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Poly (rC) binding protein 2 forms a ternary complex with the 5′-terminal sequences of poliovirus RNA and the viral 3CD proteinase

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ABSTRACT

Poly(rC) binding protein 2 (PCBP2) forms a specific ribonucleoprotein (RNP) complex with the 5′-terminal sequences of poliovirus genomic RNA, as determined by electrophoretic mobility shift assay. Mutational analysis showed that binding requires the wild-type nucleotide sequence at positions 20–25. This sequence is predicted to localize to a specific stem-loop within a cloverleaf-like secondary structure element at the 5′-terminus of the viral RNA. Addition of purified poliovirus 3CD to the PCBP2/RNA binding reaction results in the formation of a ternary complex, whose electrophoretic mobility is further retarded. These properties are consistent with those described for the unidentified cellular protein in the RNP complex described by Andino et al. (Andino R, Rieckhof GE, Achacoso PL, Baltimore D, 1993, *EMBO J 12*:3587–3598). Dicistronic RNAs containing mutations in the 5′ cloverleaf-like structure of poliovirus that abate PCBP2 binding show a decrease in RNA replication and translation of gene products directed by the poliovirus 5′ noncoding region in vitro, suggesting that the interaction of PCBP2 with these sequences performs a dual role in the virus life cycle by facilitating both viral protein synthesis and initiation of viral RNA synthesis.

Keywords: cloverleaf; 5′ noncoding region; picornavirus; ribonucleoprotein; RNA replication; translation

INTRODUCTION

Poliovirus, the prototypic member of the Picornaviridae family, contains a single-stranded, positive-sense RNA genome of ~7,500 nt (Kitamura et al., 1981; Racaniello & Baltimore, 1981). Following its release into the cytoplasm of an infected cell, the poliovirus genome functions as a messenger RNA to direct the translation of a large viral polyprotein (potentially 247 kDa) from a single open reading frame. Translation of the RNA is mediated by RNA sequences and structural domains between nt 130 and 600 of the 5′ noncoding region (5′NCR), known as the internal ribosome entry site or IRES (Pelletier & Sonenberg, 1988; for review, see Ehrenfeld & Semler, 1995), that form specific ribonucleoprotein (RNP) complexes with cellular RNA binding proteins. Once translation of the viral RNA ensues, a series of metabolic and morphological events takes place within the cell to ensure the production of progeny virus. These events include the shut-off of host cell cap-dependent translation (Etchison et al., 1982) and transcription (Clark et al., 1991) and the formation of membrane vesicles on which viral RNA synthesis takes place (Caliguiri & Tamm, 1969, 1970a, 1970b; Bienz et al., 1987).

During poliovirus RNA replication, the viral genomic RNA serves as a template for the synthesis of negative-strand intermediates that are, in turn, utilized as templates for the production of additional positive-strand genomic RNAs. The replication of picornavirus RNA is believed to be dependent on the formation of RNP complexes at the 3′ ends of the minus- strand intermediates and at both the 3′ and 5′ ends of positive-strand viral RNA. Several groups have reported the specific interaction of host-cell proteins with the 3′ and 5′ ends of picornavirus RNA sequences, and their studies have suggested a role for these interactions in viral RNA replication (Andino et al., 1990a, 1993; Harris et al., 1994; Roehl & Semler, 1995; Todd

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et al., 1995; Blair et al., 1996; Roehl et al., 1997). The identity of these cellular proteins has yet to be determined. Viral proteins that have been implicated in viral RNA replication by genetic and biochemical studies are encoded by the P2 and P3 regions of the viral genome and may participate in this process both in the form of polyprotein precursors and/or as fully mature gene products (Semler et al., 1988; Richards & Ehrenfeld, 1990). One of these precursors, 3CD, is an active viral proteinase that contains the amino acid sequences of both the viral 3C proteinase and the RNA-dependent RNA polymerase, $3D^{pol}$.

Biochemical and genetic evidence for the role of 3CD in RNA replication is based on the following. (1) 3CD (formerly known as NCVP 2) is a component of RNA replication complexes isolated from poliovirus-infected cells (Lundquist & Maizel, 1978; Dasgupta et al., 1979). (2) A temperature-sensitive defect in RNA replication correlated with the proteolytic breakdown of 3CD without the concomitant breakdown of 3CD cleavage products, 3C (formerly known as NCVP 7c) and 3D [formerly known as NCVP 4 (Bowles & Tershak, 1978)]. (3) A poliovirus mutant with a single amino acid substitution in the 3C proteinase domain (causing reduced cleavage efficiency of the 3C/3D junction) displayed a temperature-sensitive defect in RNA synthesis that was not the result of defective proteolytic processing (Dewalt & Semler, 1989). (4) Efficient positive-strand RNA synthesis correlates with the ability of 3CD to form an RNP complex with the 5′ terminal ~100 nt of genomic RNA (Andino et al., 1993). (5) Viral constructs containing site-specific mutations coding for single amino acid lesions within the 3C domain of 3CD showed temperature-sensitive phenotypes for RNA replication while displaying minimal effects on polyprotein processing (Blair et al., 1996). These same mutated 3CD proteins, when expressed and purified from bacterial extracts, showed a decreased ability in RNP complex formation with the first 108 nt of viral RNA relative to that of wild-type 3CD. These data suggested that the 3CD polyprotein plays an integral role in the replication of viral RNA, and that this function correlates with the ability of 3CD to interact with the 5′-terminal portion of viral genomic RNA.

The formation of the RNP complex containing 3CD was described initially by Andino et al. (1990a, 1993). The authors showed that poliovirus 3CD bound to the first ~100 nt of the positive strand of viral RNA in conjunction with a cellular protein (of approximately 36 kDa) to form an RNP complex that was essential for replication of genomic RNA. The formation of this complex was dependent on the integrity of a predicted cloverleaf-like secondary structure element within the RNA, designated stem-loop I of the 5′ noncoding region. Genetic data and chemical footprinting studies revealed that 3CD binds to stem-loop d within this cloverleaf (refer to Fig. 1), whereas the cellular protein interacts with stem-loop b (Andino et al., 1993). In transfected cells, RNAs containing reporter genes downstream of poliovirus sequences harboring site-specific mutations in stem-loop b of the cloverleaf showed marked impairment in the ability of the mutated RNA to be replicated (Andino et al., 1993; Rohll et al., 1994). In addition, other investigators have reported the formation of RNP complexes containing stem-loop I, 3CD, and another viral polyprotein, 3AB, and have identified a cellular proteolytic protein fragment as a potential binding partner for 3CD in the cloverleaf complex (Harris et al., 1994; Xiang et al., 1995).

