Journal of General Virology (1999), **80**, 1371–1376. Printed in Great Britain

Poly(C)-binding protein interacts with the hepatitis C virus 5′ untranslated region

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We have investigated whether poly(C)-binding protein (PCBP)-1 and PCBP-2 interact with the hepatitis C virus (HCV) 5′ untranslated region. Our results demonstrate that glutathione *S*-transferase (GST)–PCBP-1 and GST–PCBP-2 fusion proteins bind specifically to the HCV 5′ untranslated region. An antiserum raised against PCBP-2 induced a supershift after incubation with RNA–protein complexes formed between proteins in a HeLa cell cytoplasmic extract and the HCV 5′ untranslated region, indicating that this interaction occurs intracellularly. The complete internal ribosome entry site was necessary for efficient binding, suggesting that maintenance of the secondary structure was necessary for recognition of the binding site by the PCBPs.

Introduction

Hepatitis C virus (HCV), a member of the family *Flaviviridae* (Rice, 1996), is the principal causative agent of non-A non-B hepatitis and HCV infection may develop into hepatocellular carcinoma (Houghton, 1996). Although HCV is a major human pathogen, there is no vaccine available and no effective antiviral therapy exists today. The HCV genome is approximately 9·5 kb long and contains a unique open reading frame (ORF) which is translated into a polyprotein that is cleaved cotranslationally into functional products by viral and cellular proteases (Clark, 1997; Reed & Rice, 1998). The ORF is flanked by the 5′ and 3′ untranslated regions (UTRs), which are involved in transcription and translation initiation (Clark, 1997; Reed & Rice, 1998).

The HCV 5′ UTR contains an internal ribosome entry site (IRES) for internal initiation of translation (Lemon & Honda, 1997). The HCV IRES is located in the 5′ UTR and extends in to the capsid coding region (Reynolds *et al.*, 1995). This is in contrast to, for example, the poliovirus IRES, which is located in the 5′ UTR. Similarly to the poliovirus IRES, the HCV IRES is highly structured and the RNA folds into a secondary structure which is conserved among various HCV genotypes. It may be divided into four regions which, with the exception of regions I and IV, contain multiple stem–loop structures (Lemon & Honda, 1997). RNase protection analysis, mutagenesis of the IRES and analysis of sequences of naturally occurring HCV subtypes with sequences deviating from the consensus sequence support the secondary structure that has been proposed for the HCV 5′ UTR (Brown *et al.*, 1992; Bukh *et al.*, 1992). Therefore, it appears that maintenance of the secondary structure of the HCV IRES is a prerequisite for internal initiation of translation.

The poliovirus IRES has been shown to interact with multiple cellular RNA-binding proteins. The La protein and the poly(C)-binding proteins (PCBPs) are apparently essential for efficient translation initiation at the poliovirus IRES (Blyn *et al.*, 1997; Meerovitch *et al.*, 1993). Similarly, the HCV 5′ UTR has been shown to interact with cellular factors (Yen *et al.*, 1995) of which four have been identified: the La protein (Ali & Siddiqui, 1997), the polypyrimidine tract binding protein (PTB) (Ali & Siddiqui, 1995), heterogeneous ribonucleoprotein L (hnRNP L) (Hahm *et al.*, 1998) and eukaryotic initiation factor 3 (eIF3) (Sizova *et al.*, 1998). Depletion of the PTB from an *in vitro* translation reaction resulted in inhibition of HCV IRES dependent translation (Ali & Siddiqui, 1995) and addition of purified La protein to an *in vitro* translation reaction showed a dose dependent increase in HCV IRES directed translation (Ali & Siddiqui, 1997), suggesting that PTB and La may participate in the initiation of translation of the HCV mRNA. Here we have investigated whether PCBP-1 and -2 interact with the HCV 5′ UTR. Our results demonstrate that glutathione *S*-transferase (GST)–PCBP-1 and GST–PCBP-2 fusion proteins

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interact specifically with the HCV 5′ UTR. An antiserum raised against PCBP-2 induced a supershift after it was incubated with RNA–protein complexes formed between proteins in a HeLa cell cytoplasmic extract and the HCV 5′ UTR. The complete IRES was necessary for efficient binding, suggesting that maintenance of the secondary structure was necessary for recognition of the binding site by the PCBPs.

