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RNA 1999 5: 1570-1585

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Differential utilization of poly(rC) binding protein 2 in translation directed by picornavirus IRES elements

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ABSTRACT

The translation of picornavirus genomic RNAs occurs by a cap-independent mechanism that requires the formation of specific ribonucleoprotein complexes involving host cell factors and highly structured regions of picornavirus 5' noncoding regions known as internal ribosome entry sites (IRES). Although a number of cellular proteins have been shown to be involved in picornavirus RNA translation, the precise role of these factors in picornavirus internal ribosome entry is not understood. In this report, we provide evidence for the existence of distinct mechanisms for the internal initiation of translation between type I and type II picornavirus IRES elements. In vitro translation reactions were conducted in HeLa cell cytoplasmic translation extracts that were depleted of the cellular protein, poly(rC) binding protein 2 (PCBP2). Upon depletion of PCBP2, these extracts possessed a significantly diminished capacity to translate reporter RNAs containing the type I IRES elements of poliovirus, coxsackievirus, or human rhinovirus linked to luciferase; however, the addition of recombinant PCBP2 could reconstitute translation. Furthermore, RNA electrophoretic mobility-shift analysis demonstrated specific interactions between PCBP2 and both type I and type II picornavirus IRES elements; however, the translation of reporter RNAs containing the type II IRES elements of encephalomyocarditis virus and foot-and-mouth disease virus was not PCBP2 dependent. These data demonstrate that PCBP2 is essential for the internal initiation of translation on picornavirus type I IRES elements but is dispensable for translation directed by the structurally distinct type II elements.

Keywords: 5' noncoding region; cap-independent translation; coxsackievirus; encephalomyocarditis virus; foot-and-mouth disease virus; human rhinovirus; poliovirus; RNA binding proteins

INTRODUCTION

Translation initiation of a majority of cellular and viral mRNAs begins with the recognition of the m⁷GTP cap at the 5' terminus of the mRNA by cellular factors that associate with the 43S preinitiation complex to recruit the small ribosomal subunit to the mRNA. Following 5' cap recognition, the 43S preinitiation complex scans the 5' noncoding region (5' NCR) of the mRNA for an AUG codon that is in the appropriate context for the recruitment of the large ribosomal subunit and subsequent initiation of translation (Kozak, 1987, 1989). Thus, RNAs possessing long, highly structured 5' NCRs encoding multiple AUG codons in favorable contexts

upstream of the initiator AUG or RNAs lacking m⁷GTP cap structures should not be capable of efficient initiation of translation by a cap-dependent, ribosome-scanning mechanism. RNAs containing such characteristics belong to an expanding group of viral and cellular RNAs capable of initiating translation by alternative mechanisms. These mRNAs can be divided into two subsets based on the mechanism by which translation is initiated. The first subset is characterized by the cap-dependent recruitment of ribosomes followed by nonlinear migration of these ribosomes ("ribosome shunting") to the initiator AUG of the mRNA. Cauliflower mosaic virus and adenovirus RNAs have been shown to utilize ribosome shunting for the initiation of translation (Futterer et al., 1993; Yueh & Schneider, 1996). The second subset of alternatively translated RNAs is comprised of RNAs translated by a mechanism of cap-independent, direct internal ribosome bind-

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ing and entry. This group contains many cellular and viral RNAs including the genomic RNAs of a family of animal viruses, the *Picornaviridae* (Jang et al., 1988; Pelletier & Sonenberg, 1988; for reviews, see Jackson & Kaminski, 1995; Stewart & Semler, 1997).

The family *Picornaviridae*, consisting of the enterovirus, rhinovirus, cardiovirus, aphthovirus, hepatovirus, and the recently defined parechovirus genera (Stanway & Hyypia, 1999), contains members that are the causative agents of a diverse number of human and animal diseases. All members of this family of viruses have single-stranded, message-sense RNA genomes of 7–8 kb in length encoding a single, large open reading frame preceded by long, highly structured 5' NCRs of ~600–1200 nt in length. These 5' NCRs participate in important functions of the virus life cycle, including RNA replication and cap-independent translation initiation (reviewed in Wimmer et al., 1993). The minimal *cis*-acting RNA element of the picornavirus 5' NCR required for the initiation of cap-independent, internal ribosome entry is commonly known as the internal ribosome entry site (IRES; Jang et al., 1990). Based upon sequence and structural similarities shared between the IRES elements of the six different picornavirus genera, two major groups have been classified. The enteroviruses and rhinoviruses constitute the type I IRES elements, and the cardioviruses and aphthoviruses comprise the type II IRES elements (Wimmer et al., 1993). The IRES element of the only member of the hepatovirus genus, hepatitis A virus (HAV), cannot easily be classified into either of the major classes based on its primary and secondary structure (Brown et al., 1994). Similarly, the parechovirus IRES elements have not been definitively grouped into either class; however, parechovirus and HAV IRES elements are somewhat related to the type II IRES elements (Ghazi et al., 1998).

In addition to the primary and secondary structural differences that distinguish the two classes of IRES elements, additional divergence became apparent when *in vitro* translation assays were utilized to study picornavirus translation. Although picornaviruses with type II IRES elements are capable of efficient translation in a variety of *in vitro* translation extracts, type I IRES elements are more fastidious. For example, type I IRES elements inefficiently and inaccurately translate in rabbit reticulocyte lysate (RRL), whereas type II elements translate quite efficiently in RRL (Shih et al., 1978, 1979). However, the translation of type I IRES elements in RRL can be stimulated by the addition of certain cellular cytoplasmic extracts or crude initiation factor preparations (Brown & Ehrenfeld, 1979; Dorner et al., 1984; Phillips & Emmert, 1986; Svitkin et al., 1988). Thus, the distinct structural characteristics of type I and type II IRES elements manifest as differences in the *trans*-acting cellular factors that are required for type I compared to type II IRES-mediated internal ribosome entry.

In fact, much of the evidence compiled to date suggests that the internal initiation of translation on both type I and type II picornavirus IRES elements requires certain *trans*-acting cellular proteins in addition to canonical translation initiation factors (for reviews, see Jackson & Kaminski, 1995; Stewart & Semler, 1997). In this study, we investigated the process of translation as directed by the IRES elements of poliovirus (PV), coxsackievirus strain B (CVB), human rhinovirus (HRV), encephalomyocarditis virus (EMCV), and foot-and-mouth disease virus (FMDV). Specifically, we examined whether these picornaviruses, representing four of the six picornavirus genera, require the cellular protein, PCBP2, for viral translation.

PCBP2 is a cellular RNA-binding protein that interacts with the 5' NCR of poliovirus RNA (Dildine & Semler, 1992; Blyn et al., 1995, 1996). The protein contains three hnRNP K-homologous (KH) RNA-binding domains and has a binding preference for poly(rC). PCBP2 mRNA is widely expressed and has been localized both in the cytoplasm and the nucleus (Leffers et al., 1995). To date, the only known functions attributed to PCBP2 are its functional associations with the 3' NCRs of the α -globin and the tyrosine hydroxylase mRNAs in complexes that impart stability (Kiledjian et al., 1995; Paulding & Czyzyk-Krzeska, 1999). Similar complexes have also been found to form on the 3' NCRs of lipoxigenase, α (I)-collagen, and erythropoietin mRNAs (Holcik & Liebhauer, 1997; Czyzyk-Krzeska & Bendixen, 1999).

In addition to the role of PCBP2 in cellular mRNA stability, a number of additional functions relating to viral infection have been ascribed to the protein. We have previously shown that the interaction of PCBP2 with the poliovirus 5' NCR performs dual functions in the life cycle of the virus by facilitating the initiation of both viral protein synthesis and viral RNA synthesis (Blyn et al., 1997; Parsley et al., 1997). Likewise, Andino and colleagues confirmed this functional duality and proposed that PCBP could also function in a mechanism involving accessory proteins of viral origin to regulate the processes of translation and replication (Gamarnik & Andino, 1997, 1998). In addition to its documented functions in the poliovirus life cycle, PCBP2 also plays a role in the translation of HAV genomic RNA (Graff et al., 1998). Furthermore, human papillomavirus type 16 (HPV16), a member of the *Papovaviridae*, has evolved a mechanism to utilize PCBP2. For HPV16, PCBP2 acts as a regulator of late gene expression (Collier et al., 1998).

This work investigates the possibility that RNA binding by PCBP2 is a general requirement for the internal initiation of translation on picornavirus IRES elements. Competition RNA mobility-shift assays conducted with ³²P-labeled PV stem-loop IV RNA, recombinant PCBP2 (rPCBP2), and five different picornavirus 5' NCRs confirmed a conserved physical interaction between PCBP2 and the picornavirus 5' NCRs that were tested. *In vitro*

translation studies conducted in HeLa cell-free cytoplasmic translation extracts depleted of PCBP2 by RNA affinity chromatography showed a reduction in translation as directed by type I IRES elements; however, a similar reduction was not observed for type II IRES elements. Furthermore, the reduced translational capacity of the PCBP2-depleted extracts could be rescued by the addition of rPCBP2 in a dose-dependent fashion. We also demonstrated that in vitro translation reactions programmed with RNAs containing picornavirus type I IRES elements could be inhibited by the addition of poly(rC) to effectively deplete free PCBP2, whereas a similar inhibition was not observed for reactions containing type II IRES elements. Beyond the differential translational efficiencies of these IRES elements in various in vitro translation extracts, these data provide evidence for the existence of distinct mechanisms for the cap-independent, internal initiation of translation on type I versus type II picornavirus IRES elements.

