JOURNAL OF VIROLOGY, Aug. 1997, p. 6243–6246 Vol. 71, No. 8
0022-538X/97/$04.00+0

Requirement of Poly(rC) Binding Protein 2 for Translation of Poliovirus RNA

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Received 14 March 1997/Accepted 2 May 1997

Poly(rC) binding protein 2 (PCBP2) is one of several cellular proteins that interact specifically with a major stem-loop domain in the poliovirus internal ribosome entry site. HeLa cell extracts subjected to stem-loop IV RNA affinity chromatography were depleted of all detectable PCBP2. Such extracts were unable to efficiently translate poliovirus RNA, although extracts recovered from control columns of matrix unlinked to RNA retained full translation activity. Both translation and production of infectious progeny virus were restored in the PCBP2-depleted extracts by addition of recombinant PCBP2, but not by PCBP1, which is a closely related member of the protein family. The data show that PCBP2 is an essential factor, which is required for efficient translation of poliovirus RNA in HeLa cells.

The picornavirus positive-sense RNA genome contains a long, highly structured 5′ noncoding region (5′ NCR) that directs the initiation of translation by a cap-independent, internal ribosome entry mechanism (see references 12 and 20; for reviews, see references 6 and 11). By using this translation pathway, ribosomes bind directly to the internal ribosome entry site (IRES) in the RNA and initiate translation, rather than conform to the process of 5′-terminal cap recognition and scanning utilized by most cellular mRNAs. The novel translation initiation pathway used by picornaviruses appears to be mediated by a number of host cell factors that bind to structured regions of the 5′ NCR (6). Several studies focused on poliovirus, the prototypic picornavirus, have described a number of host cell proteins that bind to specific regions within the predicted stem-loop domains of the IRES (3, 9, 10, 16). Stem-loops II, IV, V, and sequences in VI have been shown to be required for poliovirus translation, although their specific roles in the process remain unknown (5, 7, 8, 21). Only three of the host cell proteins that bind to this translation domain have been identified. First, La protein, whose cellular function involves RNA polymerase III transcription termination (23), binds to stem-loop VI and appears to play a role in the correct initiation of translation of poliovirus RNA (17). Second, polypyrimidine tract binding protein, which is involved in cellular pre-mRNA splicing, binds to the poliovirus 5′ NCR as well as to the 5′ NCR of another picornavirus, encephalomyocarditis virus (9, 13). Third, poly(rC) binding protein 2 (PCBP2), which is a protein detected as part of the α-globin mRNP stability complex (14, 25), binds, as we have shown, to stem-loop IV of poliovirus RNA (nucleotides 230 to 430) (4). PCBP2 is a 39-kDa protein that is found in most mammalian tissues. It contains three K homology domains found in a number of RNA binding proteins (14, 15). Evidence that the binding of PCBP2 to poliovirus RNA affects translation derives from the identification of stem-loop IV mutations that abrogate both PCBP2 binding and poliovirus translation (3, 4).

In studies described in this report, we examined the requirement of PCBP2 for viral translation. PCBP2 was purified from bacteria harboring a previously described plasmid into which the cDNA from *pcbp2* had been cloned (pQE30-PCBP2) (4). This recombinant PCBP2 (rPCBP2) contains a hexa-histidine tag on its amino terminus. Bacteria containing pQE30-PCBP2 were induced with isopropyl-β-D-thiogalactopyranoside grown overnight, and the cells were harvested and lysed in a French press. The clarified lysate was fractionated by precipitation with ammonium sulfate, and the 40% ammonium sulfate precipitate was further purified by MonoQ ion-exchange column chromatography. Fractions containing rPCBP2 were eluted at approximately 0.15 M NaCl, dialyzed, bound to a nickel column, and eluted with imidazole. This procedure yielded rPCBP2 that was ~95% pure, which was used to induce antibody production in rabbits.

