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Two functional complexes formed by KH domain containing proteins with the 5′ noncoding region of poliovirus RNA

ANDREA V. GAMARNIK and RAUL ANDINO
Department of Microbiology and Immunology, University of California, San Francisco, California 94143-0414, USA

ABSTRACT

The 5′ noncoding region of the poliovirus genome contains RNA structures important for replication and translation. Here we show that two closely related cellular poly(rC) binding proteins ($PCBP_1$ and $PCBP_2$) bind to the terminal cloverleaf structure and facilitate the interaction of the viral protein 3CD (the uncleaved precursor of the protease–polymerase). In addition, these cellular proteins bind to stem-loop IV of the internal ribosomal entry site. The proteins are cytoplasmic and largely associated with ribosomes; they appear to dimerize in solution and to form heterodimers when binding to stem-loop IV. Initiation of viral translation in *Xenopus* oocytes is strongly inhibited by co-injection of specific antibodies directed against $PCBP_1$ or $PCBP_2$, indicating that the poly(rC) binding proteins may facilitate this process. Furthermore, PCPB-depleted HeLa extracts translate poliovirus RNA inefficiently and the activity is partially restored by addition of recombinant PCBP proteins.

Keywords: internal initiation of translation; PCBP; picornaviruses; RNA replication

INTRODUCTION

Poliovirus, an enterovirus member of the picornavirus family, has a single positive-strand RNA genome of approximately 7,500 nt (Kitamura et al., 1981; Racaniello & Baltimore, 1981). The genomic RNA functions as mRNA, directing the synthesis of a single large polyprotein, which is proteolytically processed to yield mature viral proteins. For replication, the RNA genome is used as a template to synthesize its complementary strand (negative strand), which, in turn, is transcribed into new molecules of virion RNA. Negative- and positive-strand RNA synthesis are catalyzed by an RNA-dependent RNA polymerase ($3D^{pol}$). However, because $3D^{pol}$ is a primer-dependent enzyme, several other viral factors (proteins and RNA structures), as well as cellular factors, are likely to be involved in RNA synthesis (Wimmer et al., 1993; for review see Johnson & Sarnow, 1995).

The poliovirus genome contains a long 5′ noncoding region (5′-NCR) that is highly conserved among the three poliovirus serotypes and other picornaviruses (Toyoda et al., 1984). Six domains (stem-loops I–VI) have been predicted by computer analysis and many of them have been confirmed by genetics and biochemical tests (Rivera et al., 1988; Pilipenko et al., 1989; Skinner et al., 1989). Two functional elements were defined within the 5′-NCR: (1) a short 5′-terminal structure involved in RNA replication, and (2) a longer element involved in initiation of translation. It was thought that these two elements were independent of each other; however, recent evidence suggests a functional overlap between them (Simoes & Sarnow, 1991; Borman et al., 1994; Shiroki et al., 1995).

The internal ribosomal entry site (IRES), nt 130–560 in poliovirus type 1, allows ribosomes to enter the RNA internally without scanning from the 5′ end (Jang et al., 1988; Pelletier et al., 1988; Trono et al., 1988b). The mechanism of ribosome–IRES recognition is still unknown, but it is believed that canonical initiation factors, as well as other cellular proteins, participate in the process (reviewed in Jackson & Kaminski, 1995). A number of host cell proteins have been shown to bind specifically to the IRES region, but only a few of them have been identified (Hellen et al., 1993; Meerovitch et al., 1993; Blyn et al., 1996).

Domain I of the 5′-NCR, which folds into a cloverleaf-like structure, is essential for viral RNA synthesis (Andino et al., 1990a; Rohll et al., 1994). This element forms a ternary ribonucleoprotein complex (RNP-B) with a ribosome-associated cellular factor, p36, and the un-

Reprint requests to: Raul Andino, Department of Microbiology and Immunology, Box 0414, University of California, San Francisco, California 94143-0414, USA.

cleaved precursor of the viral protease–polymerase, 3CD (Fig. 1A). The binding of p36 to stem-loop B of the cloverleaf greatly enhances the interaction of 3CD with stem-loop D to form the ternary complex (Andino et al., 1993). Mutations that disrupt complex formation, either within the cloverleaf RNA or within 3CD, impair viral RNA replication (Andino et al., 1990b, 1993; Rohll et al., 1994; Roehl & Semler, 1995). The mechanism by which the ribonucleoprotein complex promotes RNA replication remains unknown, but it was suggested that the complex may catalyze *in trans* the initiation of positive-strand RNA synthesis (Andino et al., 1993).

Using mobility shift assays, recombinant proteins, and specific antibodies, we have now demonstrated that the cellular factor p36 is the human poly (rC) binding protein (PCBP, also known as hnRNPE). We have further proved that two closely related polypeptides, $PCBP_1$ and $PCBP_2$, which associate with ribosomes, interact specifically with both the cloverleaf structure and the internal domain IV of the viral 5′-NCR. Finally, our data suggest that PCBP is required for efficient poliovirus translation.

RESULTS

Two cellular polypeptides, $PCBP_1$ and $PCBP_2$, specifically bind to the 5′-terminal cloverleaf RNA

$PCBP_1$ and $PCBP_2$ are cellular proteins with homology to the RNA binding protein hnRNP-K (Leffers et al.,