RESULTS

PCBP2 is capable of interacting with the 5' NCRs of representative members from four picornavirus genera

To examine the ability of PCBP2 to interact with the 5' NCRs of picornaviruses, we carried out competition electrophoretic mobility-shift assays. Utilizing the previously described plasmid (pT220–460) as template, T7 RNA polymerase transcription led to the production of an ~240-nt, ³²P-labeled RNA probe containing sequences that correspond to the predicted stem-loop IV structure of the poliovirus IRES (Dildine & Semler, 1992). Previous work has shown that stem-loop IV (S-L IV) RNA is capable of a specific high-affinity interaction with PCBP2 (Blyn et al., 1995, 1996). Therefore, the ³²P-labeled stem-loop IV RNA was added to a reaction containing rPCBP2 and varying amounts of competitor RNA. The RNA-protein complexes formed in these reactions were resolved on nondenaturing polyacrylamide gels, and the location of the labeled probe was detected by autoradiography (Fig. 1).

When rPCBP2 was added to a reaction containing labeled stem-loop IV RNA, a retardation in the electrophoretic mobility of the probe, indicating the formation of a ribonucleoprotein (RNP) complex, was visible (Fig. 1A–C, lanes 1 and 2). An additional rPCBP2/stem-loop IV RNA complex of further retarded electrophoretic mobility could also be observed in lanes containing greater relative amounts of rPCBP2 available for interacting with stem-loop IV RNA. To test the specificity of these complexes, the ability of a homologous competitor RNA (unlabeled S-L IV), poly(rC), or the 5' NCR of β -globin mRNA to compete with the probe for PCBP2-binding was tested (Fig. 1C). A dose-dependent de-

FIGURE 1. The interaction between recombinant PCBP2 and different picornavirus 5' NCRs. Competition RNA mobility-shift assays were conducted as described in Materials and Methods. Reactions contained final concentrations of 10 nM probe and 100 nM rPCBP2 and were resolved on native 4% polyacrylamide/5% glycerol/0.5× TBE gels at 4°C. **A:** Competition shifts were carried out with the complete 5' NCRs of poliovirus (lanes 3–6), coxsackievirus (lanes 7–10), and human rhinovirus (lanes 11–14) serving as competitor RNA. **B:** Competition reactions were conducted with the 5' NCRs of EMCV (lanes 3–6) and FMDV with (lanes 7–10) and without (lanes 11–14) sequences encoding the leader polypeptide. The poly(C) regions of the EMCV 5' NCR RNA and both of the FMDV 5' NCR RNAs were truncated to 9 nt and 25 nt, respectively. **C:** Competition electrophoretic mobility shifts were conducted with unlabeled competitor RNAs including poliovirus S-L IV RNA (lanes 3–6), poly(rC) with an average chain length of 286 residues (lanes 7–10), and the 5' NCR of β -globin mRNA (lanes 11–14). Lane 1 of panels **A**, **B**, and **C** serves as an indicator of the mobility of uncomplexed probe and contains labeled S-L IV RNA only. Lane 2 of panels **A**, **B**, and **C** is a control reaction that marks the electrophoretic mobility of the S-L IV/PCBP2 RNP complex.

crease in the formation of the rPCBP2/³²P-labeled stem-loop IV RNA RNP complexes was detected when the competitor RNA was either unlabeled stem-loop IV or poly(rC), both nucleic acids for which PCBP2 has a known high affinity (Fig. 1C, lanes 3–10). However, even at a 500-fold molar excess of the 5' NCR of β -globin mRNA, a significant decrease in RNP complex formation was not observed (Fig. 1C, lanes 11–14). Therefore, the competition RNA electrophoretic

mobility-shift assay was capable of distinguishing RNA sequences that are known to have binding capacities for PCBP2 from those that do not.

Examination of PCBP2 binding to picornavirus 5' NCRs by competition mobility-shift assays conducted with several picornavirus 5' NCRs indicated that both type I IRES elements (Fig. 1A) and type II IRES elements (Fig. 1B) could effectively compete with stem-loop IV for binding of PCBP2 in a dose-dependent fashion. Additionally, lower molar excesses of picornavirus 5' NCR RNAs containing type II IRES elements compared to those containing type I IRES elements were required to efficiently compete for rPCBP2 binding. However, quantitative interpretations based on this result must be considered tentative given that the three competitor RNAs representing picornaviruses (i.e., EMCV and the two FMDV 5' NCRs) with type II IRES elements contain poly(C) tracts that were significantly truncated compared to those of the wild-type (wt) genomic RNA. In summary, these results indicate a conservation in the ability of PCBP2 to interact with the 5' NCRs of representative members from four of the picornavirus genera.

PCBP2 is required for translation directed by picornavirus type I IRES elements

To investigate the role of PCBP2 in translation directed by picornavirus IRES elements, we modified an assay previously used to elucidate the requirement for PCBP2 in poliovirus translation (Blyn et al., 1997). In our *in vitro* translation assay, HeLa cell cytoplasmic (S10) extract supplemented with HeLa ribosomal salt wash (RSW) was used to translate T7 RNA polymerase-transcribed picornavirus 5' NCR/firefly luciferase reporter RNAs. To determine if the cellular protein (PCBP2) has a role in translation mediated by different picornavirus IRES elements, endogenous PCBP2 contained in the HeLa cell translation extracts was depleted by passage of the extracts over an affinity column containing matrix coupled to stem-loop IV of PV RNA (Fig. 2, compare lanes 1 and 3), an RNA sequence known to bind PCBP2 (Blyn et al., 1996). If the translation of the reporter RNA was impaired upon PCBP2 depletion, the specific involvement of PCBP2 in this translation could be confirmed by addition of rPCBP2 leading to the rescue of translation.

To examine the role of PCBP2 in picornavirus IRES-mediated translation, both type I and type II IRES elements from representative members of four picornavirus genera were analyzed in the translation assay. The reporter RNAs consisted of the complete 5' NCRs of PV, CVB, HRV, EMCV, and FMDV followed by a cistron encoding luciferase (Fig. 3). Additionally, constructs encoding both the 5' NCRs of PV and EMCV upstream of luciferase and the 3' NCRs of PV and EMCV, respectively, downstream of luciferase were utilized. The latter

FIGURE 2. Depletion of PCBP from HeLa cell translation extracts by PV S-L IV RNA affinity chromatography. RNA affinity chromatography was conducted as described in Materials and Methods. Western blot analysis was conducted as described (Sambrook et al., 1989). The primary antibody was polyclonal, affinity-purified α -PCBP2, and the secondary antibody was alkaline phosphatase-conjugated goat α -rabbit (Sigma). NBT/BCIP colorimetric detection (BioRad) was employed. The relative electrophoretic mobilities of rPCBP2, ovalbumin (46 kDa), and carbonic anhydrase (30 kDa) are indicated by arrows. Forty micrograms of total protein from a HeLa cell translation extract left undepleted (lane 1), depleted on a PV S-L IV RNA affinity chromatography column (lane 3), or mock-depleted on a column not coupled to RNA (lane 2) were loaded in each lane. Three distinct PCBP species representing differentially posttranslationally modified forms of PCBP2 and PCBP1, a highly homologous isoform, were resolved and detected with α -PCBP2 polyclonal antisera. The slowest migrating species can be resolved as a doublet (data not shown) as is indicated by two arrows.

two reporter RNAs were used to address the possibility that the viral 3' NCRs participate in the initiation of translation via these IRES elements. Furthermore, two FMDV 5' NCR/luciferase RNAs, FMDV(Long) and FMDV(Short), were generated. FMDV(L) contains the entire 5' NCR of the virus, including sequences that encode a leader polypeptide upstream of the initiator AUG codon that is the preferential initiation site of FMDV polyprotein synthesis. FMDV(S) contains only those 5' NCR sequences upstream of the less frequently uti-

FIGURE 3. Picornavirus 5' NCR luciferase reporter RNAs. The 5' NCRs of picornaviruses that contain type I IRES elements (PV, CVB, and HRV) and type II IRES elements (EMCV and FMDV) are represented. The 5' NCR luciferase RNAs were transcribed in vitro utilizing transcription plasmids containing the 5' NCR of each picornavirus linked in-frame to the firefly luciferase gene by two cytosine residues. FMDV(L) RNA contains the 84 nt that precede the preferentially utilized initiator AUG; however, in a small number of translation events, an initiator AUG upstream of these 84 nt is utilized as the initiator codon. When this upstream AUG is utilized as the initiator codon, a 28-amino acid leader polypeptide is translated at the amino-terminus of the FMDV L proteinase (or in this case luciferase). FMDV(S) RNA does not contain the 84 nt that encode the leader polypeptide. All of the 5' NCR luciferase reporter RNAs are uncapped and are preceded by two non-viral guanosine residues.

lized alternate initiation codon of the leader polypeptide. If this alternate codon is utilized for the initiation of translation on FMDV(L) RNA, the leader polypeptide is translated at the N-terminus of luciferase.

In vitro translation assays utilizing the luciferase reporter RNAs were performed in triplicate with each RNA, and luciferase levels were assayed in triplicate for each reaction. Reactions were carried out with extract that had been depleted by stem-loop IV RNA affinity chromatography (S-L IV-depleted) or extract that had been passed over a column of matrix that was not coupled to any RNA (mock-depleted) and was thus not depleted of PCBP2 (Fig. 2, lane 2). Although only minor reductions in translation were observed upon comparison of S-L IV-depleted reactions with mock-depleted reactions that were programmed with reporter RNAs containing type II IRES elements (Fig. 4B), significant decreases, of one log unit or greater, were recorded upon comparison of similar type I IRES-mediated translation reactions (Fig. 4A). Furthermore, some of the reduced translation ability of RNAs containing type I IRES elements in S-L IV-depleted extracts programmed with reporter could be dose-dependently restored by the addition of rPCBP2. As shown in Table 1, the translation of the type I IRES luciferase reporter RNAs in S-L IV-depleted extracts could be stimulated from ~4-fold to 30-fold over control reactions by the addition of rPCBP2. However, with some variability, we were not always able to rescue translation directed by type I IRES elements to mock-depleted levels in the S-L IV-

depleted translation reactions (Fig. 4A). This result suggested the parallel depletion of an additional factor(s) that may be required for type I IRES-directed translation. In the experiments with type II IRES elements, the depletion of endogenous PCBP2 or the addition of rPCBP2 had little effect on translation (Fig. 4B). Furthermore, the presence or absence of the 3' NCRs of PV and EMCV did not appear to significantly affect translation in the in vitro assays. Taken together, these data suggest that type I IRES elements require the cellular protein PCBP2 for translation whereas type II IRES elements do not.

