Polypyrimeidine tract-binding protein interacts with coxsackievirus B3 RNA and influences its translation

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INTRODUCTION

Coxsackievirus B3 (CVB3), a member of the family Picornaviridae, is the causative agent of virus-induced myocarditis and dilated cardiomyopathy (Baboonian et al., 1997). The CVB3 RNA, like that of a typical picornavirus, contains a single, long open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTRs). The 5' UTR of CVB3 is 741 nt long and highly structured, containing multiple AUG triplets that are not used for initiation of translation. Ribosomes are recruited upstream of the AUG triplet at position 591 (AUG591), also called the cryptic translation. Ribosomes are recruited upstream of the AUG, after which they scan downstream for about 150 nt before initiating at the initiator AUG or AUG741 (Bhattacharyya & Das, 2005; M’hadheb-Gharbi et al., 2007). The 3' UTR of CVB3 is 99 nt long and highly structured, containing conserved domains, and is followed by a poly(A) tail of variable length (Klump et al., 1990).

The 5' UTR contains an internal ribosome entry site (IRES) element that recruits ribosomes in a cap-independent manner (Chen et al., 2008). Internal initiation of translation requires interaction between the IRES RNA and a number of proteins present in the host cell. The canonical translation-initiation factors that have been shown to play a role in IRES-mediated translation are eIF3, eIF4B and a cleaved part of eIF4G (Fitzgerald & Semler, 2009). In addition, a number of non-canonical factors, known as IRES trans-acting factors or ITAFs, have been shown to have crucial influence on IRES-mediated translation. These include human La-autoantigen (p52), polypyrimeidine tract-binding protein (PTB or p57) (Kolupaeva et al., 1996), upstream-of-NRas (Unr or p97) and poly(rC)-binding protein-2 (PCBP2 or p39) (Zell et al., 2008; reviewed by Vagner et al., 2001; Fitzgerald & Semler, 2009).

PTB or heterogeneous nuclear ribonucleoprotein-I (hnRNP-I), first identified as a pre-mRNA splicing factor, has been reported to have a role in IRES-mediated translation of several picornaviruses, including poliovirus (PV), encephalomyocarditis virus (EMCV) and hepatitis A virus (HAV), and in flaviviruses, e.g. hepatitis C virus (HCV) (Jang et al., 1988; Pestova et al., 1991; Hellen et al., 1994; Schultz et al., 1996; Hunt & Jackson, 1999; Gosert et al., 2000; Venkatramanan et al., 2003). The possibility of PTB acting as an RNA chaperone that binds to different regions of the PV IRES and stabilizes the tertiary structure has been reported (Song et al., 2005). PTB has also been shown to stabilize the EMCV IRES structure (Kafasla et al., 2009). Although PTB has been shown to influence IRES-mediated translation from a wide variety of viruses, the mechanism of such activity is as yet unknown. In foot-and-mouth disease virus (FMDV), mutations in the major PTB-binding site interfere with the formation of initiation complexes and the efficiency of translation (Niepmann et al., 1997).

The secondary structure of the 3' UTR of CVB3 RNA consists of three stem–loops (X, Y and Z), followed by a poly(A) tail sequence. The role of these stem–loops is not
known, but they are suggested to play an important role during regulation of virus replication through interaction with cellular proteins. The 3′ UTR is known to enhance IRES activity, although the mechanism is not clear (Melchers et al., 1997; Dobrikova et al., 2006).

In the present report, we establish PTB as a bona fide ITAF for CVB3 by characterizing the effect of partial silencing of PTB on IRES-mediated translation ex vivo in HeLa cells. IRES activity in BSC-1 cells, reported to have a very low level of endogenous PTB (Gosert et al., 2000), was found to be significantly lower than that in HeLa cells. PTB is observed to interact with both the 5′ and 3′ UTRs of CVB3, although with different affinities. Finer mapping of the interaction between PTB and the UTRs showed that the protein interacts with multiple regions of both UTRs. We also show the cis-acting effect of the CVB3 3′ UTR on IRES-mediated translation. The PTB contact points on the 3′ UTR map to conserved regions, deletion of which abrogates the 3′ UTR-mediated enhancement of IRES activity. These results suggest that PTB plays a pivotal role in mediating the internal initiation of translation of CVB3 RNA.