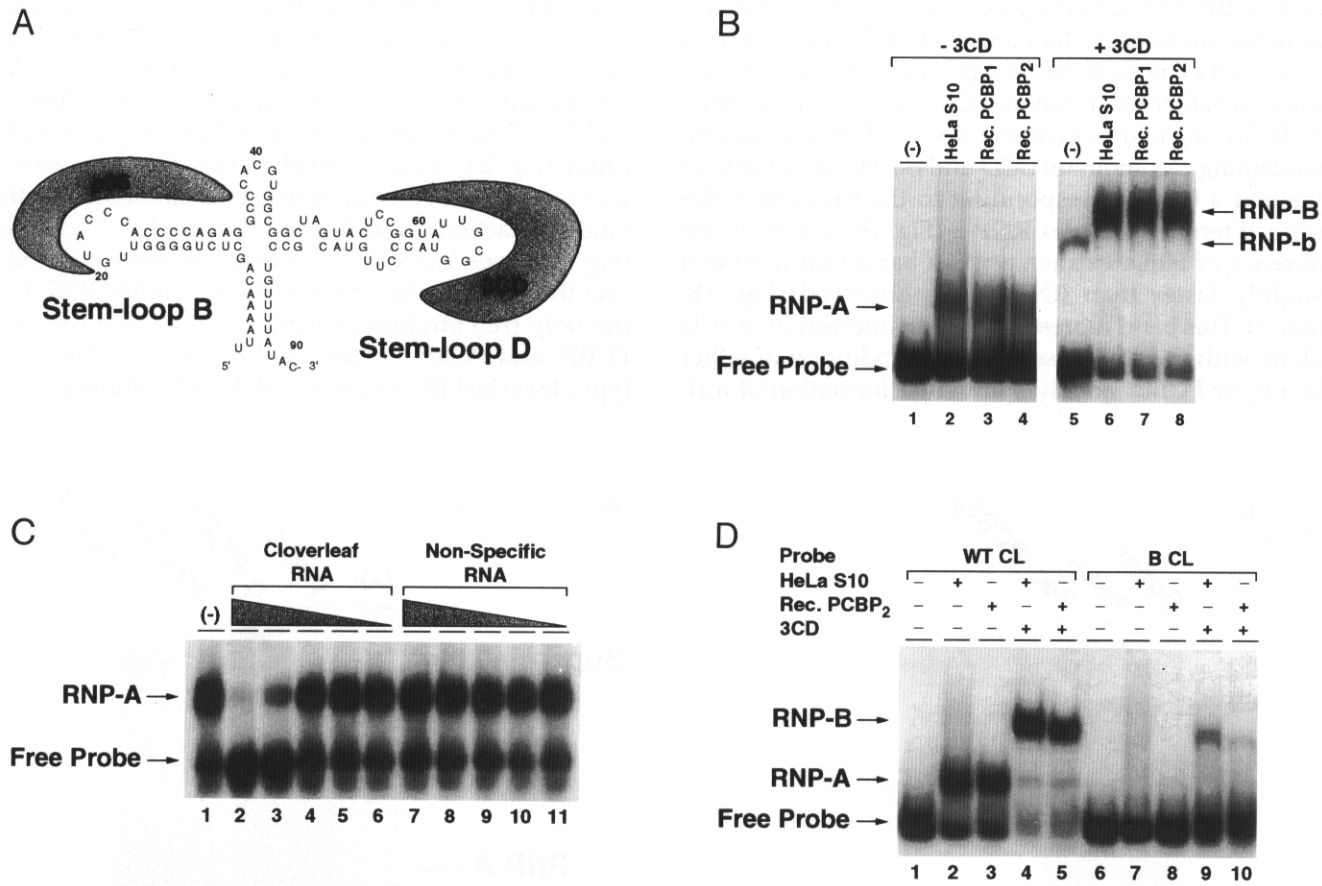

FIGURE 1. Identification of the cellular factor p36 as the human poly(rC) binding protein (PCBP). **A:** Schematic structure of the complex RNP-B. The cloverleaf structure of the 5′-most 91 nt of the poliovirus genomic RNA is shown. Stem-loop B (nt 10–34) and stem-loop D (51–78) are indicated. Viral factor 3CD and cellular factor p36 are shown interacting with their specific target sequences. **B:** RNA mobility shift analysis of recombinant $PCBP_1$ and $PCBP_2$. Uniformly labeled cloverleaf RNA (3 ng 35,000 cpm) was incubated with purified recombinant $PCBP_1$ or $PCBP_2$ (0.5 μg), or HeLa S_{10} proteins (10 μg), as indicated at the top. Binding reactions were performed in the absence (lanes 1–4) or presence of 0.5 μg of recombinant 3CD protein (lanes 5–8). Positions of specific complexes (RNP-A, RNP-b, and RNP-B) and the free cloverleaf probe (Free Probe) are indicated. **C:** Competition experiment showing that the RNP-A complex formed with the recombinant PCBP is sequence-specific. Binding reactions were performed with 0.5 μg of recombinant $PCBP_2$ protein and 3 ng of ^{32}P-labeled cloverleaf RNA in the presence of unlabeled specific (lanes 2–6) or nonspecific (lanes 7–11) RNA competitor. Decreasing amounts of competitor RNA from 400 ng to 5 ng are indicated at the top of the gel. **D:** Effect of mutations within the cloverleaf RNA probe on the interaction with the recombinant PCBP. RNA mobility shift experiments were performed with two different probes: wild-type cloverleaf (WT CL, lanes 1–5) and loop-B mutated cloverleaf, in which nt 23–26 were deleted, (B CL, lanes 6–10). Each probe was incubated with 10 μg of HeLa S_{10} or 0.5 μg of recombinant $PCBP_2$ in the presence or absence of recombinant 3CD protein (3CD), as indicated at the top of the panel.

1995). Recently, it has been shown that $PCBP_2$ binds specifically to stem-loop IV of the poliovirus IRES (Blyn et al., 1996). In addition, evidence was presented that $PCBP_1$ and $PCBP_2$ could also interact with the terminal cloverleaf structure of the poliovirus 5′-NCR (T.B. Parsley, J.S. Towner, L.B. Blyn, E. Ehrenfeld, & B.L. Semler, pers. comm.). To examine this possibility in more detail, we obtained cDNA molecular clones encoding $PCBP_2$ and its homologue $PCBP_1$ from HeLa cells (see Materials and Methods). The proteins were expressed in *Escherichia coli* as fusions to the maltose binding protein (MBP), purified by affinity chromatography, and proteolytically cleaved from MBP with factor X protease to produce $PCBP_1$ and $PCBP_2$ polypeptides. We then examined binding to the 5′-terminal cloverleaf structure of the poliovirus genomic RNA by electrophoretic mobility shift assays (EMSA). Both recombinant $PCBP_1$ and $PCBP_2$ formed complexes with identical electrophoretic mobilities to that formed by p36 obtained from HeLa cell extracts (RNP-A, Fig. 1B, lane 2–4). We next asked whether recombinant $PCBP_1$ and $PCBP_2$ affected 3CD binding to the cloverleaf RNA. A binding reaction containing recombinant 3CD and S_{10} HeLa cell extract yielded a band corresponding to the previously described ternary complex RNP-B (Fig. 1B, lane 6). In the absence of HeLa extract, a weak band that migrated slightly faster than RNP-B was observed (Fig. 1B, lane 5). This band represents a weak interaction of 3CD alone with the cloverleaf RNA. The addition of either $PCBP_1$ or $PCBP_2$ greatly stimulated formation of a ribonucleoprotein complex indistinguishable from that formed with HeLa cell extracts (Fig. 1B, compare lanes 5 with 6 and 7).

RNP-A complex formation was specific to the cloverleaf RNA because addition of specific competitor RNA (unlabeled cloverleaf) resulted in a dose-dependent reduction of the complex (Fig. 1C, lanes 2–6), whereas a nonspecific RNA (synthetic RNA containing a plasmid vector polylinker sequence) did not compete (Fig. 1C, lanes 7–11). In addition, recombinant $PCBP_2$ interacted with wild-type and mutated cloverleaf RNAs with similar specificity as the factor obtained from HeLa cells. An RNA probe with a four-base deletion at the top of stem-loop B (B CL) was unable to form RNP-A and interacted weakly with 3CD (Fig. 1D, lanes 6–10), suggesting that p36 and the recombinant protein recognize the same domain within the cloverleaf.

To further confirm these results, we used UV-irradiation to induce crosslinking between the radiolabeled cloverleaf RNA and proteins present in the complex. After crosslinking, HeLa cell extract and recombinant $PCBP_2$ produced similar bands of about 36 kDa in SDS-PAGE (Fig. 2A). Finally, the identity of these complexes was confirmed by including anti-PCBP antibodies in the binding reactions, resulting in a "super-shift" by EMSA (Fig. 2B, lanes 3 and 5). Taken together, these data indicate that the cellular component of complex RNP-B is the poly (rC) binding protein and that both proteins $PCBP_1$ and $PCBP_2$ are able to interact with the wild-type cloverleaf RNA and stimulate 3CD binding.