PCBP2 is not a general translation factor

To confirm that PCBP2 does not act as a general translation initiation factor, we conducted in vitro translation reactions with mock- and S-L IV-depleted HeLa extracts programmed with m⁷GTP-capped mRNAs. Both brome mosaic virus (BMV) RNA and capped, polyadenylated mRNA purified from rabbit reticulocyte lysates by oligo(dT) cellulose chromatography were utilized to determine if PCBP2 has a role in cap-dependent translation. In vitro translation reactions programmed with BMV RNA resulted in the synthesis of five protein products corresponding to the viral proteins 1a (110 kDa), 2a (97 kDa), 3a (35 kDa), the coat protein (20 kDa), and a coat protein-related product of 15 kDa (Fig. 5). A decrease in the translation of the BMV proteins is apparent with S-L IV depletion of the in vitro translation extracts (Fig. 5, upper panel, compare lanes 1 and 6); however, the addition of rPCBP2 to the depleted extracts does not significantly rescue translation (Fig. 5, upper panel, lanes 7–9). Therefore, although PCBP2 is not involved in the translation of the capped BMV RNA, other factors important for translation of BMV RNA appear to be depleted or modified (either directly or indirectly) by S-L IV RNA affinity chromatography.

Similar results were obtained from mock- and S-L IV-depleted extracts programmed with capped and polyadenylated mRNA purified from rabbit reticulocyte lysates. The only significant signal observed on the autoradiograph from these reactions occurred at an apparent molecular mass corresponding to that expected of α -globin and β -globin chains (Fig. 5, lower panel). The depletion of PCBP2 from in vitro translation extracts did not greatly reduce the translation of globin mRNAs, indicating that PCBP2 does not appear to be involved in the translation of capped, polyadenylated mRNA. Furthermore, the addition of rPCBP2 to the S-L IV-depleted extracts did not stimulate globin translation (Fig. 5, lower panel, lanes 7–9). This result further demonstrates that PCBP2 is not involved in the translation of capped, polyadenylated mRNA. Additional reactions were conducted with BMV and globin mRNA to control for the possibility that nonspecific protein addition could alter the observed translation. In these controls, mu-

FIGURE 4. A distinct requirement for PCBP2 in the initiation of translation on picornavirus type I IRES elements (**A**) but not for translation on type II IRES elements (**B**). In vitro translation reactions that contained mock-depleted (hatched bars) or PV S-L IV-depleted (solid bars) HeLa cell translation extracts supplemented with rPCBP2 or buffer alone were programmed with the luciferase reporter RNAs. Translation reactions were performed in triplicate and luciferase assays of each reaction were performed in triplicate. The luciferase activity in each reaction is expressed as relative light units (RLU). Error bars represent one standard deviation from the mean.

TABLE 1. Rescue of type I IRES-mediated translation in S-L IV-depleted translation extracts by the addition of rPCBP2.

Mock- or S-L IV-depleted extract + [nM] rPCBP2	Fold stimulation over control reaction ^a							
	Type I IRES				Type II IRES			
	5'PV	5'PV3'	5'CVB	5'HRV	5'EMCV	5'EMCV3'	5'FMDV(L)	5'FMDV(S)
Mock-depl. + 300 nM	1.11 ± 0.24	1.11 ± 0.24	1.09 ± 0.32	1.12 ± 0.12	1.07 ± 0.11	0.93 ± 0.18	1.00 ± 0.20	1.16 ± 0.22
Mock-depl. + 600 nM	1.44 ± 0.52	1.31 ± 0.15	1.37 ± 0.42	1.42 ± 0.13	1.03 ± 0.11	0.94 ± 0.31	1.31 ± 0.26	1.20 ± 0.14
S-L IV-depl. + 300 nM	8.17 ± 2.79	5.08 ± 1.90	12.59 ± 1.41	12.63 ± 0.89	1.04 ± 0.09	1.20 ± 0.28	1.35 ± 0.65	1.36 ± 0.41
S-L IV-depl. + 600 nM	10.46 ± 2.75	7.54 ± 2.37	20.61 ± 0.89	35.21 ± 2.06	1.05 ± 0.09	1.17 ± 0.44	1.43 ± 0.50	1.61 ± 0.44

^aFold stimulation values represent mean RLU values normalized against the mean value from a parallel buffer control reaction. Plus or minus one standard deviation from the mean of the normalized value is indicated. Figure 4 contains the corresponding raw data.

tant (mut.) rPCBP2 (mKH1) that does not bind to the PV 5' NCR due to the introduction of point mutations in its first RNA-binding domain (Parsley, 1998; T.B. Parsley, B.L. Semler, unpubl.) was added to the mock- and S-L IV-depleted reactions. In all cases, the addition of the mKH1 protein along with any co-purifying contaminants had no effect on translation (Fig. 5; both panels; lanes 5 and 10). These data suggest that PCBP2 is not a general translation factor normally involved in the initiation of cap-dependent translation. Moreover, the general decrease in the ability of S-L IV-depleted extracts to translate either capped mRNAs or luciferase reporter RNAs, coupled with the inability of rPCBP2 to fully rescue translation on these RNAs suggests that, in addition to PCBP2, another translation factor(s) is depleted or modified (either directly or indirectly) by S-L IV RNA affinity chromatography.

Poly(rC) affinity chromatography as a method for PCBP2 depletion

In an effort to better preserve the integrity of the HeLa translation extracts while specifically depleting PCBP2, we tested whether PCBP2 could be effectively depleted by poly(rC). We first supplemented *in vitro* translation reactions programmed with the 5'PV luciferase reporter RNA with various amounts of poly(rC). Increasing the amounts of poly(rC) contained in the translation reactions resulted in a dose-dependent decrease in the translation of the 5'PV luciferase reporter RNA (Fig. 6A). Furthermore, the effects of this "depletion" of free PCBP2 from solution could be reversed by the addition of rPCBP2. The translation of 5'PV reporter RNA in reactions supplemented with 100 ng of poly(rC) could be dose dependently rescued by the addition of rPCBP2 (Fig. 6B). To test the ability of poly(rC) to specifically deplete PCBP2 from HeLa translation extracts, we performed poly(rC) RNA affinity chromatography depletions of the HeLa extracts. The poly(rC) affinity column, like the S-L IV column, was capable of deplet-

FIGURE 5. PCBP2 is not essential for cap-dependent translation. *In vitro* translation reactions conducted in PV S-L IV-depleted (lanes 6–10) and mock-depleted (lanes 1–5) HeLa cell translation extracts were programmed with brome mosaic virus (BMV) RNA (upper gel) or poly(A)⁺ RRL mRNA (lower gel). The reactions were supplemented with rPCBP2 or mutant (mut.) rPCBP2 (mKH1) or with buffer alone. ³⁵S-methionine labeled translation products were analyzed by electrophoresis on 12.5% (upper gel) or 15% (lower gel) polyacrylamide gels containing SDS. The translation of BMV RNAs resulted in the synthesis of five protein products including the viral proteins 1a (110 kDa), 2a (97 kDa), 3a (35 kDa), the coat protein (20 kDa), and a coat protein related product of 15 kDa. The only significant signal(s) produced by the RRL mRNA translations resolved with the mobility expected of α -globin and/or β -globin as deduced by comparison with a poliovirus protein standard. Both experiments were conducted in duplicate at two different concentrations of RNA.

FIGURE 6. The inhibition of poliovirus IRES-mediated in vitro translation by poly(rC) and reversal of inhibition by supplementation with rPCBP2. **A:** 5'PV luciferase reporter RNA-programmed HeLa cell translation extracts were supplemented with increasing amounts of poly(rC). Each reaction was assayed for luciferase activity (RLU) in triplicate and the results were plotted as a function of the amount of poly(rC) added to each reaction. **B:** 20 μ L HeLa cell translation reactions containing 100 ng of poly(rC) were supplemented with increasing concentrations of rPCBP2. Luciferase assays were performed in triplicate for each reaction. The results of an additional reaction performed without the addition of poly(rC) or rPCBP2 is indicated by a star.

ing PCBP2 to levels undetectable by Western blot analysis (Fig. 7A).

To confirm our previous results which suggested that PCBP2 was not involved in the translation of either the capped BMV RNA or the capped, polyadenylated globin mRNA, the in vitro translation reactions conducted in mock- and S-L IV-depleted extracts (Fig. 5) were carried out in mock- and poly(rC)-depleted extracts. The results of these experiments confirmed that PCBP2 is not a general translation factor, as poly(rC) depletion did not decrease translation levels; in fact, poly(rC) depletion led to slightly increased levels of translation for these capped mRNA (data not shown). Furthermore, the addition of rPCBP2 or mutant (mut.) rPCBP2 (mKH1) did not affect translation levels.