Antibodies raised against rPCBP2 were shown to react with PCBP2 present in HeLa cell lysates. Figure 1 shows an immunoblot of proteins present in the 0 to 40% ammonium sulfate fraction (A-cut) of a HeLa cell ribosomal salt wash which was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1, lane 2). Three protein species are detected with the antiserum raised against rPCBP2. Except for the intensity of the ~40-kDa band, which was present at various levels in different isolations, this pattern is identical to that previously obtained by purification of proteins bound to a poliovirus 5′ NCR stem-loop IV affinity column (Fig. 1, lane 3). These proteins are immunologically related, and at least two have been shown to be PCBP2 by protein sequencing (data not shown). Although it has not been demonstrated directly, the three immunoreactive species are thought to be phosphorylation variants of PCBP2 (15). The slightly retarded mobility of rPCBP2 is likely due to the presence of the hexa-histidine tag (Fig. 1, lane 1).

To investigate the role of PCBP2 in poliovirus protein synthesis, we used a HeLa cell extract (S10) supplemented with additional RSW which, upon programming with virion RNA (vRNA), gives rise to viral proteins and viable poliovirus particles (2, 18, 24). The combined HeLa S10-RSW extract was depleted of PCBP2 by passage five times over a stem-loop IV RNA affinity column (4) equilibrated with translation buffer (20 mM HEPES [pH 7.4], 91 mM KOAc, 2.6 mM KCl, 3.4 mM MgKOAc, 4 mM dithiothreitol). Figure 2 shows that passage of these extracts over stem-loop IV RNA affinity columns

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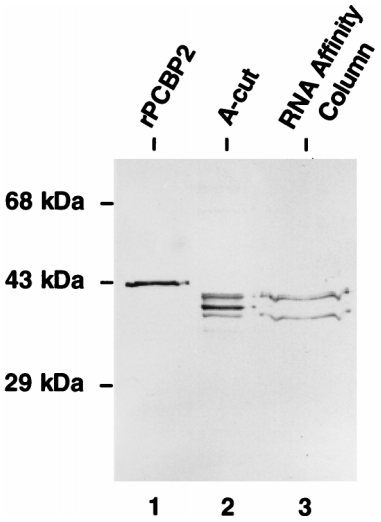

FIG. 1. Immunoblot analysis of PCBP2 from HeLa cells. Antibody raised against rPCBP2 was used in a Western blot analysis of rPCBP2 (lane 1), the 0 to 40% ammonium sulfate precipitate fraction (A-cut) of HeLa cell RSW (lane 2), or a fraction eluted from a stem-loop IV RNA affinity column to which HeLa cell A-cut had been applied (lane 3).

resulted in removal of all detectable PCBP2 (compare lanes 1 and 3), whereas a control column of matrix unlinked to any RNA produced no detectable loss of PCBP2 from the extract (compare lanes 1 and 2). Our earlier work had shown that a closely related protein, PCBP1, did not interact efficiently with stem-loop IV RNA sequences (4). However, this protein does react with the anti-rPCBP2 antibody (data not shown). Our failure to detect this protein after depletion of extracts with the stem-loop IV affinity column confirms previous reports indicating that PCBP1 mRNA is present in very low quantities in HeLa cells (1).

Depleted and control extracts were programmed with poliovirus vRNA for translation in vitro (Fig. 3). Control extracts translated vRNA (Fig. 3, lane 2) to levels similar to those of extracts not passed over any column (data not shown). Extracts recovered from a stem-loop IV affinity column and depleted of PCBP2 translated the RNA at a significantly reduced level (Fig. 3, lane 3). Depletion of extracts for PCBP2 always resulted in impaired translation; the level of impairment was somewhat variable and correlated with the amounts of residual PCBP2 detectable in different extracts after stem-loop IV RNA affinity chromatography (data not shown).