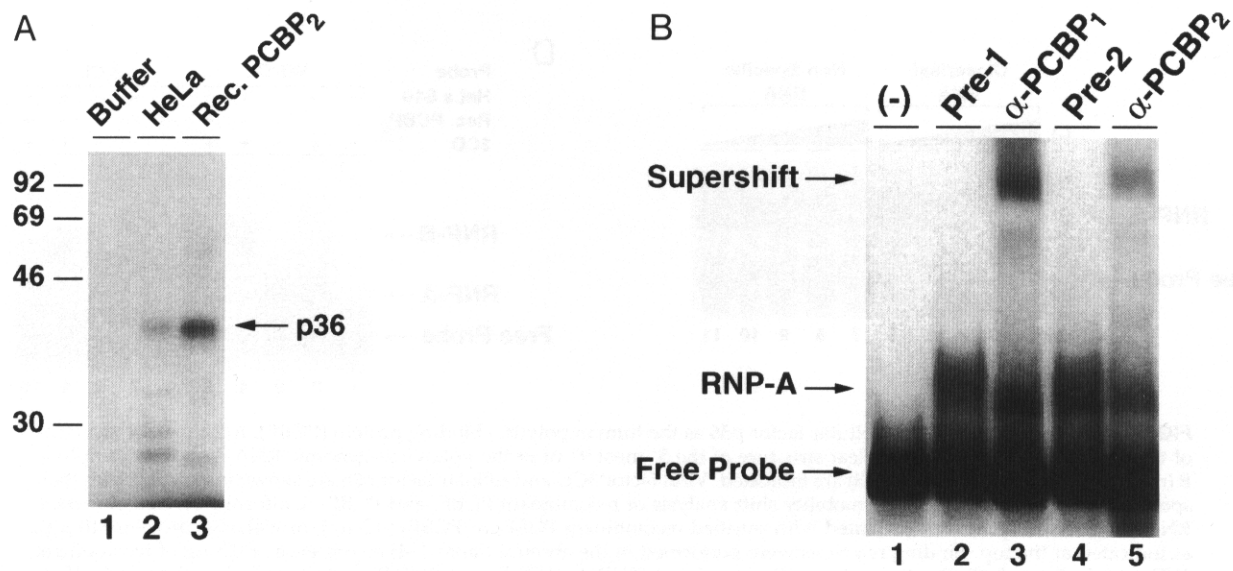

FIGURE 2. UV-crosslinking of p36/PCBP and mobility supershift of RNP-A complex by specific anti-PCBP antibodies. **A:** UV crosslinking of cloverleaf RNA to PCBP. Extraction buffer (lane 1), HeLa cell cytoplasmic S10 extract (lane 2), or recombinant $PCBP_2$ (lane 3) were incubated with ^{32}P-labeled cloverleaf RNA (20,000 cpm) for 10 min, the binding reaction was irradiated with 257-nm UV, digested with RNaseA, and analyzed by 10% SDS-PAGE. Relative mobility of molecular weight markers is indicated (kDa). **B:** Effect of anti-PCBP antibodies in the electrophoretic mobility of complex RNP-A. Lane 1 shows the free probe after incubation with extract buffer (−), the next four lanes show the resulting complexes after including in the binding reaction 10 μg of HeLa cell cytoplasmic S_{10} extract and mouse preimmune (Pre-1 and Pre-2, lanes 2 and 4), anti-$PCBP_1$ (α-$PCBP_1$, lane 3), or anti-$PCBP_2$ (α-PCBP2, lane 5) sera.

PCBP is localized mainly in the cytoplasm of HeLa cells

To determine the subcellular localization of $PCBP_1$ and $PCBP_2$, we raised polyclonal antibodies directed against synthetic peptides corresponding to nonconserved regions of the proteins to distinguish between the homologues. Western blot analysis demonstrated that antibodies raised against amino acids 231–243 of $PCBP_1$ (α-$PCBP_1$) specifically recognized recombinant $PCBP_1$, but not $PCBP_2$ (Fig. 3A, lanes 5 and 6), and antibodies raised against amino acids 200–213 of $PCBP_2$ (α-$PCBP_2$) recognized $PCBP_2$, but not $PCBP_1$ (Fig. 3A, lanes 11 and 12). Analysis of HeLa subcellular fractions indicated that $PCBP_1$ and $PCBP_2$ have a similar distribution in the cell: both were enriched in a ribosomal salt