Methods

■ **Plasmid constructions.** p5UTRS : 21 was constructed by first PCR amplifying HCV sequences from pCV-H77C (Yanagi *et al.*, 1997) with oligonucleotides HCV5S (5′ CGAGCTCCTCACTATAGCCAGCCC 3′) and HCV5A (5′ CCCATGGTGCGCAGACGGTTGGTGTTACGTT-TGG 3′), followed by insertion of the PCR fragment after the T7 promoter in plasmid pCR2.1 (Invitrogen). The HCV sequence in deletion mutant p117, which produces RNA (119-394)R, was constructed by the insertion into pCR2.1 of a PCR fragment generated from pCV-H77C (Yanagi *et al.*, 1997) with oligonucleotides HCV5S-2 (5′ GAGCT-CACCCCCCCTCCTGGGAGA 3′) and HCV5A. pGST–PCBP-1 and pGST–PCBP-2 were a generous gift from H. Leffers and have been described previously (Leffers *et al.*, 1995). pGST–PTB, pKSB2 and pKSXB have been described previously (Sokolowski *et al.*, 1997).

■ **Preparation of cell extract and recombinant GST fusion proteins.** The HeLa cell cytoplasmic extract was prepared as described previously (Zhao *et al.*, 1996) by resuspending HeLa cells in lysis buffer (10 mM HEPES pH 7·6, 60 mM KCl, 5 mM $MgCl_2$, 5 mM EDTA, 1 mM DTT, 0·2% NP40 and 1 μg/μl aprotinin). The extract was centrifuged at 10000 ***g*** for 10 min, the supernatant was collected and the protein concentration was determined.

■ ***In vitro* transcription, RNA gel shift and UV cross-linking.** *In vitro* synthesis of radiolabelled RNA was performed as described previously (Zhao *et al.*, 1996). First, p5UTRS : 21, p117, pKSB2 or pKSXB were linearized immediately adjacent to the viral insert. To create the shorter RNAs named 128R and 276R, p5UTRS : 21 was linearized with *Sma*I and *Stu*I, respectively. Then, *in vitro* transcription was performed with T3- or T7-RNA polymerase in the presence of [^{32}P]CTP. The RNA mobility shift assay was performed with radiolabelled RNA as described previously (Zhao *et al.*, 1996). Briefly, 25–40 fmol of radiolabelled RNA probe was incubated with 1 μg of GST fusion protein (or dilutions thereof), in a total volume of 20 μl binding buffer [60 mM KCl, 10 mM HEPES, 3 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 5% glycerol and 5 μg/μl heparin] for 10 min at room temperature. In competition experiments, GST fusion protein was preincubated with the RNA competitor for 5 min, prior to the addition of radiolabelled RNA probe. Complexes were resolved on native 4% PAGE gels (acrylamide : bisacrylamide ratio, 60 : 1). For supershift experiments, 4 μl of rabbit anti-PCBP serum was added to the RNA gel shift reaction and incubation continued for 10 min at room temperature. The rabbit serum towards PCBP-2 was a generous gift from E. Ehrenfeld. The UV cross-linking assay was performed as previously described (Zhao *et al.*, 1996).

Results

PCBP-1 and -2 bind specifically to the HCV 5′ UTR

It has previously been shown that PCBP-2 interacts with the poliovirus IRES (Blyn *et al.*, 1997). To investigate whether PCBP-1 and -2 bind to the HCV IRES, recombinant GST fusion proteins of PCBP-1 and -2 were purified from bacteria and subjected to RNA gel shifts with the HCV 5′ UTR as a probe. The results revealed that both proteins shifted the HCV 5′ UTR probe (Fig. 1 *a*), but not an unrelated RNA derived from the human papillomavirus (HPV) type 16 late mRNA (Fig. 1 *b*). The interaction was also observed by using UV cross-linking (Fig. 1 *c*). GST alone did not shift the RNA probe or UV cross-link to the probe (data not shown).