In this report, we describe the use of electrophoretic mobility shift assays with in vitro-synthesized RNA and bacterially expressed proteins to show that the

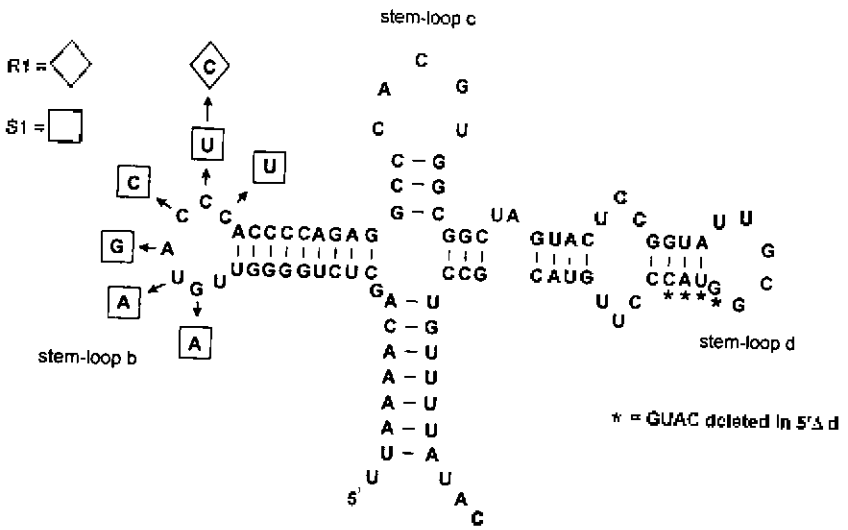

FIGURE 1. Secondary structure of the 5′-terminal cloverleaf of poliovirus RNA. The predicted secondary structure for the first 91 nt of poliovirus genomic RNA is shown along with the locations of relevant mutations. Nucleotides altered in the S1 mutation are boxed. R1 mutations are identical to those of S1 with the exception of a reversion of nt 24 back to a wild-type cytosine, as indicated by the diamond. The four nucleotides (GUAC) deleted in the 5′Δd mutation are indicated by asterisks.

cellular protein PCBP2 specifically binds to the 5′-terminal 108 nt of poliovirus genomic RNA. This protein has been shown recently to interact directly with the poliovirus IRES and to function in viral translation (Blyn et al., 1996, 1997). The PCBP2/RNA complex is further retarded in electrophoretic mobility by the addition of purified, recombinant poliovirus 3CD protein. We also show that the binding of PCBP2 to the cloverleaf structure is dependent upon the specific sequence at positions 20–25, in particular the presence of a cytosine residue at nt 24. Finally, we demonstrate that mutations in poliovirus stem-loop I that decrease its ability to interact in vitro with PCBP2 produce a decrease in both viral RNA synthesis and translation in a coupled in vitro translation/RNA replication assay, suggesting that the PCBP2/stem-loop I complex plays a role in both viral RNA replication and translation.

RESULTS

Binding of rPCBP2 to the wild-type 5′-terminal cloverleaf RNA sequences

The ability of PCBP2 to form an RNP complex with the 5′-cloverleaf of poliovirus RNA was examined by RNA electrophoretic mobility shift assays, using crude extracts from *Escherichia coli* JM109 cells transformed with the Qiagen QE30 expression vector or with the same vector containing cDNA sequences coding for human PCBP2 protein [QE30-PCBP2 (Blyn et al., 1996)]. Induction of the cells transformed with QE30-PCBP2 resulted in the expression of a recombinant histidine-tagged protein of approximately 39 kDa (rPCBP2), which was immunoreactive in western blot analysis with polyclonal rabbit anti-PCBP2 serum (data not shown). RNA mobility shift assays demonstrated that incubation of cloverleaf sequences with extracts from bacteria expressing PCBP2 resulted in the formation of a specific RNP complex (complex I; Fig. 2, lane 8). This complex was not observed when extract from bacteria transformed with the vector sequences alone (QE30) was used (lane 6), or when probe was incubated in the presence of binding buffer alone (lane 1). The complex formed by rPCBP2 is similar in mobility to that formed by a protein present in the pellet fraction of a HeLa cell lysate subjected to centrifugation at 150,000 × *g* [HeLa P-150, (lane 2)] with a slight difference due likely to the polyhistidine tract present on the bacterially expressed protein. The appearance of three complexes within this molecular weight range in lane 2 may represent the interaction of the RNA probe with cellular PCBP2 in different states of phosphorylation (Leffers et al., 1995). A further decrease in RNA mobility (complex II) was observed when purified recombinant poliovirus 3CD proteinase was added to the reactions containing either HeLa P-150 extract (lanes 3–5) or bacterial extract from cells expressing rPCBP2 (lane 9). Although there appears to be a disappearance of free probe in lanes 7 and 10, to which 3CD has been added, this addition of 3CD does not result in the formation of the stable and discrete complex seen when 3CD is incubated with the probe in the presence of either the HeLa cell extract (lanes 3–5) or the bacterial lysate containing rPCBP2 (lane 9).

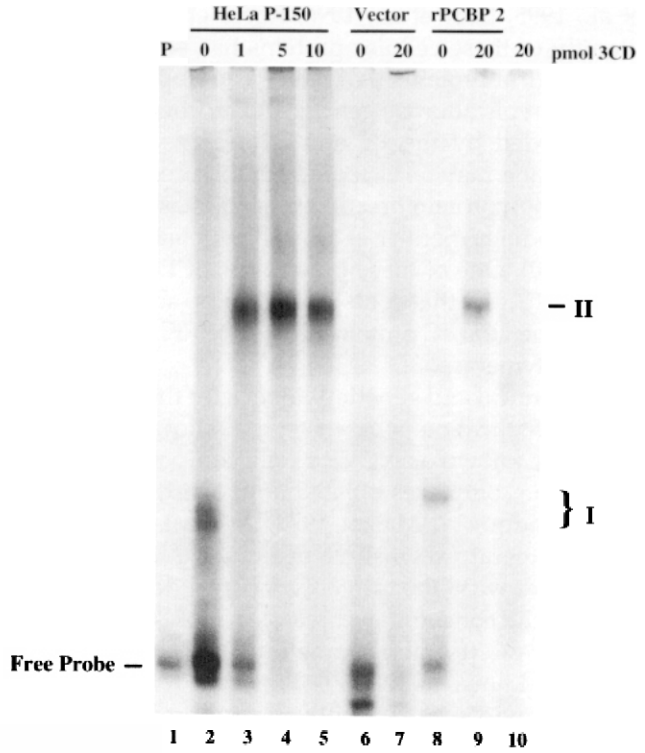

FIGURE 2. Binding of rPCBP2 to the 5′ cloverleaf of poliovirus RNA. Protein from either a HeLa P150 extract (lanes 2–5), extract from bacteria transformed with the QE-30 expression vector alone (lanes 6–7), or extract from bacteria transformed with a QE-30 expression vector containing PCBP2 cDNA coding sequences (lanes 8–9) was preincubated in the absence (lanes 2, 6, and 8) or presence (lanes 3–5, 7, and 9) of recombinant poliovirus 3CD proteinase at 30 °C for 10 min. Following pre-incubation, radiolabeled RNA probe representing the first 108 nt of the poliovirus genome was added to a final concentration of 1 nM and incubation was continued for 10 min at 30 °C. The resulting ribonucleoprotein complexes were resolved on a native 4% polyacrylamide gel. The mobilities of free probe, complex I (representing the PCBP2/viral RNA complex), and complex II (representing the 3CD containing RNP) are indicated in the figure.