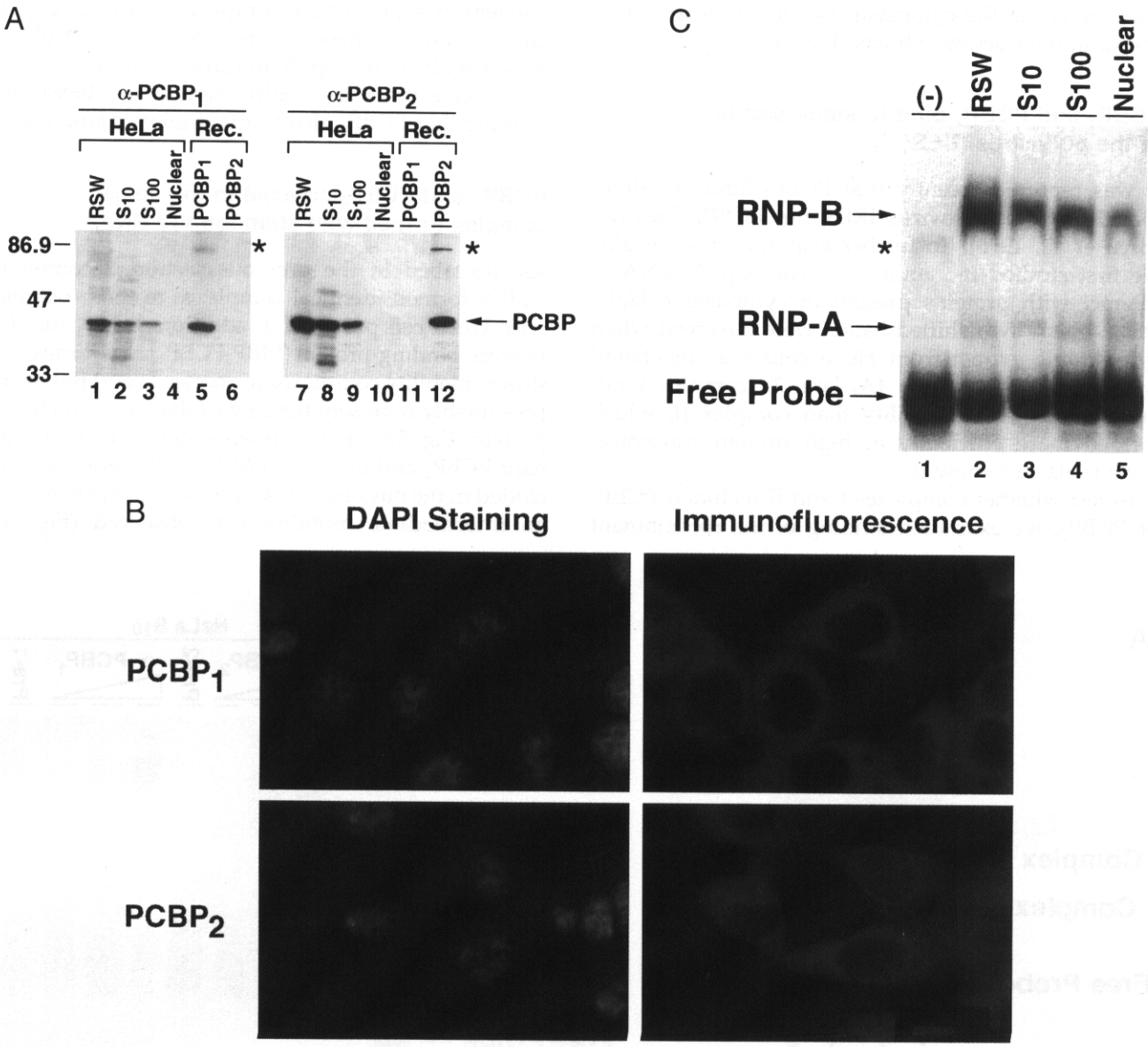

FIGURE 3. Cytoplasmic localization of $PCBP_1$ and $PCBP_2$. **A:** Western blot analysis of PCBP in HeLa subcellular fractions. Ten micrograms of HeLa proteins from ribosomal salt wash (RSW), S_{10}, S_{100}, and nuclear fractions were resolved in 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-$PCBP_1$ (left panel) or anti-$PCBP_2$ (right panel) antibodies. Ten nanograms of recombinant $PCBP_1$ (lanes 5 and 11) and $PCBP_2$ (lanes 6 and 12) were included as a control for antibody specificity. The electrophoretic mobility of PCBP proteins is indicated on the right and the asterisks indicate small amounts of uncleaved MBP-PCBP fusion remaining after factor X digestion. The mobility of molecular weight standards is shown on the left (kDa). **B:** Indirect immunofluorescence confirms cytoplasmic localization of $PCBP_1$ and $PCBP_2$. HeLa cells were fixed, probed with affinity-purified anti-$PCBP_1$ and anti-$PCBP_2$ antibodies, and visualized by staining with Texas Red conjugated anti-mouse antibody. The corresponding DAPI stained images are also shown. **C:** Subcellular distribution of the stimulating activity of p36/PCBP to form RNP-B. Binding reactions contained ^{32}P-labeled cloverleaf RNA and 0.1 μg of recombinant 3CD (lanes 1–5). In addition, lane 2 contained 10 μg of ribosomal salt wash; lane 3, 10 μg of HeLa cell S_{10}; lane 4, 10 μg of HeLa cell S_{100}; and lane 5, 10 μg of HeLa nuclear fraction.

wash fraction, were detectable readily in the cytoplasmic S_{10} and S_{100} fractions, and were undetectable in the nuclear fraction (Fig. 3A, lanes 1–4 and 7–10). The cytoplasmic localization of $PCBP_1$ and $PCBP_2$ was confirmed by indirect immunofluorescence staining (Fig. 3B). As a control, we tested preimmune serum, which did not produce any fluorescence under our experimental conditions (data not shown). Finally, we demonstrated that RNA binding activity correlated with the distribution of PCBPs. Most of the binding activity was present in the cytoplasm and less than 10% was found in the nuclear extracts (Fig. 3C).

$PCBP_1$ and $PCBP_2$ bind to stem-loop IV of the poliovirus IRES

It was reported recently that $PCBP_2$ binds to stem-loop IV of the poliovirus IRES, but $PCBP_1$ does not (Blyn et al., 1996). To further examine these results, we first studied the ability of stem-loop IV RNA to interact with proteins present in cytoplasmic HeLa cell extracts. Two shifted bands were observed when cytoplasmic extract from HeLa cells was incubated with stem-loop IV (Fig. 4A, lane 2): complex I migrated with faster mobility than complex II, which formed more efficiently at high protein concentrations (data not shown).

To test whether complexes I and II included $PCBP_1$ or $PCBP_2$, we examined binding of the recombinant proteins to stem-loop IV. Complexes very similar to those formed by the HeLa proteins were observed (Fig. 4A, lanes 3 and 4). Addition of specific or nonspecific RNA competitors indicated that the proteins interacted specifically with stem-loop IV (data not shown). Furthermore, complexes I and II formed with HeLa cell extracts could be supershifted by addition of specific anti-PCBP antibodies but not by pre-immune sera (Fig. 4B). α-$PCBP_2$ antibody supershifted most of complex I and complex II, whereas α-$PCBP_1$ was less efficient in supershifting complex I (compare Fig. 4B, lanes 3 and 7). These results confirm that $PCBP_2$ interacts with stem-loop IV to form complexes I and II, but, unlike previous results (Blyn et al., 1996), they indicate that $PCBP_1$ also forms similar complexes.

$PCBP_1$ and $PCBP_2$ co-exist in the same complex formed with stem-loop IV RNA

As described in the previous section, recombinant PCBPs formed identical complexes to those obtained with HeLa cell proteins. In addition, $PCBP_1$ fused to maltose-binding-protein (MBP-$PCBP_1$) also formed two slower mobility complexes (Complex Ia and IIa), which presumably represent binding of the larger uncleaved protein (Fig. 5A, lane 4). Interestingly, when recombinant $PCBP_2$ and uncleaved MBP-$PCBP_1$ were both included in the binding reaction, an additional band with an intermediate mobility was observed (Fig. 5A,

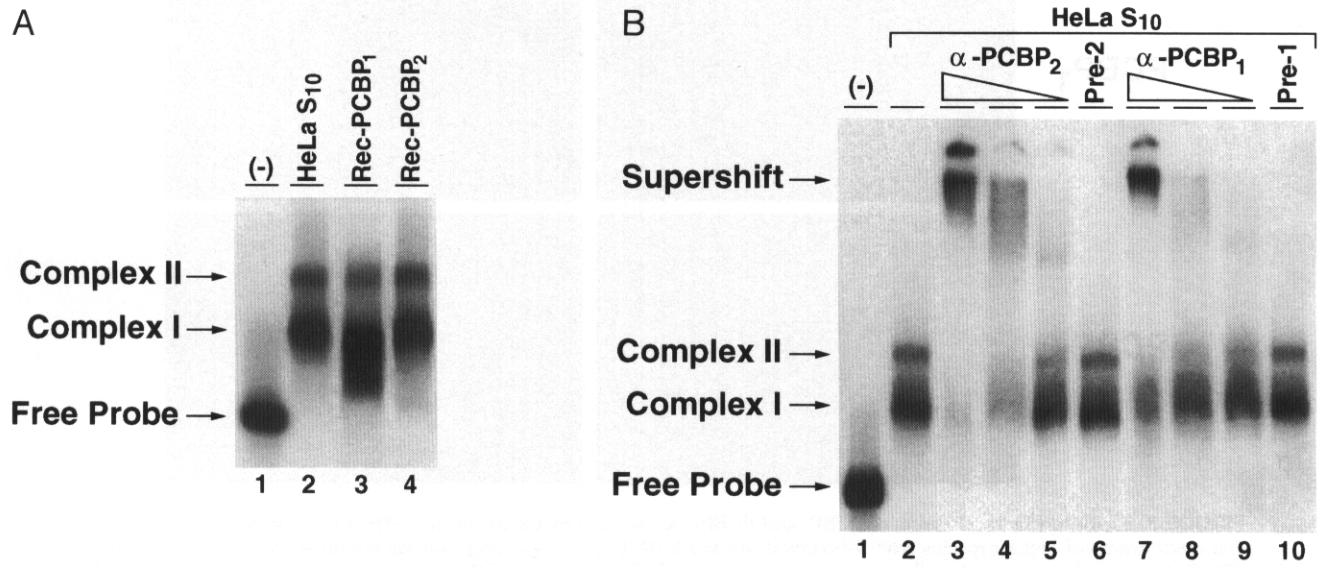

FIGURE 4. Both $PCBP_1$ and $PCBP_2$ bind to stem-loop IV of the poliovirus IRES. **A:** RNA binding analysis of recombinant proteins. Uniformly labeled (5 ng, 30,000 cpm) stem-loop IV RNA was incubated with purified recombinant $PCBP_1$ and $PCBP_2$ (2 μg) or HeLa S_{10} proteins (20 μg) as indicated. Specific complexes (Complex I and Complex II) and the free domain IV RNA (Free Probe) are indicated by arrows. **B:** RNA mobility shift assay performed in the presence of antibodies directed against $PCBP_1$ and $PCBP_2$. As in A, stem-loop IV RNA was incubated with 20 μg of HeLa S_{10} proteins (lanes 2–10). Lanes 3, 4, and 5 contain 2 μL, 1 μL, and 0.2 μL of undiluted α-$PCBP_2$ antibody, respectively. Lanes 7, 8, and 9 contain 2 μL, 1 μL, and 0.2 μL of undiluted α-$PCBP_1$ antibody, respectively. As a control, the respective preimmune sera (2 μL) were included in the binding reactions (Pre-1 and Pre-2, lanes 10 and 6, respectively). The mobility of the complexes retarded by the antibodies are shown as Supershift.