Addition of purified rPCBP2 to depleted extracts restored the abilities of these extracts to translate poliovirus vRNA (Fig. 3, lane 4). The ability of rPCBP2 to restore translation activity was dose dependent (data not shown). Increasing concentrations of rPCBP2 increased the restoration of translation until the extract appeared to saturate at about 1.2 μM. Although it was possible to restore translation completely and greater than 50% of translation activity was routinely achieved by the addition of rPCBP2 to depleted extracts, the amounts of protein required and the final levels of restoration achieved varied with different preparations of extract and protein (data not shown). It remains possible that extensive or complete depletion of PCBP2 by RNA affinity chromatography removes an additional factor(s) which is required in low amounts and not

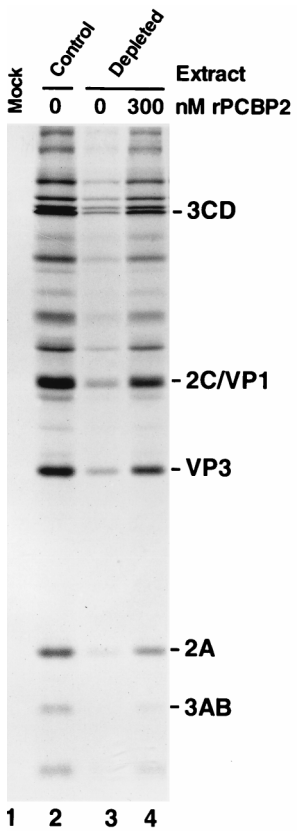

FIG. 3. Effect of PCBP2 on translation of poliovirus RNA. Extracts were depleted of PCBP2 by stem-loop IV RNA affinity column chromatography and used for in vitro translation of poliovirus vRNA in the presence of [^{35}S]methionine. Lanes: 1, products from a control extract passaged over a column not linked to stem-loop IV RNA and programmed with no RNA; 2, control extract as described for lane 1, but programmed with poliovirus vRNA; 3, an extract depleted of PCBP2 and programmed with poliovirus vRNA; 4, identical to lane 3, but supplemented with 300 nM rPCBP2. Translation reaction mixtures (10-μl volume containing 250 ng of vRNA) were incubated at 30°C for 6 h. Following translation reactions, samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

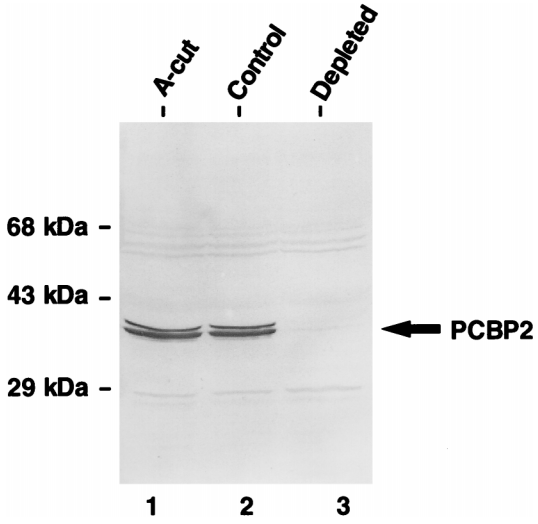

FIG. 2. Immunoblot analysis of HeLa cell translation extracts following stem-loop IV RNA affinity column chromatography. HeLa cell translation extracts were passaged five times over an immobilized stem-loop IV RNA column (1-ml bed volume) equilibrated in translation buffer (lane 3). Controls were either (i) extract not subjected to column chromatography (lane 1) or (ii) extract passaged five times over a column not linked to any RNA (lane 2).