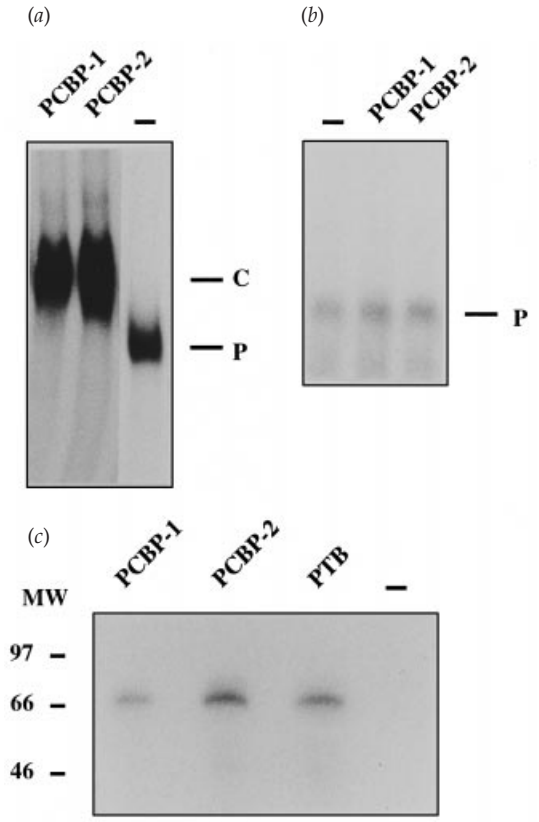

Fig. 1. (*a*) RNA gel shift with GST–PCBP-1 or GST–PCBP-2 and the radiolabelled HCV RNA 5′UTR. (*b*) RNA gel shift with GST–PCBP-1 or GST–PCBP-2 and the radiolabelled HPV RNA H3, previously shown not to interact with PCBP-1 or PCBP-2 (Collier *et al.*, 1998). (*c*) UV cross-linking of GST–PCBP-1, GST–PCBP-2 or GST–PTB to the HCV RNA probe 5′UTR. P, probe; C, complex; (–), no protein; MW, molecular mass marker.

To determine the specificity of the interaction, a competition experiment was performed. PCBP-1 was allowed to interact with the HCV 5′ UTR in the absence or presence of unlabelled, unspecific RNA competitor B2, derived from the HPV-1 3′ untranslated region (Sokolowski *et al.*, 1997), or the unlabelled HCV 5′ UTR RNA. The results revealed that the HCV 5′ UTR competed with the probe whereas the papillomavirus derived RNA did not (Fig. 2 *a*). We have previously shown that PCBP-1 and -2 interact with a sequence in the HPV-16 L2 mRNA named H2 (Collier *et al.*, 1998). This RNA sequence competed efficiently with the HCV 5′ UTR probe (Fig. 2 *b*), whereas tRNA did not.