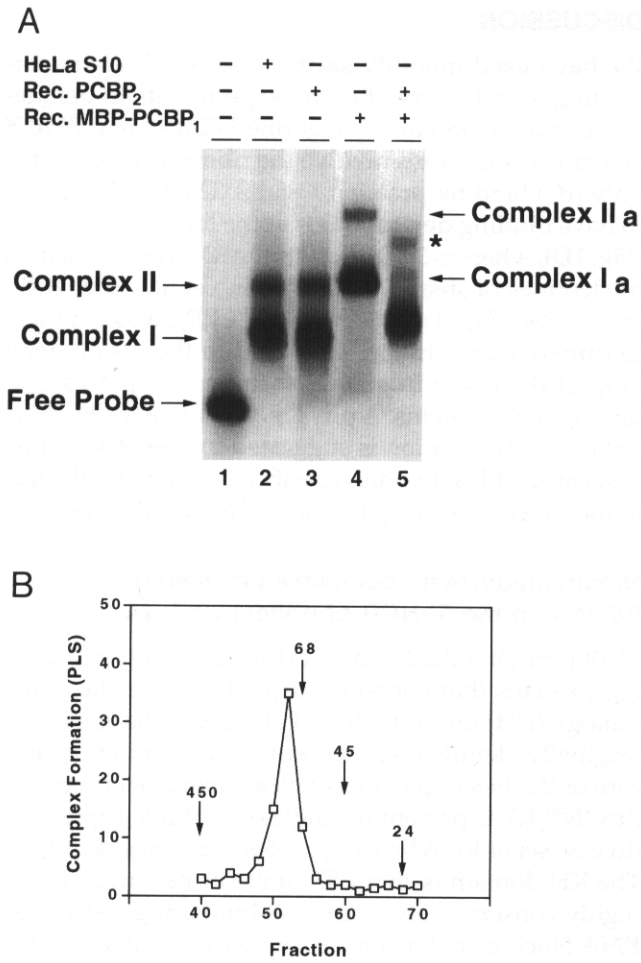

FIGURE 5. $PCBP_1$ and $PCBP_2$ form heterocomplexes with the stem loop IV of the poliovirus 5'-NCR. **A:** RNP complexes formed by different recombinant PCBP proteins. Stem-loop IV RNA probe used in Figure 4A was incubated with buffer control (lane 1), 10 μg of HeLa S_{10} extract (HeLa S_{10}, lane 2), 1 μg of recombinant $PCBP_2$ histidine tagged (Rec.$PCBP_2$, lane 3), 3 μg of uncleaved recombinant $PCBP_1$ fused to the MBP (Rec.MBP-$PCBP_1$, lane 4), or with 1 μg of Rec.$PCBP_2$ plus 3 μg of Rec.MBP-$PCBP_1$ (lane 5). Complexes formed with HeLa proteins or Rec.$PCBP_2$ (Complex I and Complex II) are indicated on the left and the ones formed with Rec.MBP-$PCBP_1$ (Complex Ia and Complex IIa) on the right. The asterisk shows the mobility of a new complex formed in the presence of both recombinants. **B:** PCBP elutes from gel filtration as a complex of approximately 70 kDa. A ribosomal salt wash fraction from HeLa cells was loaded onto a Superdex-75 gel filtration column. Fractions were assayed for their ability to stimulate *E. coli*-expressed 3CD to form complex RNP-B. Complex formation was quantified using a PhosphorImager. Elution volume of molecular weight markers is indicated by arrows.

lane 5, asterisk). This observation suggests that MBP-$PCBP_1$ and $PCBP_2$ can bind simultaneously to the same RNA. In addition, gel filtration analysis indicated that PCBPs may associate in the absence of RNA. HeLa cell extracts or recombinant proteins were loaded onto a Superdex-75 column and the activity of p36/PCBP, measured by the ability to form RNP-B in the presence of 3CD, eluted as a single peak in fractions corresponding to 70–75 kDa (Fig. 5B).

Anti-$PCBP_1$ and -$PCBP_2$ antibodies abolish translation of poliovirus RNA upon microinjection into *Xenopus* oocytes

Recently we have described a novel system to study poliovirus replication in *Xenopus* oocytes (Gamarnik & Andino, 1996). Microinjection of poliovirus RNA into *Xenopus* oocytes initiates a complete cycle of viral replication, yielding infectious viruses. By employing mutants of the poliovirus IRES, we showed that many of the same RNA elements required for translation in HeLa cells are also necessary for efficient translation in oocytes, suggesting that similar mechanisms of translation initiation are likely to be used in both cell types. Furthermore, it was suggested that $PCBP_2$ participates in initiation of translation because it interacts with the core of the IRES (stem-loop IV) and because a mutation that abrogates initiation of translation also disrupts the binding of $PCBP_2$ to stem-loop IV (Blyn et al., 1996). To test whether PCBPs are required for translation, antibodies directed against $PCBP_1$ and $PCBP_2$ were microinjected into *Xenopus* oocytes. The effect on poliovirus translation was determined by measuring luciferase activity produced by a poliovirus replicon carrying a luciferase reporter gene. Microinjection of α-$PCBP_1$ or α-$PCBP_2$ antisera inhibited translation 5–10-fold, whereas preimmune sera did not (Fig. 6A). The specificity of this inhibitory effect was demonstrated by the ability of the corresponding peptides to reverse the inhibition. As shown in Figure 6B, although the injection of peptides 1 or 2 alone had no significant effect on translation, the inhibitory effect of α-$PCBP_2$ antibody was relieved by pre-incubation with peptide 2, whereas peptide 1 was unable to prevent the effect. Because oocytes are intact cells, these data provide physiological evidence for a possible role of $PCBP_1$ and $PCBP_2$ in poliovirus translation.

PCBP proteins appear to be required for efficient poliovirus translation in HeLa cell extracts

To provide further evidence for the involvement of PCBP proteins in poliovirus translation, we employed an in vitro HeLa cell translation system. As shown in Figure 7A, a strong inhibition of viral translation was observed when specific anti-PCBP antibodies but not preimmune IgGs were included in the translation reaction.

To eliminate the possibility that the antibodies could exert their effect by sequestering PCBP–poliovirus RNA complex from the translation machinery, PCBP was removed from the HeLa cell preparations by incubating the extracts with immobilized stem-loop IV RNA (see Materials and Methods). Western blot analysis indicated