PCBP2 is a necessary factor for efficient initiation of translation on type I but not type II IRES elements

The results of in vitro translation reactions utilizing poly(rC)-depleted extracts for the translation of the luciferase reporter RNAs convincingly demonstrated a strict requirement for PCBP2 in the initiation of translation on type I IRES elements. Figure 7B displays a severe reduction in translation levels directed from both enterovirus and rhinovirus IRES elements in poly(rC)-depleted extracts compared to mock-depleted extracts. Furthermore, complete rescue of the reduced translation is possible with the addition rPCBP2, indicating that PCBP2 is specifically depleted from the translation

extracts by poly(rC) affinity chromatography. In fact, levels of rescue over poly(rC)-depleted buffer control reactions of greater than 200-fold were obtained upon the addition of the recombinant protein to depleted extracts programmed with type I IRES reporter RNAs (Table 2). The rescue of the poly(rC)-depleted extracts is specifically dependent on PCBP2 that has PV RNA binding capacity and is not dependent on a contaminating factor that copurifies with rPCBP2. This was shown by the inability of a mutant (mKH1) PCBP2 that has a severely impaired binding capacity for the PV 5' NCR to rescue translation on type I IRES elements in the poly(rC)-depleted extracts (Fig. 7B and Table 2).

While picornavirus type I IRES elements require PCBP2 to facilitate the initiation of translation, type II IRES elements do not. In translation experiments similar to those programmed with type I IRES luciferase reporter RNAs, poly(rC)-depleted extracts were programmed with type II IRES reporter RNAs. No decrease was detected in the ability of poly(rC)-depleted extracts to translate RNAs containing either cardiovirus or aphthovirus IRES elements (Fig. 7C). In fact, significant increases were observed in the translation activity of poly(rC)-depleted extracts as directed by 5'FMDV(L) and 5'FMDV(S) luciferase reporter RNAs. This result was also observed with some variability between different S-L IV-depleted HeLa cell extracts (data not shown) and correlates with the slight increases observed in the translation of BMV RNA and globin mRNA in poly(rC)-depleted extracts (data not shown, noted above).

FIGURE 7. Translation in PCBP2-depleted extracts using poly(rC) affinity chromatography. **A:** Poly(rC) RNA affinity chromatography of HeLa cell translation extracts was conducted to deplete PCBP. Western blot analysis of the depleted extracts confirmed depletion of PCBP. Western blot analysis was conducted as described in the legend to Figure 2. For molecular weight comparison, 500 ng of rPCBP2 (lane 1) and a molecular weight marker containing ovalbumin (46 kDa) and carbonic anhydrase (30 kDa) (lane 2) were loaded. Forty micrograms of total protein from a HeLa cell translation extract left undepleted (lane 3), depleted on a poly(rC) RNA affinity chromatography column (lane 5), or mock-depleted on a column not coupled to RNA (lane 4) were loaded in the remaining lanes. Three distinct PCBP species representing differentially posttranslationally modified forms of PCBP2 and PCBP1, a highly homologous isoform, were resolved and detected with α -PCBP2 polyclonal antisera. The slowest migrating species can be resolved as a doublet (indicated by two arrows). **B:** In vitro translation reactions containing mock-depleted or poly(rC)-depleted HeLa cell translation extracts supplemented with rPCBP2, mutant (mut.) rPCBP2 (mKH1), or buffer alone were programmed with 5'PV, 5'PV3', 5'CVB, and 5'HRV luciferase reporter RNAs. **C:** In vitro translation reactions were carried out as described above except 5'EMCV, 5'EMCV3', 5'FMDV(L), and 5'FMDV(S) luciferase reporter RNAs were used to program the reactions. Translation reactions were performed in triplicate and luciferase assays for each reaction were performed in triplicate. Error bars represent one standard deviation from the mean.

TABLE 2. Rescue of type I IRES-mediated translation in poly(rC)-depleted translation extracts by the addition of rPCBP2.

Mock- or poly(rC)-depleted extract + [nM] rPCBP2	Fold stimulation over control reaction ^a							
	Type I IRES				Type II IRES			
	5'PV	5'PV3'	5'CVB	5'HRV	5'EMCV	5'EMCV3'	5'FMDV(L)	5'FMDV(S)
Mock-depl. + 300 nM	1.41 ± 0.16	1.38 ± 0.21	1.04 ± 0.12	1.32 ± 0.26	1.00 ± 0.02	1.01 ± 0.10	1.03 ± 0.11	1.00 ± 0.09
Mock-depl. + 600 nM	1.71 ± 0.12	1.77 ± 0.24	1.13 ± 0.04	1.15 ± 0.25	1.04 ± 0.06	0.98 ± 0.04	1.05 ± 0.08	1.08 ± 0.28
Mock-depl. + 1200 nM	2.03 ± 0.46	2.37 ± 0.31	1.66 ± 0.19	1.47 ± 0.24	0.85 ± 0.14	0.87 ± 0.07	0.95 ± 0.09	1.01 ± 0.09
Mock-depl. + 600 nM mut. ^b	0.78 ± 0.07	1.51 ± 0.29	0.84 ± 0.04	0.94 ± 0.20	0.92 ± 0.04	1.02 ± 0.08	0.83 ± 0.07	0.94 ± 0.08
Poly(rC)-depl. + 300 nM	103.50 ± 8.64	24.49 ± 5.18	89.63 ± 13.42	30.78 ± 4.03	1.08 ± 0.03	1.01 ± 0.03	1.94 ± 0.21	1.01 ± 0.03
Poly(rC)-depl. + 600 nM	137.44 ± 45.96	55.98 ± 8.05	135.53 ± 12.51	123.13 ± 4.03	1.08 ± 0.02	1.09 ± 0.04	1.88 ± 0.16	1.09 ± 0.04
Poly(rC)-depl. + 1200 nM	198.86 ± 39.32	90.76 ± 12.22	187.31 ± 12.70	254.70 ± 17.72	1.04 ± 0.04	1.16 ± 0.05	2.26 ± 0.10	1.16 ± 0.05
Poly(rC)-depl. + 600 nM mut.	2.45 ± 0.54	1.88 ± 0.39	1.53 ± 0.17	1.24 ± 0.34	0.92 ± 0.03	0.98 ± 0.07	1.04 ± 0.14	0.98 ± 0.07

^aFold stimulation values represent mean RLU values normalized against the mean value from a parallel buffer control reaction. Plus or minus one standard deviation from the mean of the normalized value is indicated. Figure 7 contains the corresponding raw data.

^bControl reactions supplemented with a mutant (mut.) mKH1 rPCBP2 that was not capable of interacting with the poliovirus 5' NCR due to point mutations introduced into its first KH RNA-binding domain were conducted.

Finally, taking into consideration the known involvement of PCBP in the stability of some mRNAs, the possibility that poly(rC) depletion of translation extracts could affect the stability of the reporter RNAs used to program the extracts was examined. Luciferase reporter RNA stability experiments were performed in mock-depleted and poly(rC)-depleted translation extracts. The stability experiments were conducted with ³²P-labeled luciferase reporter RNAs (5'PV, 5'PV3', 5'CVB, and 5'HRV) under conditions identical to those utilized in the translation reactions. Comparison of mock-depleted and poly(rC)-depleted reactions revealed no significant difference in the stability of the luciferase reporter RNAs over a 75-min time course (data not shown).

Addition of poly(rC) inhibits *in vitro* translation on picornavirus type I IRES elements but not on type II elements

As a final test of whether PCBP2 is generally involved in picornavirus translation, we conducted *in vitro* translation reactions in mock-depleted extracts supplemented with 1.7 μ g poly(rC) per 20 μ L reaction and programmed with the luciferase reporter RNAs. In previous experiments, this concentration was sufficient to abolish translation from the 5'PV luciferase reporter RNA (Fig. 6A). The results of the translation reactions supplemented with poly(rC) clearly differentiate those 5' NCRs that are classified as possessing the primary and secondary structure of type I picornavirus IRES elements from those of type II. The presence of large quantities of poly(rC) decreases translation of luciferase from the type I reporter RNAs by greater than two log units (Fig. 8). Serving as an internal control for the specificity of this inhibition are the reactions programmed with the type II reporter RNAs. These reactions show no significant decrease in translation upon the addition of

poly(rC). As an additional confirmation of the above results, the poly(rC) inhibition experiments described above were repeated utilizing nondepleted translation extracts (data not shown). With decreases in translation directed by type I reporter RNAs of greater than one log unit in reactions supplemented with poly(rC), the results of triplicate experiments correlated perfectly with the data presented in Figure 8. Therefore, we have established a direct correlation between the introduction of a putative PCBP2-sequestering molecule and the reduction in translation from type I but not type II IRES elements.

FIGURE 8. Inhibition of picornavirus type I IRES-mediated translation initiation by the addition of poly(rC). HeLa cell translation extracts programmed with the luciferase reporter RNAs were supplemented with 1.7 μ g poly(rC) (black bars) or with equal volumes of DEPC-treated water (white bars) per 20 μ L reaction. Translation reactions were assayed in triplicate and the mean values from the triplicate assays were plotted.

DISCUSSION

In an effort to elucidate the mechanism of internal translation initiation on picornavirus RNAs, a considerable amount of work has focused on the identification of *trans*-acting cellular factors required for initiation, in addition to the canonical initiation factors. One common experimental strategy employs UV cross-linking and electrophoretic mobility-shift assays coupled with translation assays to identify proteins that interact with picornavirus 5' NCRs in a functional manner. An alternative approach exploits the observed inability of rabbit reticulocyte lysates to efficiently and accurately translate picornavirus RNAs containing type I IRES elements. The deficiencies of the lysates are utilized as the basis of a functional assay by which factors can be purified from cellular lysates (Borman et al., 1993; Hunt & Jackson, 1999; Hunt et al., 1999). Both strategies have been successful in the identification of *trans*-acting cellular proteins that are associated with picornavirus IRES activity. For example, Sonenberg and colleagues found that the La autoantigen was capable of interacting with the poliovirus 5' NCR. Furthermore, the addition of purified La to rabbit reticulocyte lysates suppressed aberrant translation initiation events and stimulated translation in *in vitro* translation reactions programmed with PV genomic RNA (Meerovitch et al., 1989, 1993; Svitkin et al., 1994).