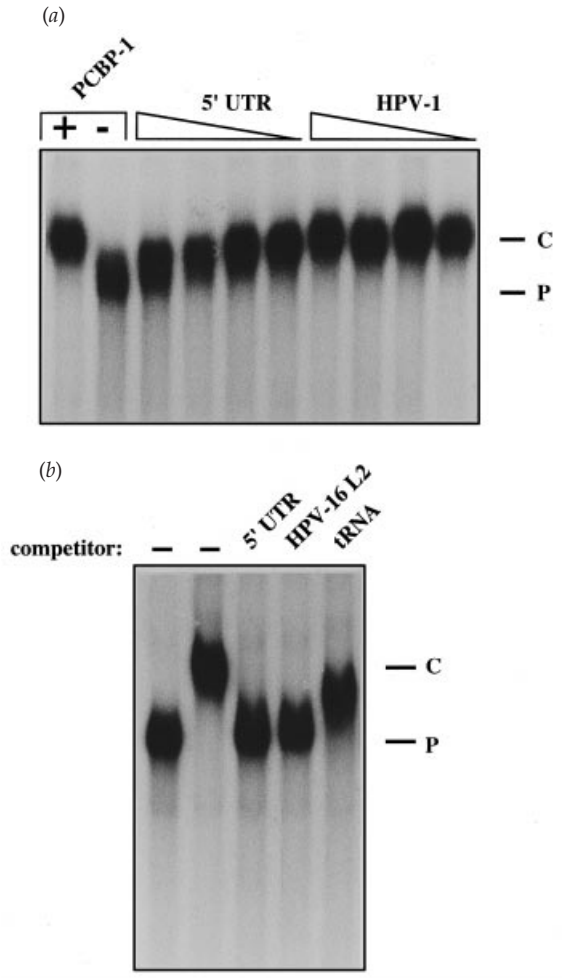

Fig. 2. (*a*) RNA gel shift with probe 5′UTR in the absence (−) or presence (+) of GST–PCBP-1 and in the presence of GST–PCBP-1 and the HCV RNA 5′UTR or the HPV-1 XB RNA (Sokolowski *et al*., 1997) as competitor. A 20-, 7-, 2- or 0·7-fold excess of each competitor was used. (*b*) RNA gel shift with probe 5′UTR in the absence or presence of GST–PCBP-1 and absence or presence of the HCV 5′UTR competitor, the HPV-16 H2 RNA competitor (Collier *et al*., 1998) or tRNA. A 20-fold excess of the competitor was used. P, probe; C, complex.

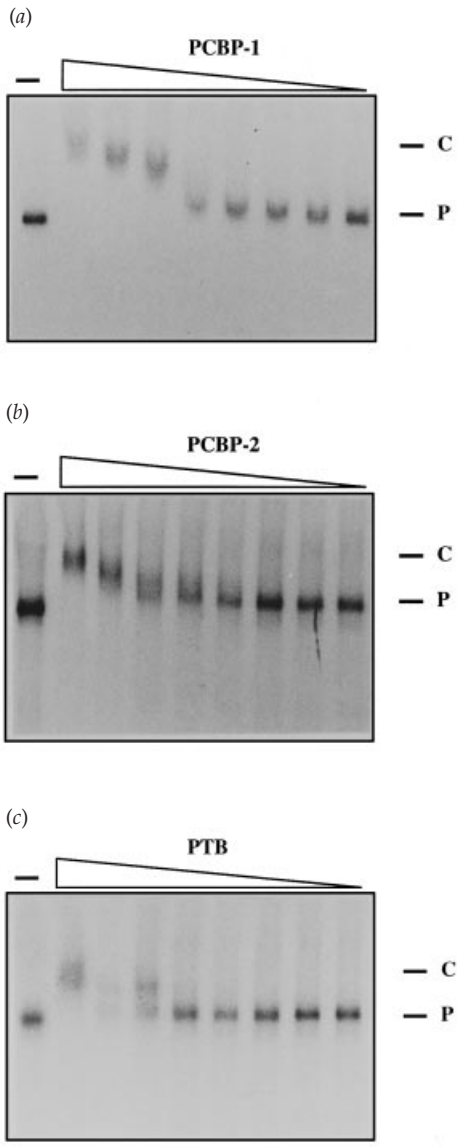

Fig. 3. RNA gel shift with the HCV 5′UTR probe in the absence (−) or presence of 1 μg of 1·5-fold serially diluted GST–PCBP-1 (*a*), GST–PCBP-2 (*b*) or GST–PTB (*c*). P, probe; C, complex.

To study the interaction between the PCBPs and the HCV 5′ UTR further, we compared the affinity of the PCBP–HCV 5′ UTR interaction with the affinity of the PTB protein for the HCV 5′ UTR (Ali & Siddiqui, 1995). PTB was previously shown to interact with the HCV 5′ UTR. Recombinant GST fusion proteins of PCBP-1, PCBP-2 and PTB were subjected to RNA gel shifts with the HCV 5′ UTR RNA probe. One μg of each protein was 1·5-fold serially diluted and incubated with the HCV 5′ UTR RNA. The results revealed that GST–PCBP-1 and GST–PCBP-2 have similar affinity for the HCV RNA (Fig. 3 *a, b*). In addition, the GST–PTB interacted with an affinity to the similar GST–PCBPs (Fig. 3 *c*). The interaction between the HCV 5′ UTR and the PTB protein could also be detected by UV cross-linking (Fig. 1 *c*). These results supported the idea that the interaction between the PCBP and HCV 5′ UTR could be functionally important.