Binding of rPCBP2 to the cloverleaf is specific for positive-strand sequences

Investigation of *cis*-acting determinants of picornavirus replication within the 3′ terminus of negative-strand viral RNA sequences identified cellular proteins similar in molecular weight to that of PCBP2, which photo-crosslink to viral RNAs (Roehl & Semler, 1995; Roehl et al., 1997). These negative-strand RNA sequences are complementary to those of the 5′ clover-

leaf and are believed to form a similar cloverleaf structure despite containing different primary sequences [although no biochemical confirmation of the proposed secondary structure has been presented (Roehl & Semler, 1995)]. To investigate the RNA determinants of PCBP2 binding and to examine the possibility that one of the cellular proteins identified in the RNA binding studies with negative-strand sequences was PCBP2, we performed RNA mobility shift assays using the 5′-terminal positive-strand RNA sequences and the 3′-terminal negative-strand RNA sequences with increasing amounts of rPCBP2. As shown in Figure 3, purified rPCBP2 formed a stable RNP complex with the positive-strand sequences at the lowest concentration of rPCBP2 used (5 nM, lane 2). However, even at the highest concentration of rPCBP2 (500 nM), no significant complex formation was observed with the negative-strand sequences (lane 10), indicating that binding of rPCBP2 to the viral RNA cloverleaf structure is positive-strand specific. A slower-migrating band can be observed with the minus strand probe (as indicated by the asterisk); however, the appearance of this band is independent of the presence of rPCBP2 and is most likely an electrophoretically distinguishable isoform of the RNA probe. Thus, PCBP2 is not one of the cellular proteins described by Roehl and Semler (1995) that interact with the 3′ end of virus negative-strand sequences. In addition, RNA mobility-shift assays using the minus strand 108-nt probe incubated in the presence of both rPCBP2 and 3CD show that these RNA sequences are unable to direct the formation of the PCBP2/3CD/RNA ternary complex (data not shown).

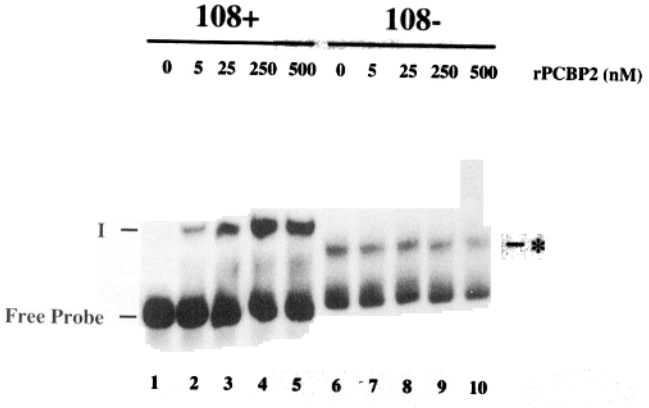

FIGURE 3. Binding of rPCBP2 to the cloverleaf is specific for positive-strand RNA sequences. Gel-purified RNA probe (0.1 nM) representing either the 5′-terminal positive-strand RNA sequences or the 3′-terminal negative-strand RNA sequences was incubated with increasing amounts of purified rPCBP2 (5–500 nM). Resulting RNP complexes were resolved on a 4% native polyacrylamide gel. Lanes 1–6, positive-strand probe; lanes 7–13, negative-strand probe. The mobilities of free probe and complex I are indicated in the figure. Asterisk indicates an isoform of the 108− probe.

Binding of PCBP2 to the 5′-terminal cloverleaf structure is specific for nt 20–25 in stem-loop b

To further define the PCBP2 binding site within the poliovirus genomic RNA stem-loop I region (i.e., the 5′-terminal cloverleaf), we performed RNA mobility shift assays with probes containing site-specific mutations in the cloverleaf (Fig. 1). Two of the mutated RNA probes, R1 and S1, contain substitutions of nt 20–25 within the loop region of stem-loop b of the cloverleaf. The third probe, 5′Δd, harbors a 4-nt deletion in the stem region of stem-loop d. A 4-nt insertion at the site of the 5′Δd mutation was described previously by Dildine and Semler (1989) as lethal to virus production. Indeed, a pseudorevertant of such a 4-nt insertion provided the first genetic evidence that poliovirus proteinase 3C contained RNA binding determinants (Andino et al., 1990b), and biochemical footprinting analysis has indicated this portion of the cloverleaf to contain the 3CD binding site (Andino et al., 1993).

The S1 mutation is a lethal lesion that was described initially by Roehl and Semler (1995). Virus recovered from HeLa cells transfected with genome-length RNAs containing the S1 mutation had a reversion of a U at nt 24 back to the wild-type C while still retaining the other four substituted nucleotides (this pseudoreverted nucleotide sequence will be referred to as the R1 mutation). Research by other groups has also implicated sequences within the loop region of stem-loop b as being critical to RNA replication, particularly the presence of the wild-type cytosine at nt 24 (Andino et al., 1993; Rohll et al., 1994). As shown in Figure 4, the presence of the S1 mutation in stem-loop b essentially abolished complex formation by rPCBP2 in an electrophoretic mobility shift assay (lanes 13–18). The presence of a cytosine at nt 24 in the R1 mutation was able to restore complex formation partially (lanes 8–12), although not to wild-type levels (lanes 2–6). These results demonstrate that rPCBP2 requires the sequences located within stem-loop b of the cloverleaf for RNA binding and that the presence of a cytosine residue at nt 24 is an important binding determinant.