Although the role of La in picornavirus translation appears to be limited to poliovirus, another cellular factor, the polypyrimidine track-binding protein (PTB), has been shown to interact with several picornavirus 5' NCRs. PTB associates with the type II IRES elements of EMCV and FMDV (Jang & Wimmer, 1990; Luz & Beck, 1991; Hellen et al., 1993). Similarly, interactions between PTB and the 5' NCRs of PV and HRV have been identified (Borman et al., 1993; Hellen et al., 1994). Some controversy exists over the functional significance of the interaction of PTB with poliovirus and EMCV RNAs (Pestova et al., 1991; Hellen et al., 1993, 1994; Haller & Semler, 1995; Gutierrez et al., 1997; Kaminski & Jackson, 1998). However, recent work utilizing translation in RRL as a functional assay for the purification of cellular factors involved in HRV translation initiation has definitively shown a requirement for PTB (Hunt & Jackson, 1999). PTB has also been found to be functionally involved in the direction of internal ribosome entry on the IRES of FMDV (Niepmann et al., 1997). Additionally, the same assay that was employed to show the involvement of PTB in HRV translation was utilized to demonstrate the participation of another cellular cytoplasmic protein (unr) in the mediation of internal ribosome entry on HRV RNA (Hunt et al., 1999).

In addition to the cellular proteins that have been newly identified as "translation factors" in picornavirus protein synthesis, it is not surprising that some of canonical eukaryotic translation initiation factors (eIFs)

have been implicated in the mediation of internal ribosome entry. Svitkin and colleagues have suggested the involvement of eIF-2 and eIF-2B in the translation of PV RNA (Svitkin et al., 1988). Another canonical translation initiation factor, eIF-4F, has been shown to play various roles in picornavirus internal ribosome entry. eIF-4G, a component of the eIF-4F complex, is cleaved by the viral-encoded 2A proteinases of PV, coxsackievirus, and HRV and the L proteinase of FMDV. The polypeptide products of these eIF-4G cleavage events have been shown to stimulate translation directed by the type I IRES elements of PV and HRV (Buckley & Ehrenfeld, 1987; Borman et al., 1997). Furthermore, eIF-4G has been shown to be involved in the recruitment of ribosomes to the EMCV IRES (Pestova et al., 1996a, 1996b; Kolupaeva et al., 1998). In addition to the roles that eIF-2, eIF-2B, and eIF-4G play in picornavirus internal ribosome entry, a number of other canonical initiation factors (eIF-2 α , eIF-4A, eIF-4B, eIF-4E, and eIF-4F) have been implicated in picornavirus translation (del Angel et al., 1989; Scheper et al., 1992; Pause et al., 1994; Meyer et al., 1995; Pestova et al., 1996b).

In this article, we provide evidence for the existence of a distinct mechanism for the internal initiation of translation on type I compared to initiation on type II IRES elements. This finding was demonstrated by the inability of *in vitro* translation extracts that were depleted of PCBP2 to efficiently translate type I IRES reporter RNAs whereas the translation of type II IRES reporter RNAs was not diminished. Moreover, the detrimental effect that PCBP2 depletion exerted on type I IRES translation was specific and reversible, as the addition of rPCBP2 to the depleted extracts could reconstitute translation in a dose-dependent manner. Additional *in vitro* translation experiments, which exploited the affinity of PCBP2 for poly(rC) to effectively deplete endogenous PCBP2 from participation in translation, substantiate the same conclusion. Although the translation of type I IRES reporter RNAs was severely compromised upon the addition of poly(rC), the translation of type II IRES reporter RNAs was not significantly influenced. Therefore, we conclude that PCBP2 is intimately involved in the internal initiation of translation on picornavirus type I IRES elements.

To address the possibility that PCBP2 is a general translation factor, we conducted PCBP2-depleted *in vitro* translation reactions programmed with two different capped RNAs. Even though S-L IV RNA affinity chromatography caused a slight decrease in the translation of both capped RNAs, this effect was not PCBP2 specific, as the addition of rPCBP2 did not affect translation. In addition, results of experiments conducted with poly(rC)-depleted extracts demonstrated that the translation of the capped mRNAs was not inhibited by poly(rC) depletion of PCBP2. We conclude that PCBP2 is not a general translation factor but is specifically

involved in the unique mechanism of internal ribosome entry on picornavirus type I IRES elements.

Our work provides evidence for the involvement of PCBP2 in internal ribosome entry on picornavirus type I IRES elements; however, the exact role that PCBP2 performs in this complex process is not well-defined. We propose three models to explain the requirement for PCBP2 in the internal initiation of translation on type I IRES elements. (1) PCBP2 that is bound to type I IRES elements interacts directly with ribosomes to facilitate ribosome recruitment and the subsequent initiation of translation. (2) The interaction of PCBP2 with type I IRES elements stabilizes RNA secondary and/or tertiary structures allowing for the direct recruitment of ribosomes. (3) The recruitment of known cellular translation factors (or factors previously not characterized as translation factors) to type I IRES elements is facilitated by direct protein-protein interactions with IRES-bound PCBP2 or by PCBP2-stabilization of IRES structures necessary for additional cellular factors to bind directly to the RNA. Although the first two models have been previously proposed (Jackson & Kaminski, 1995), the third model is potentially feasible considering the increasing number of unique factors that may function in internal ribosome entry. Bearing in mind the possible biochemical complexity of the process of internal ribosome entry, various permutations or even extensions of the proposed models may be needed to account for all of the intricacies.

Finally, it will be important to determine the precise function of the observed interactions between PCBP2 and the cardiovirus and aphthovirus 5' NCRs. We have demonstrated that PCBP2 is capable of interacting with picornavirus 5' NCRs that contain either type I or type II IRES elements; however, this interaction is functionally significant for the promotion of translation only on type I IRES elements. Given that PCBP2 has been implicated in the regulation of poliovirus RNA replication (Parsley et al., 1997; Gamarnik & Andino, 1997, 1998), this leaves open the possibility that PCBP2 binding to the cardiovirus and aphthovirus 5' NCRs is involved in viral RNA replication.

MATERIALS AND METHODS

Plasmid design

Synthetic oligonucleotides (primers) were as follows:

PV– (5'-TAATCCATGGTATGATACAATTGTCTGATT-3');
CVB3– (5'-TAATCCATGGTTTGCTGTATTCAACTAACA-3');
HRV14– (5'-TAATCCATGGGATCACAGTATATGTATATAT-3');
EMCV– (5'-TAATCCATGGATTATCATCGTGTCTTTTCAAA-3');
FMDV(L–) (5'-TAATCCATGGCTTTCCTGTAGCTCGTGA-3');
FMDV(S–) (5'-TAATCCATGGAAAGTCAGTGGCTGTAAAGAA-3');

5'NCR+ (5'-TAATCCCGGGTAATACGACTCACTATAGGTT-3');
3'PV+ (5'-GGGTCTAGACCCTACCTCAGTCGAATTGGATTG-3');
3'PV– (5'-CCCAAGCTTGTCAAACATGAGAATTCCGTTTT-3');
3'EMCV+ (5'-ATGTAGTCACTGGCACAACGCGTTA-3');
3'EMCV– (5'-CTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTCTATCTAT-3');
bg1+ (5'-CCGGGTAATACGACTCACTATAGGACATTTGCTTC-3');
bg2+ (5'-TGACACAACCTGTGTTCACTAGCAACCTCAAACAGACAC-3');
bg1– (5'-CATGGTGTCTGTTTGAGGTTGCTAGTGAACACAGTTGT-3');
bg2– (5'-GTCAGAAGCAAATGTCCTATAGTGAGTCGTATTAC-3').

pT220-460, which contains the cDNA for stem-loop IV RNA of the poliovirus IRES downstream of a T7 promoter, and the PCBP2 bacterial expression vector, pQE30-PCBP2, have been previously described (Dildine & Semler, 1992; Blyn et al., 1996).

Oligonucleotide mismatch mutagenesis was utilized for mutagenesis of regions encoding a putative RNA binding domain within the PCBP2 bacterial expression vector pQE30-PCBP2 (Blair et al., 1998).

The picornavirus 5' NCR luciferase reporter constructs were generated by cloning of polymerase chain reaction (PCR) amplified 5' NCR sequences into the *Xma*I/*Xba*I sites of pUC19 along with the *Nco*I/*Xba*I DNA fragment of pGL3-control (Promega) that encodes a modified firefly luciferase. PV–/5'NCR+, CVB3–/5'NCR+, HRV14–/5'NCR+, EMCV–/5'NCR+, FMDV(L–)/5'NCR+, and FMDV(S–)/5'NCR+ were the primer sets used in the PCR amplifications of the 5' NCRs for insertion into the luciferase reporter constructs, p5'PVLuc, p5'CVBLuc, p5'HRVLuc, p5'EMCVLuc, p5'FMDV(L)Luc, and p5'FMDV(S)Luc, respectively. Templates for the PCR 5' NCR amplifications consisted of infectious picornavirus clones (or in the case of FMDV, truncations thereof) as listed respectively: pT7PV1 (Haller & Semler, 1992), pCVB3-0 (Chapman et al., 1994), pT7-HRV14(ST) (Todd et al., 1997), pE-C9 (Hahn & Palmenberg, 1995), and a subgenomic derivative of pRMC₂₅ (Rieder et al., 1993) that was truncated in VP2 (truncated pRMC₂₅ was used as template for both FMDV 5'NCR amplifications). Whereas the amplified 5' NCRs of p5'HRVLuc, p5'EMCVLuc, p5'FMDV(L)Luc, and p5'FMDV(S)Luc were inserted as *Xma*I/*Nco*I fragments, the presence of *Nco*I sites in the PV and CVB 5' NCRs necessitated the insertion of these 5' NCRs into p5'PVLuc and p5'CVBLuc as two fragments, *Xma*I/*Eco*RI and *Eco*RI/*Nco*I.