The entire HCV 5′ IRES is required for efficient binding of GST–PCBP to the HCV RNA

In order to map the PCBP protein binding site in the HCV 5′ UTR, deletion mutants were generated and analysed in RNA gel shift assays. Unlabelled RNA of the 5′ and 3′ deletion mutants and the HCV 5′ UTR RNA were synthesized and used as competitors in RNA gel shift assays with radiolabelled HCV 5′ UTR RNA as probe. The results revealed that the both the 5′- and the 3′-end-deleted RNAs failed to compete with the probe under the same conditions as the complete HCV 5′ UTR competitor efficiently competed with the probe for GST–

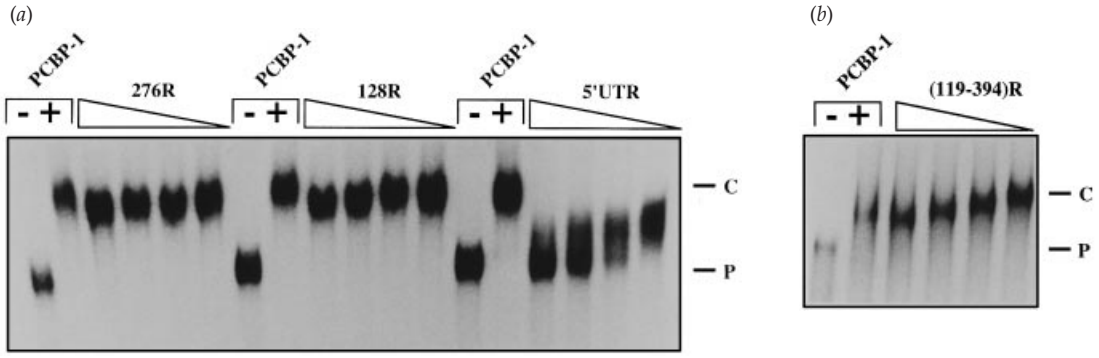

Fig. 4. (*a*) RNA gel shift with probe 5′UTR in the absence (−) or presence (+) of GST–PCBP-1 and in the presence of GST–PCBP-1 and the HCV competitor RNA 276R, 128R or 5′UTR. The names of the probes refer to the end-point of the deletions at nucleotide positions 128 and 276. A 20-, 7-, 2- or 0·7-fold excess of each competitor was used. (*b*) RNA gel shift with probe 5′UTR in the absence (−) or presence (+) of GST–PCBP-1 and in the presence of GST–PCBP-1 and the HCV competitor RNA (119-394)R. A 20-, 7-, 2- or 0·7-fold excess of the competitor was used. P, probe; C, complex.