The results of RNA mobility shift assays using either the wild-type probe or 5′Δd [a mutation that is predicted to disrupt the integrity of stem-loop d without affecting the structure of stem-loop b (data not shown)] are displayed in Figure 5. As shown in Figure 5A, rPCBP2 binds to both RNAs with similar affinities. However, the addition of purified recombinant 3CD to the binding reactions (Fig. 5B) does not result in a further retardation in RNA mobility of the 5′Δd probe (compare lanes 7–12 to lanes 1–6). These results demonstrate that the binding of rPCBP2 to the cloverleaf is specific for stem-loop b sequences and is unaffected by mutations in the cloverleaf that disrupt 3CD binding.

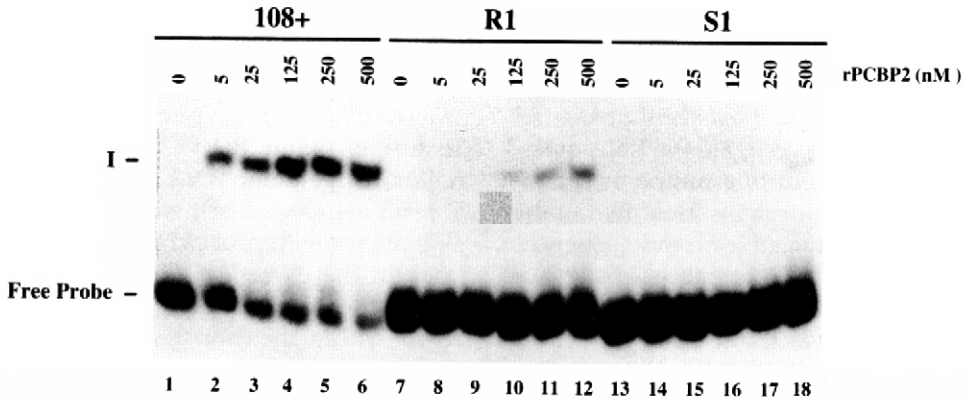

FIGURE 4. RNA mobility shift assays with probes containing site-specific mutations in stem-loop b of the 5′ cloverleaf. RNA electrophoretic mobility shift assays were performed using increasing amounts (5–500 nM) of purified rPCBP2 and 0.1 nM gel purified wild-type 108+ (lanes 1–6), R1 (lanes 7–12), or S1 (lanes 13–18) RNA probes. Resulting RNP complexes were resolved on a 4% native polyacrylamide gel. Mobility of free probe and complex I are indicated on the right-hand side of the figure.

Mutations in the 5′ cloverleaf structure display defects in RNA replication and translation in an in vitro replication assay programmed with mutated dicistronic RNAs

To provide evidence of a functional role for PCBP2 in active poliovirus RNA replication, in vitro translation/replication assays were performed using dicistronic RNAs generated in vitro from transcription templates containing the S1, R1, and Δd mutations in stem-loop I of the poliovirus 5′ NCR (Fig. 6A). Use of dicistronic RNAs in this assay system allows for the translation of the viral enzymatic proteins (P2 and P3 proteins) independently of mutations in the poliovirus 5′ NCR (Molla et al., 1992). Thus, any noted defects in RNA replication are presumed to be independent of translation and therefore viral enzyme concentrations. As controls, similar reactions were performed with in vitro-transcribed full-length poliovirus RNA (monocistronic) or in the absence of any exogenous RNA (mock). Figure 6B shows the results of in vitro translation of the dicistronic RNAs. In these assays, translation of the structural viral gene products, directed by the poliovirus 5′ NCR, was decreased ~5–7-fold when programmed with dicistronic RNA containing either the S1 or R1 mutations in stem-loop I of poliovirus relative to dicistronic RNA containing wild-type poliovirus 5′ NCR sequences (compare the level of VP3 in lanes 4

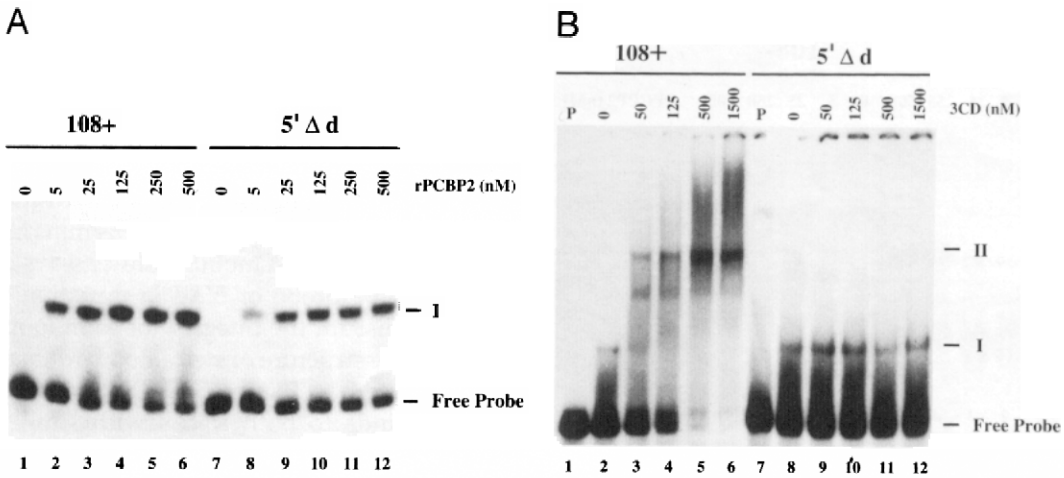

FIGURE 5. RNA mobility shift assays with RNA probe containing a 4-nt deletion in stem-loop d of the 5′ cloverleaf. RNA mobility shift assays were performed with either the wild-type probe or a probe containing a 4-nt deletion in stem-loop d of the 5′ cloverleaf in the absence or presence of increasing amounts of recombinant poliovirus 3CD proteinase. **A:** Wild-type 108+ (lanes 2–6) or 5′Δd (lanes 8–12) probe was incubated with increasing amounts of purified rPCBP2. **B:** Wild-type 108+ or 5′Δd probe was incubated with 125-nM rPCBP2 and increasing amounts of purified recombinant 3CD proteinase (lanes 2–6 and lanes 8–12, respectively). Lanes 1 and 7 show probe incubated in the presence of buffer alone. Mobilities of free probe, complex I, and complex II are indicated on the figure.

and 5 to that in lane 6). The same relative decrease in translation of the viral nonstructural gene products, directed by the EMCV IRES, was not observed for these mutated S1 and R1 RNAs (compare the level of 3CD in lanes 4 and 5 to that in lane 6). The presence of the Δd mutation in stem-loop I of the poliovirus 5′ NCR did not result in a drastic decrease in translation of the structural gene products and appeared to result in an increase in translation from the downstream EMCV IRES (Fig. 6B, lane 3). The nature of this increase is unknown. Translation levels of both the wild-type and Δd dicistronic RNAs (lanes 6 and 3, respectively) appear to be equivalent to that seen when the reaction is programmed with in vitro-transcribed full-length poliovirus RNA (lane 1). The relative levels of translation for all four dicistronic RNAs are shown graphically in Figure 6C. For these graphs, the levels of VP3 and 3CD (as determined by densitometric scanning) for each of the dicistronic RNAs were plotted relative to the wild-type dicistronic levels. This analysis indicated that translation of the P1 gene products directed by the poliovirus 5′ NCR was at 83% for the Δd mutation, 14% for the S1 mutation, and 21% for the R1 mutation. Translation of the viral nonstructural proteins, directed by the EMCV IRES, was at 130% for the Δd mutation, 92% for the S1 mutation, and 93% for the R1 mutation.