To construct p5'PVLuc3' and p5'EMCVLuc3', primer sets 3'PV+/3'PV– and 3'EMCV+/3'EMCV– were used to amplify the PV and EMCV 3' NCRs from pT7PV1 and pE-C9 templates for subcloning into p5'PVLuc and p5'EMCVLuc. Following amplification, the PV 3' NCR PCR product was treated with *Xba*I and *Hind*III to produce sticky ends for direct insertion into the *Xba*I/*Hind*III sites of p5'PVLuc. After treatment with T₄ polynucleotide kinase, the EMCV 3' NCR PCR product was blunt-end ligated into the *Xba*I/*Sa*I sites (which were both blunt-ended and dephosphorylated) of p5'EMCVLuc.

For the construction of p5'βgLuc, the synthetic oligonucleotides bg1+, bg2+, bg1−, and bg2− were phosphorylated with T₄ polynucleotide kinase. After phosphorylation, bg1+ and bg2− along with bg2+ and bg1− were annealed. The resulting synthetic inserts were ligated into the *Xma*I/*Xba*I sites of pUC19 along with the *Nco*I/*Xba*I DNA fragment of pGL3-control. The orientation, sequence, and junctions of all plasmid constructs were confirmed by dideoxynucleotide sequencing.

Transcription and purification of RNA

RNAs for use in in vitro translation reactions were generated by run-off transcription utilizing T7-based MAXIscrip in vitro transcription kits (Ambion). Plasmids were linearized by digestion with *Xba*I (p5'PVLuc, p5'CVBLuc, p5'HRVLuc, p5'EMCVLuc), *Acc*I (p5'FMDV(L)Luc, p5'FMDV(S)Luc), *Sal*I (p5'EMCVLuc3'), or *Eco*RI (p5'PVLuc3'). Following transcription, each reaction was treated with DNase I, phenol/chloroform extracted twice, chloroform extracted, ethanol precipitated in the presence of 625 mM ammonium acetate, and subjected to gel filtration on a spin column. Plasmid templates for use in the transcription of unlabeled competitor 5' NCR RNAs for RNA competition mobility-shift analyses were linearized with *Bbs*I (p5'PVLuc, p5'CVBLuc), *Nco*I (p5'HRVLuc, p5'EMCVLuc, p5'FMDV(L)Luc, p5'FMDV(S)Luc, p5'βgLuc), or *Hind*III (pT220–460). For the generation of the S-L IV RNA probe for use in the competition electrophoretic mobility-shift analysis, *Hind*III-linearized pT220–460 was transcribed as described for the competitor RNAs; however, the reaction contained 50 μM unlabeled UTP and 50 μCi [α -³²P]UTP (specific activity: 3,000 Ci/mmol) (Amersham Pharmacia Biotech). S-L IV RNA for coupling to the RNA affinity column was generated using T7-MEGAscript (Ambion) starting with 4 μg of *Hind*III-linearized pT220–460 per 20 μL reaction.

Poly(A)⁺ RNA was purified from rabbit reticulocyte lysates (Green Hectares). Initial isolation of total RNA followed by duplicate sequential selection on oligo(dT)-cellulose type 7 was carried out as described (Sambrook et al., 1989). Poly(rC) with an estimated average chain length of 286 nt, based on sedimentation analysis, was purchased from Pharmacia.

Competition RNA mobility-shift analyses

The competition RNA mobility-shift analyses were performed essentially as described by Dildine and Semler (1992) with some modifications. Briefly, purified rPCBP2 (final reaction concentration was 100 nM) was preincubated for 10 min at 30 °C with or without unlabeled competitor RNA (at the appropriate molar ratio to probe) in the presence of RNA binding buffer [5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH, pH 7.8, 25 mM KCl, 2 mM MgCl₂, 0.1 mM disodium ethylenediamine tetraacetate (EDTA), 3.8% glycerol, 2 mM dithiothreitol (DTT), 1 mg/mL *Escherichia coli* tRNA (Sigma), 0.5 mg/mL bovine serum albumin (BSA)] in a total volume of 9 μL. Following preincubation, 1 μL of ³²P-labeled poliovirus S-L IV RNA probe was added to a final concentration of 10 nM and incubation was continued for 10 min at 30 °C. After the addition of 2.5 μL 50% glycerol to each reaction, the resulting complexes were resolved

at 4 °C on native 5% polyacrylamide (40:1 acrylamide-bisacrylamide)-5% glycerol-0.5× TBE (45 mM Tris-borate, 1 mM EDTA) gels and were visualized by autoradiography.

Purification of rPCBP2

Purification of histidine-tagged PCBP2 was performed essentially as described by Parsley et al. (1997) with the following modifications. Briefly, PCBP2 was purified from IPTG-induced *E. coli* JM109 cells carrying pQE30-PCBP2 (Blyn et al., 1996). The conditions for growth, induction of protein expression, and purification of PCBP2 were identical to the previously described methods; however, the lysis of the IPTG-induced *E. coli* JM109 cells that were expressing histidine-tagged PCBP2 was achieved by incubation in lysozyme lysis buffer [25 mM Tris-HCl, pH 8.0, 2.0 × 10⁵ U/mL chicken egg white lysozyme (Sigma), 2 mM phenylmethylsulfonyl fluoride (PMSF) solubilized in 100% ethanol] for 30 min at 0 °C followed by sonication. Prior to use in RNA mobility-shift assays or in vitro translation reactions, the purified protein was dialyzed overnight at 4 °C against 4 L initiation factor buffer [5 mM Tris-HCl, pH 7.4, 100 mM KCl, 0.05 mM EDTA, 1 mM DTT, 5% glycerol (Brown & Ehrenfeld, 1979)]. Purity and quantity of PCBP2 was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis and Bradford protein assay (Bio-Rad). The fraction of rPCBP2 used in these experiments was determined to be >95% pure by Coomassie-blue staining.

HeLa S10 preparation

Extracts were prepared essentially as described by Barton and Flanagan (1993), which includes adaptations of methods originally described by Brown and Ehrenfeld (1979), with the following modifications: (1) HeLa cells were resuspended in a volume of hypotonic buffer equal to that of the cell pellet, (2) the cells were lysed by as few strokes of the glass Dounce homogenizer as possible to obtain 95% lysis as assayed by trypan blue exclusion, (3) the extract was treated with 120 U micrococcal S7 nuclease (Worthington Biochemical) per milliliter of S10 supernatant at 13 °C for 15 min, and (4) ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) was added to a final concentration of 4 mM for inactivation of the nuclease. The HeLa S10 translation extracts were stored under liquid nitrogen.

HeLa cell IF (RSW) preparation

HeLa cell initiation factor (IF), ribosomal salt wash (RSW), was prepared essentially by protocols adapted from Brown and Ehrenfeld (1979) by Barton and Flanagan (1993). The only modification was the clarification of the dialyzed IF by centrifugation for 10 min at 3,000 rpm in a Beckman JA-20 rotor. The IF preparation was then stored under liquid nitrogen.

Preparation of RNA affinity columns

The poliovirus S-L IV and the poly(rC) RNA affinity columns were prepared by methods adapted from Fahnestock and Nomura (1972) and Metspalu et al. (1978). Briefly, 1 mg RNA

[either S-L IV RNA or poly(rC)] in 0.1 M Na(CH₃CO₂) (pH 5.0) was oxidized by treatment with a 120-fold molar excess of NaIO₄. After incubation in the dark at room temperature for 1 h, a 10,000-fold molar excess of glycerol over NaIO₄ was added to inactivate the excess NaIO₄. After inactivation for 20 min at room temperature, the RNA was precipitated with ethanol, collected by centrifugation, redissolved in 0.1 M Na(CH₃CO₂), and precipitated again by the addition of ethanol. The precipitated RNA was then collected by centrifugation, washed in 70% ethanol, and resuspended at a concentration of 1 mg RNA per milliliter 0.1 M Na(CH₃CO₂) (pH 5.4).

To prepare the affinity column resin, 2 mL of agarose-adipic acid hydrazide (Pharmacia) was washed with two volumes of wash buffer I [0.1 M Na(CH₃CO₂), pH 4.0, 0.5 M NaCl] followed by two volumes of wash buffer II [0.1 M Tris-acetate, pH 8, 0.5 M NaCl]. These alternate washes were repeated two times followed by three two-volume washes with 0.1 M Na(CH₃CO₂) (pH 5.4). During the final wash, the matrix was divided into two equal volumes.

The oxidized RNA was then added to one-half of the matrix, while an equal volume of 0.1 M Na(CH₃CO₂) (pH 5.4) was added to the "mock column" matrix. After overnight incubation in the dark with gentle rocking at 4 °C, a 10-fold molar excess of sodium cyanoborohydride over RNA was added to each reaction and incubation was continued for 24 h at 4 °C in the dark. Following confirmation of the efficient coupling of the RNA to the column by ethidium bromide-stained agarose gel electrophoresis of the reaction supernatant, the column was washed extensively in 0.1 M Na(CH₃CO₂) (pH 5.4) followed by a 20-volume TE [10 mM Tris-HCl, pH 8.0, 1 mM EDTA] wash. The column was stored in TE at 4 °C.

