragments of the FMDV 5′UTR cDNA (see Fig. 1) were constructed by PCR using the template pT7S3ΔApa (Belsham et al., 2000) with the primers (MWG-biotech) indicated in Table 1. Fragment 1ΔS was generated using a two-step overlap PCR. For the first round, one reaction employed primers IS3F and IS1ΔSB with pT7S3ΔApa as template and the second reaction used the same template with primer ISΔSF and the M13 reverse primer. The two PCR fragments were purified and mixed, and a second round of PCR was performed using the IS1F and IS1B primers. The 46-nt deletion produced a new *Sma*I site within the cDNA. In all cases, the PCR products were gel purified and ligated into the pGEM-T vector (Promega) and then *Acc*I-*Sac*I fragments (these sites were specified by the PCR primers) from the resultant plasmids were inserted into similarly digested pSP64Poly(A). The structures of the plasmids were verified by restriction enzyme digestion and sequence analysis using a Cy5-labeled SP6 primer with an ALF sequencer (Pharmacia).

Plasmid pQE30-P2 (Blyn et al., 1996; kindly provided by B.L. Semler) encoding PCBP-2 was digested with *Eco*RI and *Pst*I and the 1.2-kbp fragment was ligated into similarly digested pGEM3Z (Promega) to produce pGEM-PCBP-2.

TABLE 1. PCR primers used to generate FMDV cDNA fragments.

Primer I.D.		Sequence (5′–3′)[a]	Fragments
First round PCR	Second round PCR		
IS1F			
+		CCGTCGACTGTACTCAAACACTGGTG	
IS1B		CCGAGCTCATAGTGGTAAAAGGAAA	1
	IS1F		
IS3F + **ISΔSB**	+	CCAGTCCCCGGGCATCCTTAGCCT	
ISΔSF + M13(B)	IS1B	AAGGATGCCCGGGGACTGGGACTTCT	1ΔS
IS3F			
+		CCGTCGACTTTTGTACTGTGTGTTTGACTCC	
IS2B		CCGAGCTCAGCCTGTCACCAGTGCTTG	3
IS2F			
+		CCGTCGACCCAAGTTTTACCGTCGT	
1S5B		CCGAGCTCGTCAAGCACAGTACAAAG	5
IS6F + IS2B		CCGTCGACCTGACACAAACGTCAAACTT	6

[a] Sequences given refer to the primer in bold.

Maxi-prep DNA was purified using the BIO 101 RPM-1G kit (Anachem).

In vitro transcription

Plasmids derived from the pSP64Poly(A) vector were linearized with *Eco*RI. The DNAs were purified by phenol extraction and ethanol precipitation and then used as templates for transcription reactions. RNA transcripts (containing a 3′ poly(A) tail of 30 residues) were produced and purified according to the manufacturer's instructions (Ambion, SP6 Megascript). The plasmids pGEM-CAT/FMD/LUC and pGEM-CAT/EMC/LUC (Roberts et al., 1998) were linearized with *Xho*I and transcribed as above but using the T7 AMBION Megascript kit to generate uncapped dicistronic mRNAs. Capped dicistronic mRNAs were produced from the linearized pGEM-CAT/FMD/LUC and *Eco*RI linearized pXL40-372NS (containing the HCV IRES; Reynolds et al. (1995); kindly provided by A. Kaminski and R.J. Jackson) using cap-scribe buffer (Roche) and T7 RNA polymerase (Ambion), and are identified as cCAT/FMD/LUC and cXL/HCV/NS, respectively. The RNAs were purified by phenol extraction and ethanol precipitation. The transcripts were analyzed by agarose electrophoresis and their concentration determined by spectrophotometry.

Depletion of IRES-binding proteins from RRL

Oligo dT Dynabeads (Dynal, 0.5 mL unless otherwise stated) were captured on a magnetic stand and the supernatant removed. The beads were washed in 0.5× SSC (0.5 mL) and binding buffer (10 mM Tris, pH 7.5, 100 mM KCl, 2 mM $MgCl_2$, 0.2 mL) and resuspended in binding buffer (50 μL). In vitro-transcribed polyadenylated RNA transcripts (5 μg, an excess) were incubated with the oligo dT dynabeads at 4 °C for 30 min on a rotating wheel. Unbound RNA was removed and the beads–RNA complex washed twice with binding buffer. The immobilized RNA transcripts were then incubated with RRL (Promega, 25 μL unless otherwise stated) at 4 °C for 60 min on a rotating wheel. The magnetic beads were captured and the depleted RRL was removed. The beads–RNA–protein complex was washed twice in binding buffer, resuspended in SDS sample buffer, and incubated at 4 °C for 10 min. These samples were analyzed by SDS-PAGE and Western blot analysis for specific proteins, as indicated in figure legends. The depleted RRL (1 μL) was analyzed in the same way. Western blot analysis for eIF4G was performed using 7% SDS-PAGE, whereas all other proteins were analyzed using 10% SDS-PAGE.

In vitro translation reactions

Dicistronic RNA transcripts (1 μg) were added to depleted or undepleted (control) RRL (6.5 μL) with [^{35}S]-methionine (10 μCi, Amersham) in a total volume of 10 μL. Translation reactions were incubated at 30 °C for 60 min and then analyzed by SDS-PAGE (10%) and autoradiography.

Coupled transcription/translation reactions were performed using TNT systems (Promega) with [^{35}S]-methionine and undigested plasmid DNA essentially as described by the manufacturer.

Binding of [^{35}S]-labeled proteins to the FMDV IRES

[^{35}S]-labeled proteins were produced in TNT reactions using specific plasmids as templates and then the RRL was incubated with oligo dT magnetic beads–RNA complexes as described above. After incubation at 4 °C (60 min), the depleted RRL was removed and 1 μL was mixed with SDS sample buffer. The beads–RNA–protein complex was washed twice with binding buffer (200 μL) and proteins were then eluted in SDS sample buffer as above. All samples were analyzed by SDS-PAGE and autoradiography.

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