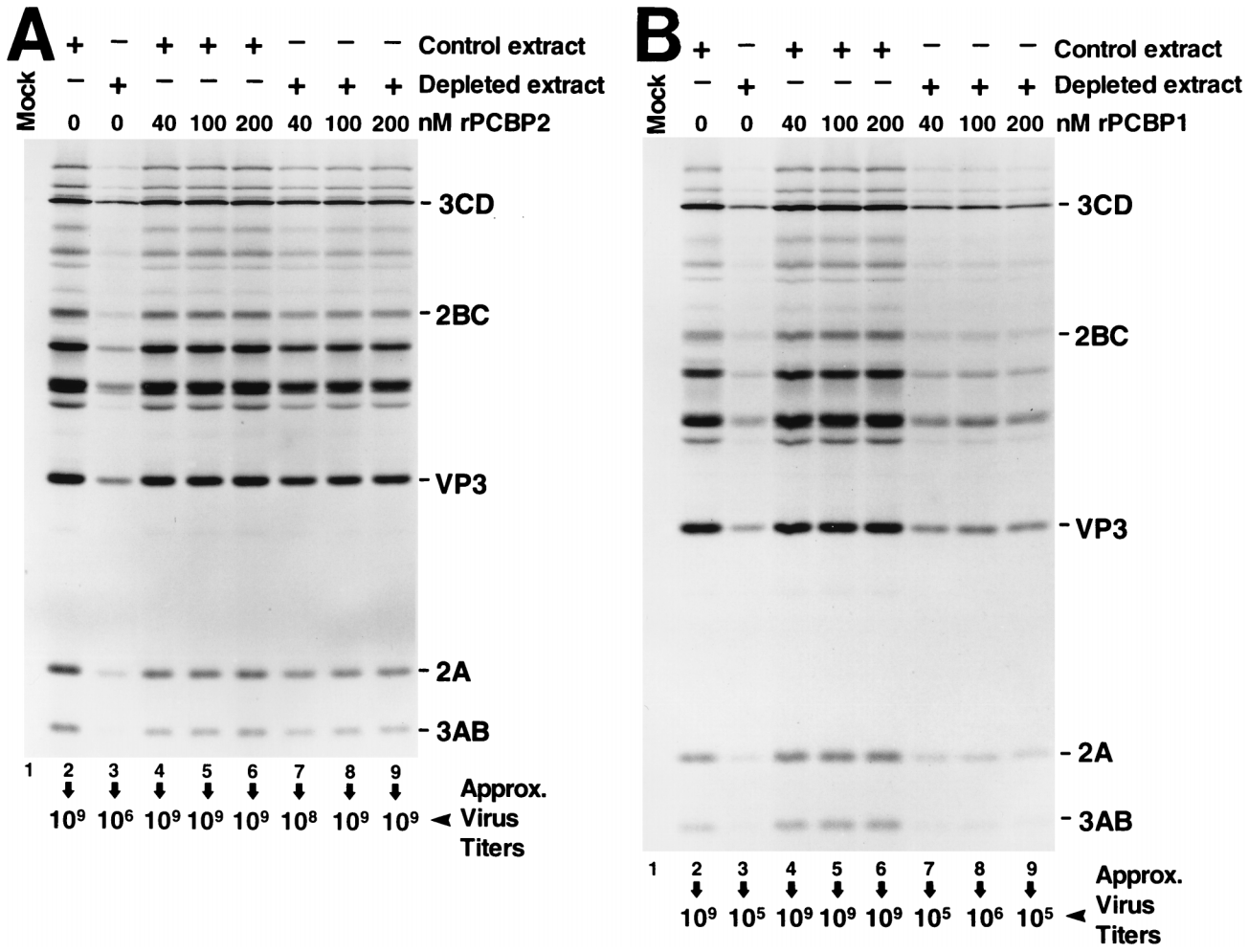

FIG. 4. Restoration of translation and virus production by rPCBP2 or rPCBP1 in extracts depleted of PCBP2. (A) Lanes: 1, a nondepleted reaction programmed with no RNA; 2, a nondepleted control reaction programmed with poliovirus vRNA (the migration of some poliovirus proteins is indicated to the right); 3, a depleted reaction programmed with poliovirus vRNA; 4 to 6, the effects of the addition of rPCBP2 to nondepleted reactions programmed with poliovirus vRNA; 7 to 9, the effects of the addition of rPCBP2 to depleted reactions programmed with poliovirus vRNA. Translation reaction mixtures (10-μl volume containing 250 ng of vRNA) were incubated at 30°C for 6 h, while parallel cell-free virus production reactions (50-μl volume containing 1.25 μg of vRNA) were incubated at 30°C for 6 h followed by incubation at 34°C for 12 h. The reaction mixtures were then freeze-thawed and treated with 20 μg of RNase A and 0.35 U of RNase V1 for 25 min at 34°C prior to carrying out of plaque assays on HeLa cell monolayers. Approximate (approx.) virus titers in PFU per ml are indicated at the bottom of the panel. (B) Lanes are identical to those shown in panel A, except that rPCBP1 was used in place of rPCBP2.