RNA affinity chromatography depletion of HeLa cell translation extracts

Poliovirus S-L IV RNA and poly(rC) RNA affinity columns were washed extensively with binding buffer [20 mM HEPES-KOH, pH 7.4, 1.3 mM Tris-HCl, pH 7.5, 33 mM KCl, 80 mM K(CH₃CO₂), 3.7 mM Mg(CH₃CO₂)₂, 4.3 mM DTT, 13 μM EDTA, 1.3% glycerol] before use. Following the wash, a small aliquot of a mixture containing 74% HeLa S10 and 26% HeLa IF (RSW) was saved (nondepleted extract), whereas the remaining mixture was divided into two equal volumes. One aliquot of the remaining HeLa cell extract was applied to a S-L IV or a poly(rC) RNA affinity column, whereas the other volume was applied to the respective "mock column" to which no RNA was coupled. Each flow through was collected and was reapplied to the column four additional times. Following the fifth pass over the RNA affinity or mock columns the depleted or mock-depleted HeLa cell translation extracts were stored under liquid nitrogen. The columns were recycled for additional depletions by extensive washes in 2 M KCl followed by TE. All depletion experiments were conducted at 4 °C. Multiple HeLa cell extracts that were separately depleted were later combined to obtain sufficient quantities of uniform extract for the translation reactions.

In vitro translation

Preliminary RNA titration, translation time course, and rPCBP2 dose-response experiments (data not shown) were con-

ducted with the 5'PV luciferase reporter RNA to establish reaction parameters for the in vitro translation reactions. The translation reactions (20 μL) contained 68% (by volume) HeLa S10/RSW (S-L IV, poly(rC), or mock-depleted), 50 fmol luciferase reporter RNA, 16 mM HEPES-KOH (pH 7.4), 60 mM K(CH₃CO₂), 1 mM ATP, 250 μM GTP, 250 μM CTP, 250 μM UTP, 30 mM creatine phosphate (Boehringer Mannheim), 400 μg of creatine-kinase (Boehringer Mannheim) per milliliter, and 15% (by volume) initiation factor buffer (Brown & Ehrenfeld, 1979) that did or did not contain rPCBP2 or mutant rPCBP2 (mKH1). These reactions were incubated at 30 °C for 75 min, at which time translation was terminated by two-fold dilution with 1× Reporter Lysis Buffer (Promega) and transfer to 0 °C.

The in vitro translation reactions of the BMV RNA and the poly(A)⁺ RRL mRNA were conducted in mock- and S-L IV-depleted HeLa cell translation extracts under similar conditions with the following modifications: (1) the BMV reactions were programmed with either 200 ng or 400 ng BMV RNA whereas the poly(A)⁺ RRL mRNA reactions were programmed with either 250 ng or 500 ng RNA, (2) the reactions were incubated for 3.5 h, and (3) the reactions were supplemented with 10.5 μCi ³⁵S-methionine (specific activity: >1,000 Ci/mmol) (Amersham Pharmacia Biotech). These reactions were also conducted in mock- and poly(rC)-depleted extracts at 400 ng BMV RNA and 500 ng poly(A)⁺ RRL mRNA concentrations per 20-μL reaction. To control for RNA saturation of extracts, 200 ng and 600 ng BMV RNA reactions and 250 ng and 750 ng poly(A)⁺ RRL mRNA reactions were conducted in parallel.

The conditions for poly(rC)-inhibited translation reactions (conducted in mock-depleted extracts) were the same as for the other luciferase reporter reactions except varying amounts of poly(rC) (7 ng to 1.7 μg) were added in place of the 15% (by volume) initiation factor buffer that did or did not contain rPCBP2. Similarly, the experiments displaying the rescue of poly(rC)-inhibited translation were conducted under conditions identical to the poly(rC)-inhibited (100 ng poly(rC)) reactions; however, these reactions were supplemented with 12% (by volume) initiation factor buffer that did or did not contain varying amounts of rPCBP2.

Luciferase assay

Luciferase assays were performed on three separate 5-μL samples from each of the 1× Reporter Lysis Buffer-diluted translation reactions. A Monolight 2010 Analytical Luminescence Laboratory was utilized with automatic injection of 100 μL of reconstituted Luciferase Assay Substrate (Promega) followed by a 10 s read to quantitate luciferase activity.

ACKNOWLEDGMENTS

We are grateful to Gwendolyn Jang and David Brown for their critical reading of the manuscript and helpful scientific discussions. We also thank Nora Chapman and Steven Tracy for the gift of pCVB3-0, Ann Palmenberg for the gift of the pE-C9 plasmid, Peter Mason for the gift of the subgenomic derivative of pRMC₂₅, Louis Leong for valuable experimental advice and subcloning of p5'PVLuc3', and Todd Parsley for the construction of pQE30-PCBP2(mKH1) and the purification of mutant (mKH1) PCBP2.

This work was supported by Public Health Service grants AI 26765 and AI 12387 from the National Institutes of Health. B.L.W. is supported by a research fellowship from the American Heart Association, Western States Affiliate.

Received July 12, 1999; returned for revision August 10, 1999; revised manuscript received August 25, 1999

REFERENCES

- Barton DJ, Flanagan JB. 1993. Coupled translation and replication of poliovirus RNA in vitro: Synthesis of functional 3D polymerase and infectious virus. *J Virol* 67:822–831.
- Blair WS, Parsley TB, Bogerd HP, Towner JS, Semler BL, Cullen BR. 1998. Utilization of a mammalian cell-based RNA binding assay to characterize the RNA binding properties of picornavirus 3C proteinases. *RNA* 4:215–225.
- Blyn LB, Chen R, Semler BL, Ehrenfeld E. 1995. Host cell proteins binding to domain IV of the 5' noncoding region of poliovirus RNA. *J Virol* 69:4381–4389.
- Blyn LB, Swiderek KM, Richards O, Stahl DC, Semler BL, Ehrenfeld E. 1996. Poly(rC) binding protein 2 binds to stem-loop IV of the poliovirus RNA 5' noncoding region: Identification by automated liquid chromatography-tandem mass spectrometry. *Proc Natl Acad Sci USA* 93:11115–11120.
- Blyn LB, Towner JS, Semler BL, Ehrenfeld E. 1997. Requirement of poly(rC) binding protein 2 for translation of poliovirus RNA. *J Virol* 71:6243–6246.
- Borman A, Howell MT, Patton JG, Jackson RJ. 1993. The involvement of a spliceosome component in internal initiation of human rhinovirus RNA translation. *J Gen Virol* 74:1775–1788.
- Borman AM, Kirchweber R, Ziegler E, Rhoads RE, Skern T, Kean KM. 1997. eIF4G and its proteolytic cleavage products: Effect on initiation of protein synthesis from capped, uncapped, and IRES-containing mRNAs. *RNA* 3:186–196.
- Brown BA, Ehrenfeld E. 1979. Translation of poliovirus RNA in vitro: Changes in cleavage pattern and initiation sites by ribosomal salt wash. *Virology* 97:396–405.
- Brown EA, Zajac AJ, Lemon SM. 1994. In vitro characterization of an internal ribosomal entry site (IRES) present within the 5' non-translated region of hepatitis A virus RNA: Comparison with the IRES of encephalomyocarditis virus. *J Virol* 68:1066–1074.
- Buckley B, Ehrenfeld E. 1987. The cap-binding protein complex in uninfected and poliovirus-infected HeLa cells. *J Biol Chem* 262:13599–13606.
- Chapman NM, Tu Z, Tracy S, Gauntt CJ. 1994. An infectious cDNA copy of the genome of a non-cardiovirulent coxsackievirus B3 strain: Its complete sequence analysis and comparison to the genomes of cardiovirulent coxsackieviruses. *Arch Virol* 135:115–130.
- Collier B, Goobar-Larsson L, Sokolowski M, Schwartz S. 1998. Translational inhibition in vitro of human papillomavirus type 16 L2 mRNA mediated through interaction with heterogenous ribonucleoprotein K and poly(rC)-binding proteins 1 and 2. *J Biol Chem* 273:22648–22656.
- Czyzyk-Krzeska MF, Bendixen AC. 1999. Identification of the poly(C) binding protein in the complex associated with the 3' untranslated region of erythropoietin messenger RNA. *Blood* 93:2111–2120.
- del Angel RM, Papavassiliou AG, Fernandez-Tomas C, Silverstein SJ, Racaniello VR. 1989. Cell proteins bind to multiple sites within the 5' untranslated region of poliovirus RNA. *Proc Natl Acad Sci USA* 86:8299–8303.
- Dildine SL, Semler BL. 1992. Conservation of RNA-protein interactions among picornaviruses. *J Virol* 66:4364–4376.
- Dorner AJ, Semler BL, Jackson RJ, Hanecak R, Duprey E, Wimmer E. 1984. In vitro translation of poliovirus RNA: Utilization of internal initiation sites in reticulocyte lysate. *J Virol* 50:507–514.
- Fahnestock SR, Nomura M. 1972. Activity of ribosomes containing 5S RNA with a chemically modified 3'-terminus. *Proc Natl Acad Sci USA* 69:363–365.
- Futterm J, Kiss-Laszlo Z, Hohn T. 1993. Nonlinear ribosome migration on cauliflower mosaic virus 35S RNA. *Cell* 73:789–802.
- Gamarnik AV, Andino R. 1997. Two functional complexes formed by KH domain containing proteins with the 5' noncoding region of poliovirus RNA. *RNA* 3:882–892.
- Gamarnik AV, Andino R. 1998. Switch from translation to RNA replication in a positive-stranded RNA virus. *Genes & Dev* 12:2293–2304.
- Ghazi F, Hughes PJ, Hyypia T, Stanway G. 1998. Molecular analysis of human parechovirus type 2 (formerly echovirus 23). *J Gen Virol* 79:2641–2650.
- Graff J, Cha J, Blyn LB, Ehrenfeld E. 1998. Interaction of poly(rC) binding protein 2 with the 5' noncoding region of hepatitis A virus RNA and its effects on translation. *J Virol* 72:9668–9675.
- Gutierrez AL, Denova-Ocampo M, Racaniello VR, del Angel RM. 1997. Attenuating mutations in the poliovirus 5' untranslated region alter its interaction with polypyrimidine tract-binding protein. *J Virol* 71:3826–3833.
- Hahn H, Palmenberg AC. 1995. Encephalomyocarditis viruses with short poly(C) tracts are more virulent than their mengovirus counterparts. *J Virol* 69:2697–2699.
- Haller AA, Semler BL. 1992. Linker scanning mutagenesis of the internal ribosome entry site of poliovirus RNA. *J Virol* 66:5075–5086.
- Haller AA, Semler BL. 1995. Stem-loop structure synergy in binding cellular proteins to the 5' noncoding region of poliovirus RNA. *Virology* 206:923–934.
- Hellen CU, Pestova TV, Litterst M, Wimmer E. 1994. The cellular polypeptide p57 (pyrimidine tract-binding protein) binds to multiple sites in the poliovirus 5' nontranslated region. *J Virol* 68:941–950.
- Hellen CU, Witherell GW, Schmid M, Shin SH, Pestova TV, Gil A, Wimmer E. 1993. A cytoplasmic 57-kDa protein that is required for translation of picornavirus RNA by internal ribosomal entry is identical to the nuclear pyrimidine tract-binding protein. *Proc Natl Acad Sci USA* 90:7642–7646.
- Holcik M, Liebhauer SA. 1997. Four highly stable eukaryotic mRNAs assemble 3' untranslated region RNA-protein complexes sharing *cis* and *trans* components. *Proc Natl Acad Sci USA* 94:2410–2414.
- Hunt SL, Hsuan JJ, Totty N, Jackson RJ. 1999. unr, a cellular cytoplasmic RNA-binding protein with five cold-shock domains, is required for internal initiation of translation of human rhinovirus RNA. *Genes & Dev* 13:437–448.
- Hunt SL, Jackson RJ. 1999. Polypyrimidine-tract binding protein (PTB) is necessary, but not sufficient, for efficient internal initiation of translation of human rhinovirus-2 RNA. *RNA* 5:344–359.
- Jackson RJ, Kaminski A. 1995. Internal initiation of translation in eukaryotes: The picornavirus paradigm and beyond. *RNA* 1:985–1000.
- Jang SK, Krausslich HG, Nicklin MJ, Duke GM, Palmenberg AC, Wimmer E. 1988. A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. *J Virol* 62:2636–2643.
- Jang SK, Pestova TV, Hellen CU, Witherell GW, Wimmer E. 1990. Cap-independent translation of picornavirus RNAs: Structure and function of the internal ribosomal entry site. *Enzyme* 44:292–309.
- Jang SK, Wimmer E. 1990. Cap-independent translation of encephalomyocarditis virus RNA: Structural elements of the internal ribosomal entry site and involvement of a cellular 57-kD RNA-binding protein. *Genes & Dev* 4:1560–1572.
- Kaminski A, Jackson RJ. 1998. The polypyrimidine tract binding protein (PTB) requirement for internal initiation of translation of cardiovirus RNAs is conditional rather than absolute. *RNA* 4:626–638.
- Kiledjian M, Wang X, Liebhauer SA. 1995. Identification of two KH domain proteins in the alpha-globin mRNP stability complex. *EMBO J* 14:4357–4364.
- Kolupaeva VG, Pestova TV, Hellen CU, Shatsky IN. 1998. Translation eukaryotic initiation factor 4G recognizes a specific structural element within the internal ribosome entry site of encephalomyocarditis virus RNA. *J Biol Chem* 273:18599–18604.
- Kozak M. 1987. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res* 15:8125–8148.
- Kozak M. 1989. The scanning model for translation: An update. *J Cell Biol* 108:229–241.