RESULTS

PTB interacts specifically with the CVB3 5′ UTR

The CVB3 5′ UTR has been known to interact with various cellular trans-acting factors, some of which have been characterized, e.g. La-autoantigen, while many others are as yet unidentified (Ray & Das, 2002). To explore other 5′ UTR-interacting proteins, we performed a UV cross-linking assay using body-labelled 5′ UTR RNA with HeLaS10 extract. The results indicated that polypeptides with molecular masses of 117, 100, 95, 90, 75, 72, 58, 52, 50, 47, 42 and 39 kDa interact with the CVB3 5′ UTR (Fig. 1a, lane 2). In order to investigate the specificity of these interactions, unlabelled self RNA was allowed to compete with the 5′ UTR probe for the interacting proteins. Most of these proteins were outcompeted by a 500-fold molar excess of unlabelled self RNA (Fig. 1a, compare lanes 3 and 4 with lane 2). Interestingly, a 500-fold molar excess of unlabelled CVB3 3′ UTR RNA competed for all of the proteins interacting with the 5′ UTR, although to a much lesser extent. Further, the 3′ UTR RNA competed with a higher affinity for p95 than for the other proteins (Fig. 1a, compare lanes 5 and 6 with lanes 2, 3 and 4). As expected, unlabelled non-specific RNA did not compete significantly (Fig. 1a, lane 7). Among the proteins that interact with the CVB3 5′ UTR, the 52 kDa protein has been characterized as La-autoantigen and shown to be important for CVB3 IRES function (Ray & Das, 2002; Bhattacharyya & Das, 2005). Also, a 58 kDa polypeptide was observed to interact specifically with the 5′ and 3′ UTRs (Fig. 1a, compare lanes 5 and 6 with lanes 2, 3 and 4). In order to investigate whether the 58 kDa polypeptide is PTB (as observed in the HAV IRES), we immunoprecipitated the UV cross-linked ribonucleoprotein complex with an antibody against PTB. The results suggest that the 58 kDa polypeptide can be immunoprecipitated by anti-PTB antibody (Fig. 1b, compare lanes 1 and 2). For further confirmation, UV cross-linking was performed using labelled 5′ and 3′ UTRs of CVB3 riboprobe, with an increasing concentration of recombinant PTB protein. The results demonstrated clearly that PTB indeed interacts with both the 5′ and 3′ UTRs of CVB3 RNA, although the affinity of interaction with the 3′ UTR was lower than that with the 5′ UTR (Fig. 1c, compare lanes 4 and 5 with lanes 2 and 3). A filter-binding experiment, performed to find the relative retention of riboprobe by increasing the quantity of purified protein, indicated the $K_d$ for PTB binding to be approximately 350 nM for the 5′ UTR and 520 nM for the 3′ UTR (data not shown). The specificity of interaction between PTB and the 5′ UTR or 3′ UTR RNA was confirmed by competition experiments, wherein a molar excess of unlabelled RNA was allowed to compete with labelled RNA for binding to PTB. The results showed that 250- and 500-fold molar excesses of unlabelled 5′ UTR RNA competed with the CVB3 5′ UTR riboprobe for binding to PTB, whereas non-specific RNA could not (Fig. 1d). Similarly, 250- and 500-fold molar excesses of unlabelled 3′ UTR RNA were able to compete with the CVB3 3′ UTR riboprobe for PTB binding (Fig. 1e), whereas non-specific RNA could not compete.