present in the rPCBP2 preparation; however, the contribution of PCBP2 to the translation of poliovirus RNA is clearly demonstrated.

Translation of viral RNA in the HeLa cell extracts used in this study has been shown previously to result in the efficient production of viral proteins which, together with cellular factors endogenous to the extract, can replicate and package viral RNA to generate infectious viral particles (2, 18, 24). The effect of PCBP2 on virus production was examined by measuring plaque-forming activity generated by control, depleted, and rPCBP2-restored extracts (Fig. 4A). The passage of extracts over a control column had no significant effect on the abilities of those extracts to produce virus (Fig. 4A, lane 2). Extracts that had been depleted of PCBP2 had a 3- to 4-log-unit reduction in the yield of viable virus (Fig. 4A, lane 3). Some variability in plaque reduction, which appeared to correlate with the level of depletion of PCBP2 in the extract, was observed. Addition of increasing amounts of rPCBP2 (to a maximum of 200 nM) restored translation to near control levels, restored the yield of viable virus, and gave rise to plaques identical to those produced in nondepleted extracts (Fig. 4A, lanes 7 to 9; data not shown). Addition of rPCBP2 to control extracts had no effect on translation or virus yields (Fig. 4A, lanes 4 to 6).

The specificity of rPCBP2 restoration activity was examined in experiments similar to the ones described above with purified rPCBP1 (Fig. 4B). PCBP1 is unable to efficiently bind stem-loop IV of poliovirus RNA (4), although both PCBP1 and PCBP2 bind stem-loop I RNA (reference 19 and data not shown). Addition of PCBP1 had no effect on translation or virus production in control extracts (Fig. 4B, lanes 4 to 6) and was unable to restore translation or virus production in stem-loop IV RNA-depleted extracts (Fig. 4B, lanes 7 to 9). However, the binding activity of the rPCBP1 preparation to stem-loop I of the poliovirus 5′ NCR was confirmed (data not shown), indicating that the protein preparation was active.

Recently, we suggested that PCBP2 plays a role in the initiation of viral RNA synthesis, as a result of its binding to the 5′ terminal stem-loop I sequences in poliovirus RNA (19).

These results, together with the work presented here and previous genetic studies implicating specific sequences in stem-loop I in poliovirus translation (22), suggest that PCBP2 plays multiple roles in the poliovirus life cycle. Such roles may represent direct and independent functions at two different sites on the viral RNA, or they may affect more than one process by contributing a general structural function. In agreement with multiple functions for PCBP2 during a poliovirus infection, we have observed that production of infectious virus in vitro appears to be more sensitive to PCBP2 depletion than translation of viral RNA (Fig. 4). For example, intermediate levels of PCBP2 may partially restore translation activity but fail to restore virus production to similar levels (data not shown). Although there are numerous potential explanations for the lack of a linear relationship between translation activity and virus production, competition for PCBP2 in two essential reactions would explain the apparent discrepancy and may provide a regulatory linkage of these steps in virus growth.

We are grateful to Todd B. Parsley for preparation of purified PCBP1 and PCBP2, Oliver Richards for experimental advice, and Hung Nguyen for expert assistance.

This work was supported by Public Health Service grants AI 26765 and AI 12387 from the National Institutes of Health. L.B.B. was supported by a postdoctoral fellowship from the Bank of America-Giannini Foundation.

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