The effects of stem-loop I mutations on RNA synthesis are shown in Figure 6D. For this analysis, half of the RNA extracted from the in vitro reactions was treated with glyoxal and resolved on an agarose gel. The migration of the single-stranded viral RNA (ssRNA) product is indicated on the right side of the figure. Densitometric scanning of the ssRNA products resulting from synthesis of the S1, R1, and wild-type dicistronic RNAs (lanes 4, 5, and 6) revealed that the level of RNA synthesis in the reactions programmed with RNA harboring the S1 or R1 mutation was 17% and 55% of that of the wild-type dicistronic RNA, respectively, correlating the binding of PCBP2 to these sequences with RNA replication. Reactions programmed with the Δd dicistronic RNA (Fig. 6D, lane 3) showed no evidence of viral RNA synthesis. These results indicate that the interaction of PCBP2 with stem-loop I of the poliovirus 5′ NCR plays a role in both translation and viral RNA synthesis during the course of viral replication. RNA synthesis of wild-type poliovirus RNA (lane 1) was much greater than that exhibited by the wild-type dicistronic RNA (lane 6), most likely due to the presence of ~100 nonviral nucleotides at the 3′ end of the dicistronic RNA, which result from transcription of the *Dra* I linearized DNA template. The appearance of a higher molecular weight RNA species (migrating more slowly than ssRNA) in lanes 1, 4, 5, and 6 of Figure 6D is consistent with the presence of double-stranded RI/RF isoforms that were not denatured completely by treatment with glyoxal. All other bands in Figure 6D most likely result from nonspecific labeling of endogenous RNAs, because they are present in the mock incubated reaction (lane 2) in near equal proportions to those programmed with specific RNA templates. Additionally, as a control for quantities of RNA loaded from each of the RNA synthesis reactions, 1 μL from each reaction was resolved on a 1% nondenaturing agarose gel. Ethidium bromide staining of the gel revealed that concentrations of ribosomal RNAs were equivalent for each of the individual reactions (data not shown).

DISCUSSION

PCBP2 is a mammalian RNA binding protein that contains three hnRNP-K homologous domains [KH-domains] (Siomi et al., 1993; Leffers et al., 1995). It is characterized by its ability to interact preferentially with polycytidylic acid sequences (both rC and dC). It also binds weakly to sequences containing poly(rG) and poly(rU). PCBP2 has been shown by northern blot analysis to be expressed in most human tissues tested (Leffers et al., 1995) and has been proposed to play a functional role in stabilization of globin mRNA through the formation of a sequence-specific complex with the 3′ untranslated region of the globin mRNA (Kiledjian et al., 1995). Recently, PCBP2 was identified as a HeLa cell factor that binds to stem-loop IV of the poliovirus RNA 5′ noncoding region and is essential for efficient poliovirus translation (Blyn et al., 1996, 1997). A 3-nt insertion in stem-loop IV, which is lethal for viral growth and interferes with viral translation (Trono et al., 1988; Blyn et al., 1995), also abrogates PCBP2 binding to the stem-loop (Blyn et al., 1996). Both translation and virus production in an in vitro replication assay are diminished greatly in extracts depleted of PCBP2 (Blyn et al., 1997). Although it is assumed that PCBP2 binding to stem-loop IV facilitates internal ribosome entry or some step in the translation initiation process, the precise mechanism by which PCBP2 facilitates viral gene expression has yet to be determined. Binding of PCBP2 to the IRES element may serve to form an RNP scaffold onto which other cellular factors, or 40S ribosomes, necessary for translation initiation are loaded.

In this manuscript, we provide evidence that PCBP2 is required for poliovirus RNA replication. Our evidence shows that PCBP2 participates in the formation of an RNP complex with stem-loop I, the 5′ cloverleaf, of poliovirus genomic RNA. Formation of an RNP complex composed of the 5′ cloverleaf, poliovirus 3CD, and a cellular RNA binding factor has been shown previously to be essential for the initiation viral RNA synthesis (Andino et al., 1993; Blair et al., 1996). Our mutagenesis studies have demonstrated that the PCBP2 binding determinants are located within stem-loop b of the 5′ cloverleaf, and that the presence of a cytosine at position 24 is a determinant for PCBP2/cloverleaf