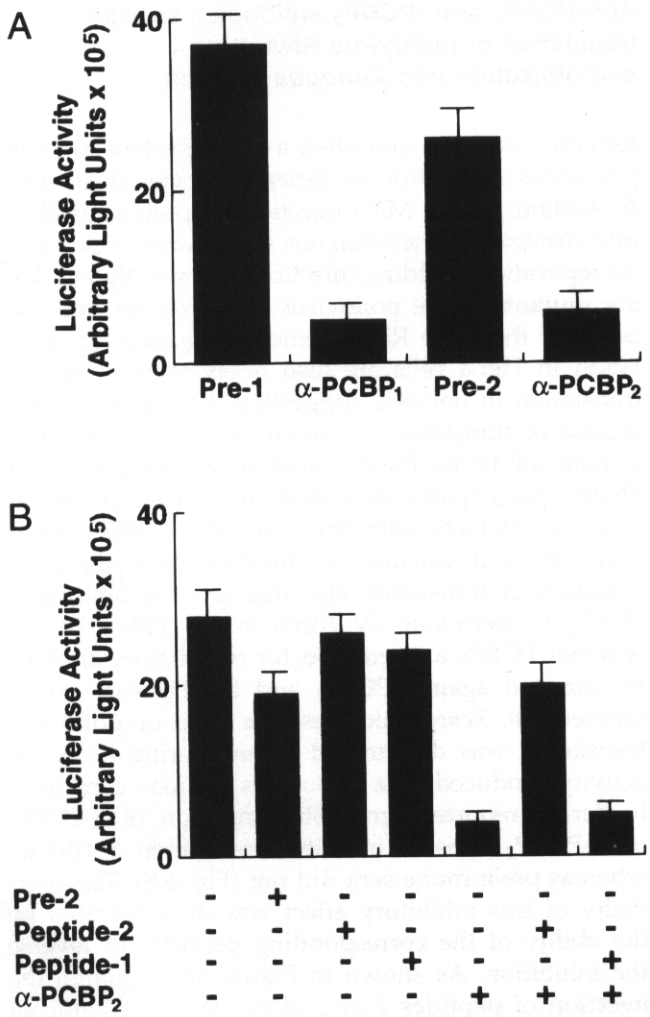

FIGURE 6. Anti-PCBP antibodies inhibit poliovirus translation in *Xenopus* oocytes. **A:** Effect of microinjected anti-PCBP antibodies on poliovirus translation. *Xenopus* oocytes were injected with 20 ng of reporter Polio-Luc RNA plus 200 ng of HeLa cell proteins from the RSW fraction, together with anti-PCBP antibodies (αPCBP$_1$ or αPCBP$_2$). Control oocytes were injected with Polio-Luc RNA, HeLa cell proteins, and preimmune sera (Pre-1 or Pre-2). Luciferase activity was determined after 5 h of incubation at 25 °C. **B:** Reversion of antibody effect on viral translation by specific peptide. Oocytes were injected with 20 ng of Polio-Luc RNA, 200 ng of HeLa proteins, along with anti-PCBP$_2$ antibodies, which were preincubated with 2 ng of the specific peptide (Peptide 2, corresponding to amino acids 200–213 in PCBP$_2$) or with 2 ng of nonspecific peptide (Peptide 1, corresponding to amino acids 231–243 of PCBP1). Control oocytes were injected with Polio-Luc RNA and HeLa proteins, with peptides alone or with preimmune serum, as indicated at the bottom. Luciferase activity was determined as in A. Data represent the average of three independent injections (error bars indicate standard deviations).

that the PCBP-depleted HeLa cell extracts retained less than 15% of PCBP proteins (Fig. 7B). Depletion of PCBP correlates with a threefold decrease in poliovirus translation. Importantly, addition of ribosomal-associated factors or PCBP$_2$ recombinant protein restores poliovirus translation (to 100% and 80% of control levels, respectively) (Fig. 7C).

DISCUSSION

We have used mobility shift assays, antibody supershifting, and UV crosslinking experiments to demonstrate that the previously identified RNP-A and RNP-B cloverleaf complexes involve the ribosomal-associated poly (rC) binding proteins 1 and 2. The PCBPs appear to have binding determinants at the top of stem-loop B (Fig. 1D), whereas 3CD contacts the RNA near unpaired nucleotides of stem-loop D (Andino et al., 1993; Rohll et al., 1994) (Fig. 1A). PCBP$_1$ and PCBP$_2$, which appear to dimerize in solution, also bind to the major stem-loop of the poliovirus IRES, forming complexes containing both proteins. A physiological role for PCBP in poliovirus translation is suggested by the observation that anti-PCBP antibodies specifically inhibit viral translation in *Xenopus* oocytes and in HeLa cell extracts.

Ribonucleoprotein complexes formed by PCBPs on the 5′-NCR of poliovirus RNA

PCBPs are abundant human cytoplasmic poly(rC) binding proteins that contain a triple repeat of the K homology (KH) motif (Leffers et al., 1995). The KH motif, originally identified as the most striking structural feature of the heterogeneous nuclear ribonucleoprotein K (hnRNP-K), is present in single or multiple copies in a diverse set of RNA binding proteins (Siomi et al., 1993). The KH domain is about 50 amino acids long, and is a highly conserved structure that binds single-stranded RNA (such as polycytosine sequences), but with little previously described sequence specificity. The three-dimensional structure of a KH domain shows a stable $\beta\alpha\alpha\beta\beta\alpha$ fold, with a potential surface for RNA contact located on the loop between the first two helices (Musco et al., 1996). PCBP$_1$ and PCBP$_2$ were first reported to bind to the 3′ untranslated region of the human α-globin mRNA, which appears to affect mRNA stability, but the regions of the proteins involved in RNA recognition have not yet been mapped (Kiledjian et al., 1995).

In poliovirus, formation of the RNP-B complex is greatly stimulated by PCBP$_1$ or PCBP$_2$ (Fig. 1B). It is not yet clear whether PCBP enhances the RNP-B complex formation by inducing a conformational change in the RNA, or by forming protein–protein interactions with 3CD. The binding of PCBP requires specific nucleotides that are phylogenetically conserved among enteroviruses, suggesting that this group of viruses may share a requirement for the same cellular factor.

Recently, PCBP$_2$ was shown to interact with stem-loop IV of the poliovirus IRES (Blyn et al., 1996). We have confirmed these results and also found that PCBP$_1$ binds to the same stem-loop. This domain of the IRES forms two complexes with PCBP proteins (complex I and II, Fig. 4A), and it appears that these complexes may contain both PCBP$_1$ and PCBP$_2$ (Fig. 5). It is possible that there are two separate sites of interaction for PCBPs within stem-loop IV, but, because PCBP$_1$ and PCBP$_2$

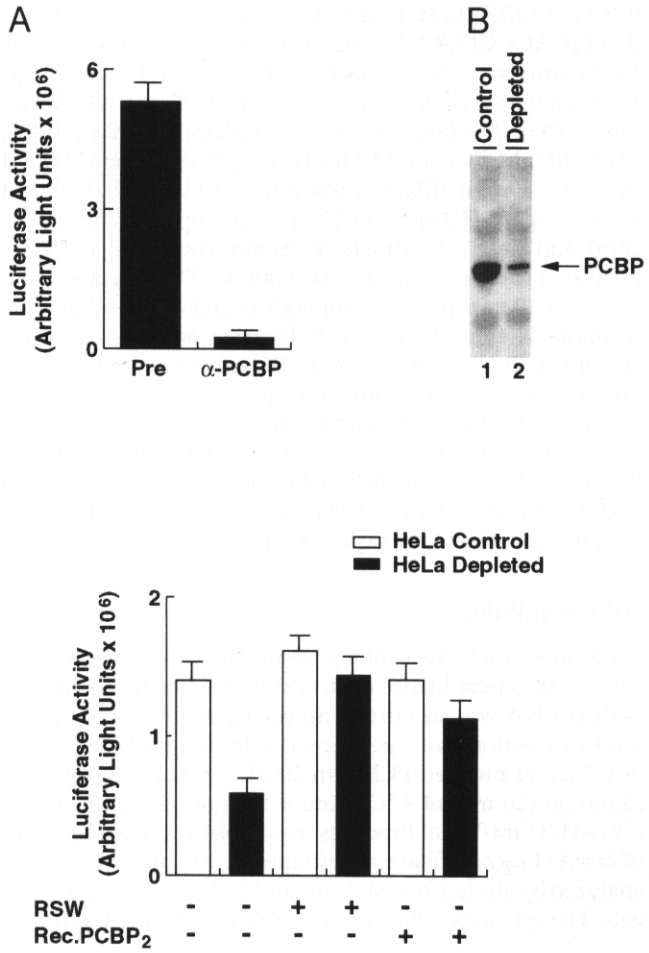

FIGURE 7. Efficient translation of poliovirus RNA in HeLa cell extracts requires PCBP proteins. **A:** Anti-PCBP antibodies inhibit poliovirus translation in HeLa cell extracts. HeLa cell extracts were programmed with 0.5 μg of Polio-Luc RNA in the presence of anti-PCBP antibodies or purified preimmune IgGs (10 μg). Luciferase activity was determined after 1 h of incubation at 30 °C. **B:** Western blot analysis of PCBP in HeLa cell translation extracts. Twenty micrograms of HeLa proteins from PCBP-depleted or control extracts were resolved in 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with rabbit anti-PCBP antibodies, which recognized both PCBP proteins (PCBP1 and PCBP2). The electrophoretic mobility of PCBP is indicated. **C:** Effect of PCBP depletion on poliovirus translation. Forty microliters of HeLa control or PCBP-depleted extracts were used to translate Polio-Luc RNA as described in A. The reactions were supplemented with 20 μg of a ribosomal salt wash preparation (RSW) or 3 ng of $PCBP_2$ recombinant protein as indicated at the bottom of the panel.