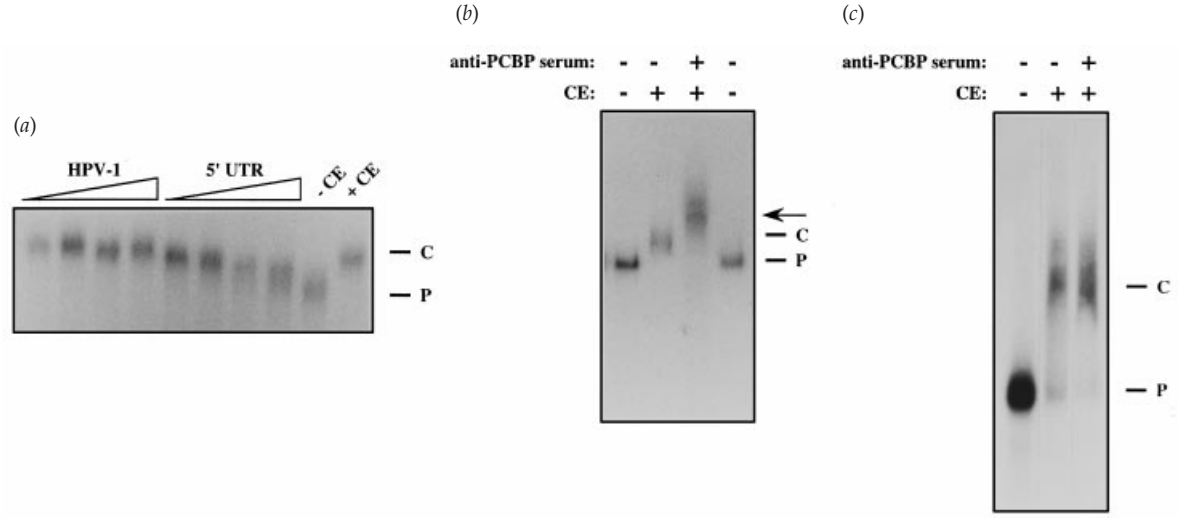

Fig. 5. (*a*) RNA gel shift with probe 5′UTR in the absence (−) or presence (+) of HeLa cell cytoplasmic extract (CE) and in the presence of CE and the HCV RNA 5′UTR or the HPV-1 XB RNA (Sokolowski *et al*., 1997) as competitor. A 20-, 7-, 2- or 0·7-fold excess of each competitor was used. (*b*) RNA gel shift with probe 5′UTR in the absence (−) or presence (+) of HeLa cell extract (CE) and in the absence or presence of anti-PCBP-2 serum. (*c*) RNA gel shift with probe H3 (Collier *et al*., 1998) in the absence (−) or presence (+) of HeLa cell extract (CE) and in the absence or presence of anti-PCBP-2 serum.

PCBP-1 (Fig. 4 *a, b*). These results demonstrated that the entire HCV IRES was necessary for efficient binding of GST–PCBP-1, strongly suggesting that maintenance of the HCV 5′ UTR RNA secondary or tertiary structure is required for efficient recognition of the PCBP-1 binding site.

Antiserum reactive with PCBP-1 and -2 supershifts complexes formed between cytoplasmic factors and the HCV 5′ UTR

In order to investigate if this interaction occurs in extracts of human cells, cytoplasmic extract was prepared from HeLa cells as described in Methods and subjected to RNA gel shifts with the HCV 5′ UTR RNA probe. As can be seen in Fig. 5 (*a*), complexes were formed between cytoplasmic factors and the HCV RNA probe. Competition experiments using the HPV-1 XB RNA (Sokolowski *et al*., 1997) or the HCV 5′ UTR RNA as competitor demonstrated that the HCV 5′ UTR competed with the radiolabelled probe whereas the papillomavirus derived RNA did not (Fig. 5 *a*). Therefore, under the conditions used here, the detected RNA–protein complexes were specific for the HCV 5′ UTR RNA sequence.

To determine if the PCBPs were present in the RNA–protein complexes, RNA gel shifts with cytoplasmic extract and the HCV 5′ UTR RNA probe were performed in the absence or presence of a rabbit serum towards PCBP-2. This serum induced a supershift of the RNA–protein complexes (Fig. 5 *b*). A supershift was not seen when HPV-16 L2 RNA was used under the same conditions (Fig. 5 *c*). Antisera raised against other RNA binding proteins did not induce supershifts of the complexes formed between the HCV 5′ UTR RNA and cytoplasmic factors (data not shown). In conclusion, the PCBPs

are present in the complexes formed between HCV 5′ UTR RNA and cytoplasmic cellular factors.