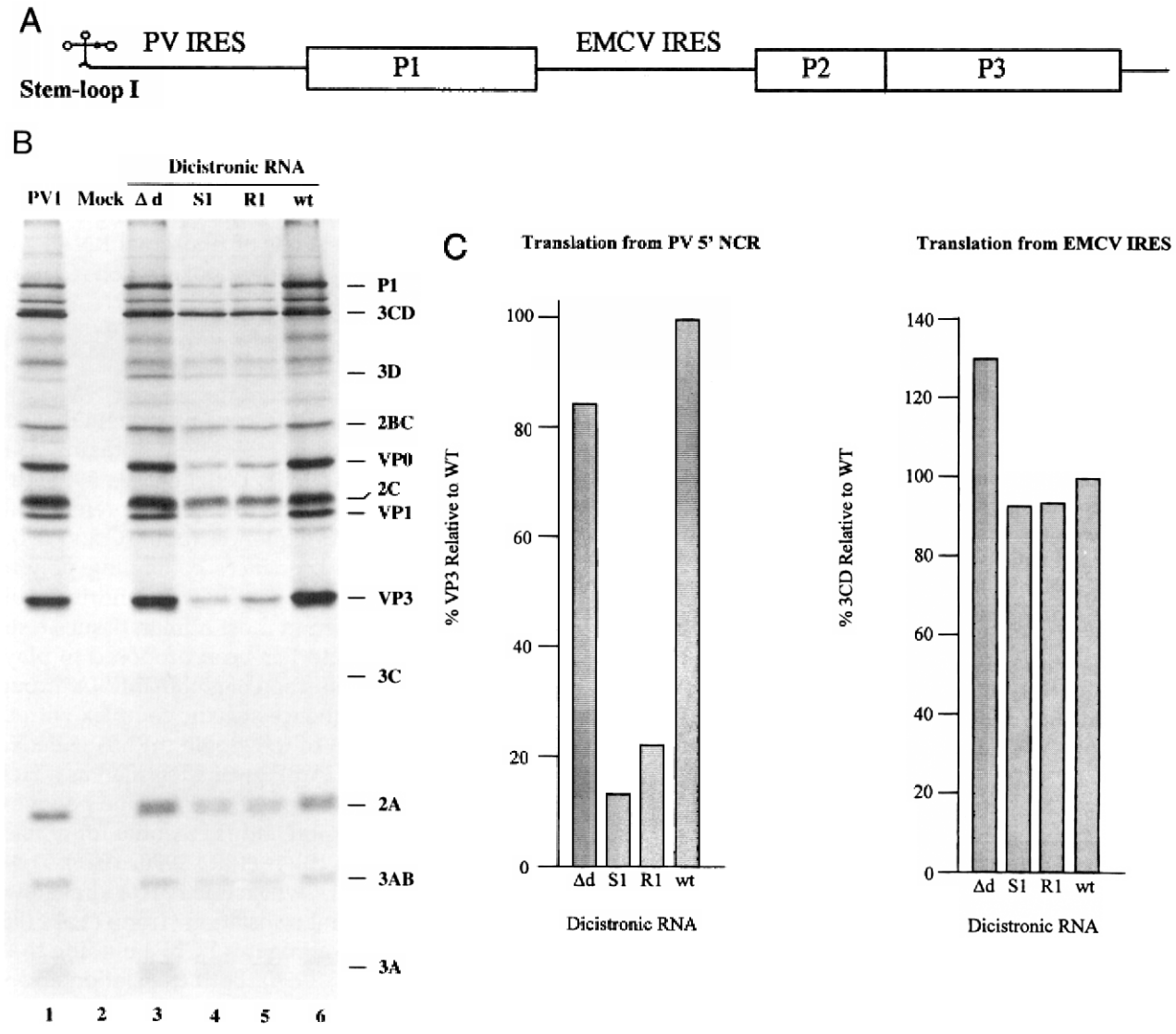

FIGURE 6A, B, and C. *(Figure continues on facing page.)*

complex formation. These data correlate the ability of PCBP2 to form a specific RNP complex with sequences of the 5′ cloverleaf implicated by others to be essential for RNA replication (Racaniello & Meriam, 1986; Andino et al., 1993; Rohll et al., 1994). We have also used dicistronic RNAs harboring mutations within stem-loop b of the cloverleaf to show that the ability of PCBP2 to interact with these sequences in in vitro RNA binding assays correlates with the ability of these sequences to be both translated and replicated in an in vitro translation/replication assay. Our data strongly suggest that PCBP2 is the previously unidentified cellular binding partner of poliovirus 3CD proteinase, and formation of an RNP complex containing PCBP2 and the poliovirus 5′ cloverleaf participates in both translation and RNA replication of the virus. It can also be concluded from the data using the Δd dicistronic RNA that the role of the PCBP2/stem-loop I interaction in viral translation is independent of 3CD binding. In contrast, 3CD binding and ternary complex formation are essential for viral RNA synthesis. The observation that PCBP2 interacts with both the cloverleaf and stem-loop IV of the 5′ noncoding region suggests that the virus has evolved to utilize a single cellular RNA binding protein for more than one function. In addition, other investigators have come to broadly similar conclusions regarding PCBP2 using a *Xenopus* oocyte expression assay (A.V. Gamarnik & R. Andino, pers. comm.).

The mechanism by which PCBP2 functions in RNA replication has yet to be elucidated; however, the addition of purified rPCBP2 to RNA polymerase reactions catalyzed by poliovirus $3D^{pol}$ does not affect RNA chain elongation (O. Richards, L.B. Blyn, & E. Ehrenfeld, unpubl. data). This observation is consistent with genetic data indicating that mutations in the viral genome (either within the 5′ cloverleaf or in 3CD) that inhibit RNP complex formation affect positive-strand