may dimerize in solution (Fig. 5B), it seems more plausible that homo- and heterodimers of PCBP are involved in binding. Additional experiments are needed to define more rigorously the nature of the interactions.

Possible functions of PCBPs in the poliovirus life cycle

The precise mechanism by which poliovirus initiates RNA replication remains unknown, but we have shown previously that mutations that affect RNP-B complex formation completely abolish RNA replication without affecting RNA stability (Andino et al., 1990a, 1993). As shown here, PCBP is an essential part of the RNP-B structure, and perhaps its main role in RNA synthesis is to facilitate the binding of 3CD to the cloverleaf RNA. However, because the poliovirus genome is genetically diverse, it seems that 3CD could have co-evolved with the cloverleaf RNA to interact efficiently in the absence of cellular factors. The fact that PCBPs are required for binding of 3CD suggests both a structural and a functional role for PCBPs during RNA replication.

The finding that PCBPs interact with the core of the IRES (stem-loop IV) suggests that, in addition to a role in RNA replication, these proteins may also participate in viral translation. We present evidence for a possible role of PCBPs in cap-independent initiation of translation: addition of antibodies against $PCBP_1$ and $PCBP_2$ inhibited viral translation in *Xenopus* oocytes and in a HeLa cell translation system (Figs. 6 and 7A). Furthermore, depletion of PCBP proteins induced a reduction of poliovirus translation that could be reverted by the addition of recombinant $PCBP_2$ (Fig. 7C). Although the binding of PCBPs to stem-loop IV could be involved directly in translation, we observed that a four-base deletion at the top of stem-loop B of the cloverleaf seriously compromises poliovirus translation (manuscript in prep.). These data, together with previously reported evidence (Simoes & Sarnow, 1991), indicate that the interaction of PCBP with the cloverleaf RNA is also important for translation. It seems plausible that PCBPs may bridge the cloverleaf and stem-loop IV RNA to form a high-order structure, which, in turn, could enhance internal ribosomal entry; however, we have not yet been able to detect a direct interaction between RNP-A and stem-loop IV in vitro (data not shown). It is possible that additional IRES elements or components of the translation machinery are also required for such association. The specific role of PCBPs in initiation of translation requires further studies, nevertheless, their association with ribosomes suggests that they may be part of the cellular translation machinery.

The commonly held view that the *cis*-acting elements required for translation and RNA replication are independent structures within the 5′-NCR is changing; several lines of evidence now suggest that these structures act in concert with each other (Simoes & Sarnow, 1991; Borman et al., 1994; Shiroki et al., 1995). Here we present evidence that *trans*-acting factors may also have a bifunctional activity, consistent with the view that translation and RNA replication may be intimately coupled. Because negative-strand RNA synthesis proceeds with the opposite 5′ to 3′ polarity of translation, the virus must have mechanisms to coordinate the two processes. Overlapping translation and replication signals might provide one strategy to halt translation when RNA synthesis must begin.

MATERIALS AND METHODS

Expression of recombinant proteins

$PCBP_1$ and $PCBP_2$ cDNA sequences were amplified by PCR from a HeLa cell library. Primers included restriction sites to facilitate the insertion into pMAL vectors (New England Biolabs). $PCBP_1$ coding region was amplified with primers 1 and 2 (1, GCTAGCGAATTCGATGCCGGTGTGACTGA; 2, TAGCAGGTCGACCTACTAGCTGCACCCCA), and $PCBP_2$ with primers 3 and 4 (3, GCTAGCGAATTCGACACCGGT GTGATTGAA; 4, TAGCAGGGATCCCTAGCTGCTCCCCAT GCC). The PCR fragments were digested with *Eco*R I and *Sal* I (for PCBP1) or *Eco*R I and *Bam*H I (for $PCBP_2$) and ligated into the pMALc2 vector digested with the same enzymes. Histidine-tagged $PCBP_2$ was prepared by amplification of the coding region using primers 5 and 4 (5, GCTAGC GGTACCCACCATCACCATCACCATGACACCGGTGT GATTGA). The PCR fragment was digested with Asp 718 and *Bam*H I and ligated to pT7-48 expression vector (Andino et al., 1993). Recombinant T7-expression plasmids were transformed into the *E. coli* strain BL21 (DE3), which contained the T7 polymerase gene under the control of the *lac*UV5 promotor. For protein production, overnight culture of freshly transformed bacteria was diluted 1/10, and the culture was grown to OD_{550} = 0.5 before IPTG was added to a final concentration of 0.4 mM. The overexpression was conducted for an additional 2 h. Cells were harvested, washed once with phosphate-buffered saline (PBS), and resuspended in lysis buffer (10 mM HEPES, pH 7.9, 20 mM KCl, 25 mM EDTA, 5 mM DTT, 0.1 mM PMSF, 1% Triton X-100). The suspension was frozen and thawed three times, sonicated for about 30 s to reduce viscosity, and centrifuged at 15,000 × *g* for 15 min to remove debris. Glycerol was added to a final concentration of 20% and the supernatant was stored at −70 °C (Andino et al., 1993). Fusion proteins with MBP were purified by affinity chromatography through a column prepared with amylose resin (New England Biolabs). MBP-PCBP fusion proteins were digested with factor X to cleave PCBP sequences from MBP.

To analyze PCBPs by gel filtration chromatography, HeLa cell high-salt ribosomal wash or recombinant proteins were loaded onto Superdex-75 column. Fractions were assayed for their ability to stimulate RNP-B complex formation with recombinant 3CD as described below.

RNA binding assays

RNA binding reactions and electrophoretic mobility shift assays were performed as described (Andino et al., 1990a). Uniformly [^{32}P]-labeled RNA probes were generated by in vitro transcription using T7 RNA polymerase. The cloverleaf probe (CL) represented the first 108 nt of the poliovirus genome and the stem-loop IV probe corresponded to a fragment from nt 220 to 440 from the poliovirus genome. Cloverleaf RNA probe with a four-base deletion in loop B (nt 23–26) was generated by PCR using oligonucleotides 6 and 7 (6, TACGTCGACTAATACGACTCACTATAGGTTAAAAC AGCTCTGGGGTTGTACCCCAGAGG; 7, TAATGTGCGCG CTAAGTTACGGGAAGG). The PCR fragment was cloned using *Sal* I and *Bss*H II into a plasmid vector. For stem-loop IV RNA probe, a PCR fragment generated by primers 8 and 9 (8, GCTAATACGACTCACTATAGGCTTATGTACTTCGAG; 9, ATTCAGGGGCCGGAGGATTC) was used as a template for in vitro transcription as described above. HeLa extracts were prepared essentially as described (Gamarnik & Andino, 1996). The binding reaction contained 5 mM HEPES, pH 7, 40 mM KCl, 2 mM $MgCl_2$, 4% glycerol, 2 mM DTT, 1 mg/mL of yeast tRNA, uniformly [^{32}P]-labeled cloverleaf RNA (3 ng, 35,000 cpm) or [^{32}P] stem-loop IV (5 ng, 30,000 cpm), and HeLa cell extracts or recombinant $PCBP_1$ or $PCBP_2$ purified proteins in 20 μL final volume. The mix was incubated for 10 min at room temperature and analyzed by electrophoresis through native polyacrylamide gels (5% for cloverleaf and 4% for stem-loop IV) supplemented with 5% glycerol. Gels were pre-run for 30 min at 4 °C at 100 mV, then 15 μL of samples were loaded and electrophoresis was allowed to proceed for 4 h at constant voltage. RNA competitors or antibodies were included in the preincubation reaction as described in each case. The gels were dried and visualized by autoradiography or exposed on a PhosphoImager plate.