Discussion

Four proteins have been shown to bind to the HCV 5′ UTR: PTB (Ali & Siddiqui, 1995), La (Ali & Siddiqui, 1997), hnRNP L (Hahm *et al*., 1998) and eIF3 (Sizova *et al*., 1998). The interactions between these proteins and the HCV 5′ UTR have been proposed to affect translation of mRNAs that depend on the HCV 5′ UTR for efficient translation. For example, immunodepletion of PTB from rabbit reticulocyte lysates or from HeLa S10 extracts resulted in abrogation of HCV 5′ UTR dependent translation, but addition of GST–PTB did not restore translation (Ali & Siddiqui, 1995). The La protein was shown to stimulate translation of HCV 5′ UTR containing mRNAs in a dose dependent manner when added to rabbit reticulocyte lysates (Ali & Siddiqui, 1997). Deletion of the 3′ end of the HCV 5′ UTR which included the hnRNP L binding site resulted in loss of function of the HCV IRES (Hahm *et al*., 1998), thereby suggesting that hnRNP L is involved in translation of HCV mRNAs. Similarly to PTB and La, the PCBP-2 protein has previously been shown to bind to the poliovirus IRES (Blyn *et al*., 1997). The PTB and La appear to be required for translation of mRNAs that depend on the poliovirus IRES. It was recently shown that depletion of PCBPs from HeLa S10 extract resulted in abrogation of poliovirus IRES dependent translation and that the activity of the IRES could be restored by addition of recombinant His-tagged PCBP-2 (Blyn *et al*., 1997). Therefore, one may speculate that PCBPs binding to the HCV 5′ UTR may be involved in initiation of translation at the HCV IRES.

eIF3 binds to the internal loop in the apical stem IIIb (Sizova *et al*., 1998), and the hnRNP L binds to an unknown site in the 3′ end of the HCV IRES (Hahm *et al*., 1998). The PTB protein binds to multiple sites within the HCV IRES, all with high pyrimidine content (Ali & Siddiqui, 1995). In attempts to map the La binding site within the HCV IRES, it was found that 3′ and 5′ deletions all failed to compete with the entire IRES for binding to the La protein, indicating that secondary structure of the HCV IRES is important for recognition of the HCV sequence by the La protein (Ali & Siddiqui, 1997). This is similar to our observation that none of the deletion mutants competed with the complete IRES for binding to the HCV IRES. Therefore, PCBP binding also appears to depend on the integrity of the higher order structure of the HCV IRES.

It has been shown that binding of the PCBPs to an mRNA may affect translation in a negative manner. The 15-lipoxygenase mRNA 3′ UTR contains an RNA element that prevents translation of the mRNA in undifferentiated red blood cells. In response to differentiation, the repression of translation of the 15-lipoxygenase mRNA is relieved and translation of the mRNA proceeds. It was shown that binding of PCBPs and hnRNP K to the 15-lipoxygenase mRNA inhibited its translation *in vitro* (Ostareck *et al*., 1997). Similarly, the HPV-16 L1 and L2 genes are expressed in a differentiation dependent manner in squamous epithelial cells (Stanley, 1994). This is partly due to the presence on the L1 and L2 mRNAs of *cis* acting negative regulatory RNA elements (Schwartz, 1997). One of these elements is located in the 3′ end of the HPV-16 L2 coding region (Sokolowski *et al*., 1997) and interacts with PCBP-1 and -2 (Collier *et al*., 1998). The interaction between PCBP and the HPV-16 L2 mRNA resulted in the specific inhibition of translation *in vitro* of the L2 mRNA (Collier *et al*., 1998). Therefore, the PCBPs may stimulate translation or inhibit translation. One possible explanation for these opposite effects may come from the known effect of the poliovirus protease on eIF4G, which is cleaved and inactivated by poliovirus protease 2A. One may speculate that PCBPs take the place of the eIF4G to attract ribosomes in poliovirus and HCV infected cells, whereas in cells in which the PCBPs have a negative effect on translation, they may prevent eIF4G from binding to the ribosomes. Further experiments are needed to determine the role of the PCBPs in the HCV replication cycle.

Thanks to M. Sokolowski, B. Collier, E. Ehrenfeld and S. Pedersen-Mart for materials and L. Goobar-Larsson for critically reading the manuscript. Research sponsored by Medivir AB.

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Received 3 December 1998; Accepted 5 February 1999