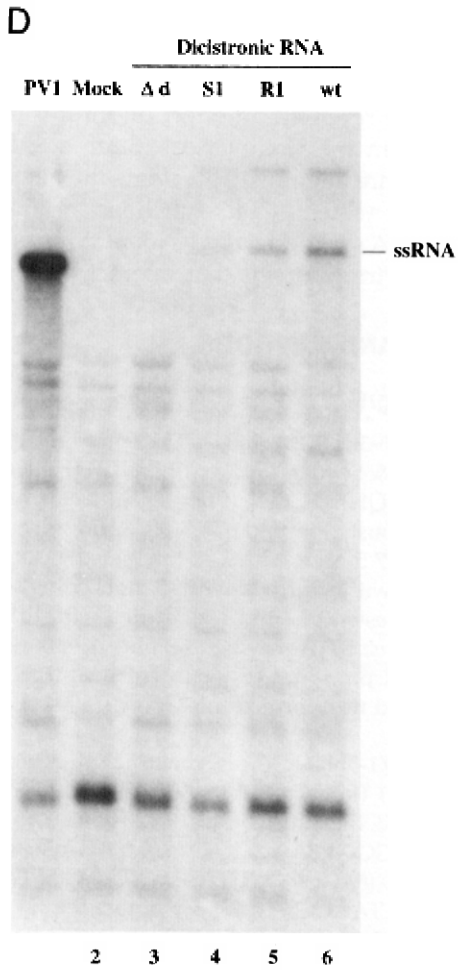

FIGURE 6. In vitro translation/replication programmed with dicistronic RNAs. **A:** Linear representation of the dicistronic RNAs (Molla et al., 1992). These constructs contain the entire poliovirus 5′ NCR followed by the coding region for the structural P1 precursor protein. The P1 coding region is separated from the coding region of the nonstructural P2/P3 protein precursor by the EMCV IRES. This allows for translation of the viral enzymatic proteins independent of mutations in the poliovirus 5′ NCR. The location of stem-loop I of the poliovirus 5′ NCR into which the S1, R1, and Δd mutations were placed is detailed in the figure. Reaction mixtures programmed with in vitro-synthesized RNA (60 μL total) were divided into aliquots containing 10 μL and 50 μL. The 10-μL aliquots contained [^{35}S]methionine and were used subsequently for translation analysis (**B**), and the 50-μL reaction was used for analysis of RNA synthesis (**D**). All reactions were incubated for 6 h at 30 °C, at which time the translation reactions were diluted in Laemmli sample buffer, boiled, and subjected to SDS-PAGE. Replication reactions were centrifuged at 15,000 × *g* for 15 min at 4 °C and resuspended in 5 μL of HEPES buffer containing 25 μCi of [α-^{32}P] CTP and incubated for an additional hour at 37 °C. Total RNA was then extracted from each sample, ethanol-precipitated, and resuspended in 20 μL of H_2O. Half of the resuspended RNA was treated with glyoxal and resolved on a 1.3% agarose–10-mM sodium phosphate gel. For both panels B and D, lane 1 is the incubation in the presence in vitro-synthesized full-length poliovirus RNA (monocistronic), lane 2 is incubation in the absence of any added exogenous RNA, and lanes 3–6 are incubation in the presence of the indicated dicistronic RNA. Electrophoretic mobilities of viral proteins resulting from the in vitro translation reactions are indicated on the right-hand side of panel B. The mobility of single-stranded viral RNA (ssRNA) is indicated on the right-hand side of panel D. **C:** Graphic representation of the relative levels of translation directed by the poliovirus 5′ NCR and the EMCV IRES for each of the dicistronic RNAs expressed as percentage of VP3 and 3CD, respectively, relative to that measured for the wild-type dicistronic RNA. Relative levels of VP3 are 83% (Δd), 14% (S1), and 21% (R1), and those of 3CD are 130% (Δd), 92% (S1), and 93% (R1).

RNA synthesis at the initiation step (Andino et al., 1990a, 1993; Blair et al., 1996). A model of strand exchange in which a pre-initiation complex containing cellular and viral factors forms on the cloverleaf of the positive-sense RNA strand of the duplex replicative form of viral RNA has been proposed (Andino et al., 1993; Harris et al., 1994). According to the model, once 3CD is bound to the positive-strand cloverleaf, and other necessary viral and/or cellular proteins have been recruited to form a competent pre-initiation complex, 3CD is processed proteolytically to release 3D^{Pol}, which then utilizes a VPg-primed minus-strand template for the synthesis of genomic viral RNA. The strand transfer model would thus require that *cis*-acting determinants of viral RNA replication be contained within a double-stranded RNA intermediate with portions of a pre-initiation complex assembled on the positive-sense strand and portions on the negative-sense strand. In this model, PCBP2 might be recruited into viral replication complexes early during the course of infection and serve as a scaffolding protein. Once bound to the RNA, PCBP2 could stabilize the positive-sense cloverleaf in a conformation that is recognizable by 3CD. In addition, PCBP2 may interact directly with the viral or cellular proteins necessary for the initiation of viral RNA synthesis. Other investigators have demonstrated the ability of the viral protein 3AB, a proposed precursor to the genome-linked protein VPg (3B), to interact with 3CD and poliovirus stem-loop I sequences (Xiang et al., 1995). It is possible that the interaction of PCBP2 with stem-loop I sequences not only stabilizes the secondary structure of the cloverleaf, but may also play a role in directing the formation of pre-initiation complexes containing other viral proteins, such as 3AB. Consistent with this hypothesis are preliminary data showing that the addition of anti-PCBP2 antibodies to the coupled in vitro translation/replication assay after replication complexes have been allowed to form does not inhibit viral RNA synthesis (data not shown). This would indicate that either PCBP2 was already in a pre-initiation complex that is inaccessible to antibodies, or that PCBP2 functions early in infection to recruit factors necessary for the initiation of RNA synthesis and that once these initiation complexes are formed, the function of PCBP2 is no longer needed for viral RNA synthesis.

We have also demonstrated that the interactions of PCBP2 with the 5′ cloverleaf have a role in viral trans-

lation. The PCBP2/5′ cloverleaf complex may function in translation through interactions with other regions of the 5′ NCR or proteins bound to the IRES element. One study has reported an effect on viral translation by mutations in the 5′ cloverleaf (Simoes & Sarnow, 1991). Studies are ongoing to elucidate the biochemical mechanism by which PCBP2 functions in viral translation and RNA synthesis and to identify possible cellular and/or viral proteins that interact with PCBP2.

MATERIALS AND METHODS

Plasmid design

Plasmids pET15b-3CDμ10 (Blair et al., 1996), pT7N108-, pPV1-5NC-S1 (Roehl & Semler, 1995), pT7-5′NCR (Haller & Semler, 1992), and QE30-PCBP2 (Blyn et al., 1996) have been described previously. Plasmid pT7-5′NCRΔd was constructed by digesting pT7 5′NCR with *Kpn* I, which digests the plasmid DNA once within the poliovirus 5′ noncoding sequences (nt 66). The digested plasmid was treated with the Klenow fragment of *E. coli* DNA polymerase, phenol-chloroform extracted, ethanol precipitated, incubated overnight with T4 DNA ligase, and transformed into *E. coli* C600 cells for amplification.

Plasmid pPV1-5NC-R1 was constructed essentially as pT7PV1-ΔN5-10 (Roehl et al., 1997), except the synthetic double-stranded oligonucleotides PV20-25R1+ (5′-TTAAA ACAGCTCTGGGGTTAAGCCTACCCCAGAGGCCCA-3′) and PV20-25R1- (5′-GGGCCTCTGGGGTAGGCTTAACCCC AGAGCTGTTTTAACCTAA-3′) containing the revertant sequences within the poliovirus 5′NCR (underlined) were used in place of HRT4 and HRT4′.

Dicistronic plasmids containing the S1, R1, and Δd mutations were constructed by digesting pPV1-5NC-S1, pPV1-5NC-R1, and pT7-5′NCRΔd with *Age* I and *Stu* I. The resulting 360-bp fragments were gel isolated and incubated with the large fragment of pT7-PV1-E2A (Molla et al., 1992) digested with *Sal* I and *Age* I in the presence of T4 DNA ligase and transformed into *E. coli* C600 cells for amplification. The nucleotide sequences of relevant regions of all plasmids were verified by dideoxynucleotide sequencing.

Transcription and purification of RNA probes

pT7-based plasmids containing poliovirus sequences were digested with either *Dde* I (pT7-5′NCR, pPV1-5NC-S1, pPV1-5NC-R1, and pT7 5′NCRΔd) or *Mse* I (pT7N108-) and transcriptions were performed in vitro as described previously (Blair et al., 1996). Where indicated in the figure legends, RNA probes were gel-purified on an 8% polyacrylamide urea gel. Following overnight elution in 0.5 M NH_4OAc, 1 mM EDTA, 0.1% SDS, the probes were ethanol precipitated, resuspended in DEPC-treated H_2O, and quantified based on specific activity.