- Leffers H, Dejgaard K, Celis JE. 1995. Characterisation of two major cellular poly(rC)-binding human proteins, each containing three K-homologous (KH) domains. *Eur J Biochem* 230:447–453.
- Luz N, Beck E. 1991. Interaction of a cellular 57-kilodalton protein with the internal translation initiation site of foot-and-mouth disease virus. *J Virol* 65:6486–6494.
- Meerovitch K, Pelletier J, Sonenberg N. 1989. A cellular protein that binds to the 5'-noncoding region of poliovirus RNA: Implications for internal translation initiation. *Genes & Dev* 3:1026–1034.
- Meerovitch K, Svitkin YV, Lee HS, Lejbkowitz F, Kenan DJ, Chan EK, Agol VI, Keene JD, Sonenberg N. 1993. La autoantigen enhances and corrects aberrant translation of poliovirus RNA in reticulocyte lysate. *J Virol* 67:3798–3807.
- Metspalu A, Saarma M, Vilems R, Ustav M, Lind A. 1978. Interaction of 5-S RNA, 5.8-S RNA, and tRNA with rat-liver ribosomal proteins. *Eur J Biochem* 91:73–81.
- Meyer K, Petersen A, Niepmann M, Beck E. 1995. Interaction of eukaryotic initiation factor eIF-4B with a picornavirus internal translation initiation site. *J Virol* 69:2819–2824.
- Niepmann M, Petersen A, Meyer K, Beck E. 1997. Functional involvement of polypyrimidine tract-binding protein in translation initiation complexes with the internal ribosome entry site of foot-and-mouth disease virus. *J Virol* 71:8330–8339.
- Parsley TB. 1998. Functional analysis of poliovirus 3CD proteinase in viral gene expression and RNA replication. Ph.D. Dissertation. Irvine, California: University of California.
- Parsley TB, Towner JS, Blyn LB, Ehrenfeld E, Semler BL. 1997. Poly (rC) binding protein 2 forms a ternary complex with the 5'-terminal sequences of poliovirus RNA and the viral 3CD proteinase. *RNA* 3:1124–1134.
- Paulding WR, Czyzyk-Krzeska MF. 1999. Regulation of tyrosine hydroxylase mRNA stability by protein-binding, pyrimidine-rich sequence in the 3'-untranslated region. *J Biol Chem* 274:2532–2538.
- Pause A, Methot N, Svitkin Y, Merrick WC, Sonenberg N. 1994. Dominant negative mutants of mammalian translation initiation factor eIF-4A define a critical role for eIF-4F in cap-dependent and cap-independent initiation of translation. *EMBO J* 13:1205–1215.
- Pelletier J, Sonenberg N. 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature* 334:320–325.
- Pestova TV, Hellen CU, Shatsky IN. 1996a. Canonical eukaryotic initiation factors determine initiation of translation by internal ribosomal entry. *Mol Cell Biol* 16:6859–6869.
- Pestova TV, Hellen CU, Wimmer E. 1991. Translation of poliovirus RNA: Role of an essential *cis*-acting oligopyrimidine element within the 5' nontranslated region and involvement of a cellular 57-kilodalton protein. *J Virol* 65:6194–6204.
- Pestova TV, Shatsky IN, Hellen CU. 1996b. Functional dissection of eukaryotic initiation factor 4F: The 4A subunit and the central domain of the 4G subunit are sufficient to mediate internal entry of 43S preinitiation complexes. *Mol Cell Biol* 16:6870–6878.
- Phillips BA, Emmert A. 1986. Modulation of the expression of poliovirus proteins in reticulocyte lysates. *Virology* 148:255–267.
- Rieder E, Bunch T, Brown F, Mason PW. 1993. Genetically engineered foot-and-mouth disease viruses with poly(C) tracts of two nucleotides are virulent in mice. *J Virol* 67:5139–5145.
- Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning. A laboratory manual*. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
- Scheper GC, Voorma HO, Thomas AA. 1992. Eukaryotic initiation factors-4E and -4F stimulate 5' cap-dependent as well as internal initiation of protein synthesis. *J Biol Chem* 267:7269–7274.
- Shih DS, Shih CT, Kew O, Pallansch LA, Rueckert R, Kaesberg P. 1978. Cell-free synthesis and processing of the proteins of poliovirus. *Proc Natl Acad Sci USA* 75:5807–5811.
- Shih DS, Shih CT, Zimmermann D, Rueckert RR, Kaesberg P. 1979. Translation of encephalomyocarditis virus RNA in reticulocyte lysates: Kinetic analysis of the formation of virion proteins and a protein required for processing. *J Virol* 30:472–480.
- Stanway G, Hyypia T. 1999. Parechoviruses. *J Virol* 73:5249–5254.
- Stewart SR, Semler BL. 1997. RNA determinants of picornavirus cap-independent translation initiation. *Semin Virol* 8:242–255.
- Svitkin YV, Meerovitch K, Lee HS, Dholakia JN, Kenan DJ, Agol VI, Sonenberg N. 1994. Internal translation initiation on poliovirus RNA: Further characterization of La function in poliovirus translation in vitro. *J Virol* 68:1544–1550.
- Svitkin YV, Pestova TV, Maslova SV, Agol VI. 1988. Point mutations modify the response of poliovirus RNA to a translation initiation factor: A comparison of neurovirulent and attenuated strains. *Virology* 166:394–404.
- Todd S, Towner JS, Semler BL. 1997. Translation and replication properties of the human rhinovirus genome in vivo and in vitro. *Virology* 229:90–97.
- Wimmer E, Hellen CU, Cao X. 1993. Genetics of poliovirus. *Annu Rev Genet* 27:353–436.
- Yueh A, Schneider RJ. 1996. Selective translation initiation by ribosome jumping in adenovirus-infected and heat-shocked cells. *Genes & Dev* 10:1557–1567.