UV crosslinking

Ultraviolet photocrosslinking treatment was performed according to Garcia-Blanco et al. (1989). Uniformly labeled cloverleaf RNA was in vitro synthesized as described above. Binding reactions were performed with 10 μg of HeLa cell S_{10} or 0.5 μg of purified $PCBP_2$ in 20-μL reaction. After UV irradiation (20 min at 4 °C using a Mineralight lamp model UVG-11, Ultra-Violet Products Inc.), RNA was digested with RNaseA (1 μg/μL final concentration) for 15 min at 30 °C and analyzed by electrophoresis through 10% SDS-polyacrylamide gels. The gels were dried and visualized by autoradiography.

Antibody preparation

Two peptides from regions of $PCBP_1$ and $PCBP_2$ where amino acid residues are not conserved were synthesized at the Biomolecular Resource Center at UCSF: Peptide-1 contained amino acids 231–243 in $PCBP_1$ and Peptide-2 contained amino acids 200–213 in $PCBP_2$. For sulfhydryl coupling to the carrier protein (keyhole limpet hemocyanin, KLH, Sigma), an N- or C-terminal cysteine was added to the sequence. Ten milligrams of each peptide was coupled to KLH as described (Sawin et al., 1992). After the coupling reaction, products were sonicated to disrupt aggregations and mixed 1:1 with Freund's complete adjuvant for mice immunization.

For each peptide–KLH conjugate, six mice were first bled to obtain preimmune sera (Pre-1 or Pre-2), then immunized with 400 μL of conjugate-adjuvant mix (200 μg of protein), and boosted after 4 weeks. Immune sera were screened initially by ELISA, using recombinant $PCBP_1$ or $PCBP_2$ to coat plates. Antisera with high titers (more than 2,000 of reciprocal dilution) were affinity purified using immobilized specific peptide. Briefly, peptides were coupled to *N*-hydroxysuccinimide-activated Sepharose as recommended by the manufacturer (HiTrap NHS-activated, Pharmacia). After several washes, antibodies were eluted with 0.15 M KCl, 0.2 M glycine-HCl, pH 2, and collected in 100 μL of 1 M Tris, pH 8.8, to neutralize. High-affinity antibodies were eluted with a buffer containing 6 M guanidine, 0.5 M NaCl, 0.2 M $NaHCO_3$, pH 8.3. Purified antibodies were dialyzed against Tris-buffered saline and concentrated by ultrafiltration.

Recombinant $PCBP_1$ and $PCBP_2$ proteins were used for the production of rabbit antibodies (Berkeley Antibody). Rabbit IgGs from pre and immune sera were affinity purified using HiTrap-protein G columns (Pharmacia) as recommended by the manufacturer. These antibodies recognized both PCBP proteins.

Western blot and immunofluorescence microscopy

For western blot analysis, 1 μL of each HeLa cell fraction was mixed with an equal volume of 2× SDS-PAGE sample buffer, loaded onto a 10% SDS-polyacrylamide gel, and, after electrophoresis, blotted onto nitrocellulose filters by electrotransfer. The membranes were probed with anti-$PCBP_1$ and anti-$PCBP_2$ antibodies (prepared as described above) as described (Trono et al., 1988a).

For immunofluorescence, HeLa cells were grown on coverslips to 60% confluency. Coverslips were rinsed with PBS, cells were fixed with 2% formaldehyde for 20 min at room temperature, permeabilized with 0.2% of saponin in 3% BSA, and rinsed once again with PBS. Primary anti-PCBP antibodies (serial dilutions in PBS with 3% BSA) were applied, rinsed several times with PBS, and incubated for 1 h with secondary Texas Red-conjugated anti-mouse antibodies (dilution 1:100). DNA was counterstained with DAPI included in the mounting solution.

Microinjections in *Xenopus* oocytes

Oocytes were isolated surgically (Marcus Sekura & Hitchcock, 1987) and defolliculated enzymatically by incubation with 2 mg/mL of collagenase (Wothington CSL-1) for 3 h at room temperature. Defolliculated oocytes were washed five times with modified Barth's solution (MBS: 7.5 mM Tris, pH 7.6, 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 8.2 mM $MgSO_4$, 100 units/mL of penicillin, 100 mg/mL of streptomycin, and 2% Ficoll-400) and kept in MBS plus 0.33 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$ at 17 °C. Stage VI sorted oocytes were injected with 20 nL of in vitro transcribed RNA (1 μg/μL), 20 nL of HeLa cell extract (10 μg/μL of protein), and 5 nL pre- and immune sera diluted 1:30 before injection, or buffer control. A plasmid encoding for a subgenomic poliovirus replicon where P1 was replaced by the luciferase reporter gene, Polio-Luc, has been described previously (Andino et al., 1993). To determine the specificity of the antibody effect, 5 nL of synthesized peptides (0.4 mg/mL) were preincubated with the antibodies before injection as indicated.

To determine translation of subgenomic Polio-Luc RNA or Cap-Luc carrying the luciferase reporter gene, 10 oocytes were lysed in lysis buffer (20 μL per oocyte; Promega) and spun 5 min at 10,000 × *g*. The supernatant (5 μL) was assayed using a luciferase system as recommended by the manufacturer (Promega) and quantified using an Optocomp I luminometer.

HeLa cell translation extracts

HeLa cells were grown in suspension with 5% fetal calf serum and 5% horse serum. Cytoplasmic translation extracts were prepared from 3 L of HeLa cells. Cells were harvested by centrifugation, washed three times with cold PBS, and collected by centrifugation. The pellet was resuspended in 2 volumes of hypotonic buffer (20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM $Mg(CH3CO_2)_2$, and 2 mM DTT), incubated on ice for 20 min, and broken with 20 strokes of glass Dounce homogenizer. A post-nuclei supernatant was obtained by centrifugation at 5,000 × *g* for 10 min at 4 °C, and then submitted to a second centrifugation at 15,000 × *g* for 10 min. The extract was adjusted to 1 mM $CaCl_2$, and treated with 75 U of *Staphylococcus aureus* nuclease per milliliter for 20 min at room temperature. The nuclease was inactivated by addition of EGTA at 2 mM final concentration. To deplete PCBP proteins, HeLa translation extracts were incubated with immobilized stem-loop IV biotinylated RNA/streptoavidin beads. Briefly, 50 μL of streptoavidin beads were incubated with 100 μg of biotinylated stem-loop IV RNA, and washed 5 times with hypotonic buffer. Two hundred microliters of HeLa extracts were incubated with RNA-beads for 1 h on ice, spun at 10,000 × *g* for 5 min, and used in the translation reactions. Control extracts were treated with streptoavidin beads without stem-loop IV RNA. Translation reactions were performed as described (Barton & Flanegan, 1993).

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