Purification of 3CD and PCBP2

Recombinant histidine-tagged poliovirus 3CD was purified from IPTG-induced bacterial extracts of BL21(DE3) cells harboring the expression plasmid pET15b-3CDμ10 (Blair et al., 1996). Two liters of transformed BL21(DE3) cells were grown at 37 °C to an OD_{600} of 0.6, at which time IPTG was added to 0.5 mM. The cultures were then incubated overnight at room temperature. Following overnight incubation, the bacteria were pelleted and resuspended in 30 mL of buffer A (20 mM Tris-HCl, pH 7.6, 25 mM NaCl, 1 mM EDTA, 1 mM DTT, 2 mM PMSF, and 5% glycerol). The resuspended cells were lysed in a French pressure cell (Amicon) with two passes at 8,000 psi, then sonicated to reduce viscosity. The bacterial lysates were subjected to centrifugation for 20 min at 15,000 rpm in a JA20 rotor. The resulting supernatant was discarded and the pellet fraction was washed three times with 20 mL of buffer A. Following the final wash, recombinant 3CD was extracted from the insoluble pellet fraction by resuspending the pellet in 20 mL of high-salt I_{60} buffer [20 mM Tris-HCl, pH 7.9, 1 M NaCl, 60 mM imidazole, 0.5% 1-O-*n*-Octyl-β-D-glucopyranoside (ACROS), and 10% glycerol] and incubating on ice for 30 min. The resuspended pellet was then centrifuged for 30 min at 15,000 in a JA20 rotor and the resulting supernatant was loaded onto a 1-mL Hi-Trap chelating column (Pharmacia) charged with nickel. The column was washed with 10 volumes of high-salt I_{60} buffer minus 1-O-*n*-Octyl-β-D-glucopyranoside, and recombinant 3CD was eluted with high-salt I_{200} buffer (20 mM Tris-HCl, pH 7.9, 1 M NaCl, 200 mM imidazole, and 10% glycerol). Peak nickel affinity chromatography fractions were then subjected to FPLC size-exclusion chromatography on a Superdex 75 Hi-Load column (Pharmacia) with constant buffer flow (20 mM Tris-HCl, pH 7.9, 1 M NaCl, 5 mM imidazole, and 5% glycerol). 3CD-containing gel filtration fractions were pooled and 3CD was concentrated by nickel affinity chromatography on a 1-mL Hi-Trap chelating column (Pharmacia).

Recombinant histidine-tagged PCBP2 was purified from 2 L of IPTG-induced *E. coli* JM109 cells carrying the QE30-PCBP2 plasmid (Blyn et al., 1996). Two liters of transformed JM109 cells were grown at 37 °C to an OD_{600} of 0.7, at which time IPTG was added to a final concentration of 1.0 mM. The cultures were then incubated for 14 h at room temperature. Following overnight incubation, the cells were pelleted and resuspended in 30 mL of PCBP2 lysis buffer (5 mM HEPES, pH 7.4, 25 mM KCl, 0.5 mM EDTA, 0.05% β-mercaptoethanol, and 5% glycerol). Resuspended bacterial pellets were then subjected to lysis in a French pressure cell with two passes at 8,000 psi, then sonicated to reduce viscosity. The lysates were centrifuged for 20 min at 15,000 rpm in a JA20 rotor and the supernatant fraction was adjusted to 40% ammonium sulfate. Precipitated proteins were resuspended in 15 mL of I_{30} buffer (20 mM Tris-HCl, pH 7.9, 250 mM NaCl, 30 mM imidazole, 10% glycerol) containing 0.5% NP-40 and dialyzed overnight at 4 °C against 1 L of the same buffer. The dialyzed proteins were clarified by centrifugation for 10 min at 15,000 rpm in a JA20 rotor, and the clarified lysate was loaded onto a 1-mL Hi-Trap chelating column charged with nickel. The column was washed with 10 volumes of I_{30} buffer and recombinant PCBP2 was eluted with I_{200} buffer (20 mM Tris-HCl, pH 7.9, 250 mM NaCl, 200 mM imidazole, 10% glycerol). Prior to use in biochemical assays, purified PCBP2 was dialyzed for 2 h at 4 °C against two 500-mL changes of initiation factor buffer [5 mM Tris-HCl, pH 7.4, 100 mM KCl, 0.05 mM EDTA, 1 mM DTT, and 5% glycerol (Brown & Ehrenfeld, 1979)]. Purity and quantity of purified 3CD and PCBP2 were determined by SDS-PAGE analysis and the Bio-Rad protein assay.

RNA electrophoretic mobility shift analysis

RNA electrophoretic mobility shift analysis was performed as described previously (Andino et al., 1990a; Blair et al., 1996). Briefly, purified protein or the pellet fraction of a HeLa cell lysate subjected to centrifugation at 150,000 × *g* (HeLa P150) was preincubated for 10 min at 30 °C in the presence of RNA binding buffer [5 mM HEPES-KOH, pH 7.4, 25 mM KCl, 2.5 mM $MgCl_2$, 20 mM DTT, and 3.8% glycerol, 1 mg/mL *E. coli* tRNA (Sigma), 8 U RNasin (Promega), and 0.5 mg/mL BSA (New England Biolabs)] in a total volume of 9 μL. Following preincubation, 1 μL of radiolabeled RNA probe was added (to a final concentration of either 1 nM or 0.1 nM) and incubation was continued for 10 min, after which 2.5 μL of 50% glycerol was added and the resulting complexes were resolved at 4 °C on a native 4% polyacrylamide gel.

In vitro translation/replication assay of dicistronic poliovirus RNAs containing mutations in stem-loop I of the poliovirus 5′ NCR

Conditions for the in vitro translation/replication assay were as described by Todd et al. (1997), which include modifications of conditions described by Barton et al. (1995, method 2), and Molla et al. (1991). Reaction mixtures (60 μL), programmed with in vitro-transcribed dicistronic (Molla et al., 1992) or full-length poliovirus RNA, were divided into aliquots containing 10 μL and 50 μL. The 10-μL aliquots (containing an additional 10.5 μCi of [^{35}S]methionine) were used for translation analysis, whereas the 50-μL reaction was used for analysis of RNA synthesis. All reactions were incubated for 6 h at 30 °C, at which time the translation reactions were diluted in Laemmli sample buffer, boiled, and subjected to SDS-PAGE. The replication reactions were centrifuged at 15,000 × *g* and resuspended subsequently in 5 μL of HEPES buffer (50 mM HEPES, pH 8.0, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM each of ATP, GTP, and UTP) containing 25 μCi of [α-^{32}P]CTP and incubated for an additional hour at 37 °C. Total RNA was then extracted from each sample, ethanol precipitated, and resuspended in 20 μL of DEPC-treated H_2O. Ten microliters of each resuspended RNA was treated with glyoxal for 40 min at 50 °C and then resolved on a 1.3% agarose, 10 mM sodium phosphate gel.

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