To demonstrate conclusively the interaction of PTB with the CVB3 5′ UTR, an RNA–protein complex consisting of PTB protein and bicistronic RNA containing the CVB3 5′ UTR, assembled in HeLa cells, was precipitated by using an anti-PTB antibody. The result showed specific amplification of the CVB3 5′ UTR from the immunoprecipitate, obtained by using the anti-PTB antibody but not by using pre-immune serum (Fig. 1f, compare lanes 3 and 5). As a control for precipitation of the bicistronic-encoding plasmid, PCR was performed with the immunoprecipitate without reverse transcription; as expected, this did not show any significant amplification (Fig. 1f, compare lanes 2 and 3). A PCR performed without addition of any cDNA also did not show any amplification (Fig. 1f, lane 1).

Mapping of putative PTB-binding sites in the CVB3 5′ and 3′ UTRs

PTB has been implicated to function as an RNA chaperone that interacts with different regions of the RNA and holds them in a particular conformation. In order to broadly map the binding sites of the PTB protein on the 5′ UTR RNA, recombinant PTB was UV cross-linked to riboprobes corresponding to the full-length 5′ UTR (nt 1–741) or fragments representing nt 1–250, 250–533 or 533–741 (Fig. 2a). The results suggested that PTB interacts with different affinities with the 5′ UTR fragments, showing a higher affinity for fragment 533–741 than for fragment 1–250 or 250–533 (Fig. 2b, compare lanes 3, 5, 7 and lanes 4, 6, 8). However, the binding affinity for all deletions was lower than that for the full-length 5′ UTR (Fig. 2b,
Fig. 1. Cellular protein binding to the untranslated regions of CVB3 RNA. (a) $^{32}$P-labelled CVB3 5’ UTR riboprobe was UV cross-linked to proteins in HeLaS10 extract in the absence (lane 2) or presence of a 250- or 500-fold molar excess of unlabelled 5’ UTR or 3’ UTR RNA or a 500-fold molar excess of non-specific (Nsp) RNA (lanes 3–7, as indicated). Lane 1 shows cross-linking in the absence of HeLaS10 extract. Numbers on the left indicate the molecular masses of marker proteins (in kDa) and numbers on the right represent riboprobe-interacting proteins. (b) Immunoprecipitation of UV cross-linked ribonucleoprotein complexes of $^{32}$P-labelled CVB3 5’ UTR RNAs and rabbit reticulocyte lysate (RRL) extract with anti-PTB antibody or rabbit pre-immune serum, as indicated. (c) $^{32}$P-labelled CVB3 3’ UTR or 5’ UTR RNA (lanes 2–5, as indicated) was UV cross-linked to an increasing concentration of recombinant PTB and analysed by SDS-PAGE (10 % gel) followed by phosphorimaging. Lane 1 shows the UV cross-linking of recombinant La with $^{32}$P-labelled CVB3 5’ UTR. (d) $^{32}$P-labelled CVB3 5’ UTR RNA was UV cross-linked to recombinant PTB in the absence (lane 2) or presence of a 250- or 500-fold molar excess of unlabelled self or non-specific (Nsp) RNA (lanes 3–6, as indicated). Lane 1 shows cross-linking without any protein added. (e) $^{32}$P-labelled CVB3 3’ UTR RNA was UV cross-linked to recombinant PTB in the absence (lane 1) or the presence of a 250- or 500-fold molar excess of unlabelled self or non-specific (Nsp) RNA (lanes 2–5, as indicated). (f) Immunoprecipitation of CVB3 bicistronic RNA–PTB complex from HeLa cell extract. HeLaS3 lysate transfected with CVB3 bicistronic plasmid was immunoprecipitated with pre-immune (PI) serum or anti-PTB antibody (as indicated), followed by RT-PCR using primers specific for the CVB3 5’ UTR. The immunoprecipitates were used for PCR without reverse transcription (RT; lanes 2 and 4) as a negative control. Lane 1 shows PCR without addition of any template.
was UV cross-linked to 32P-labelled probes corresponding to 533–741. (b) An increasing concentration of recombinant PTB and deletion fragments representing nt 1–250, 250–533 and 533–741 of the 5′ UTR RNA was UV cross-linked to 32P-labelled full-length CVB3 5′ UTR RNA. (a) Schematic diagram showing full-length 5′ UTR and deletion fragments representing nt 1–250, 250–533 and 533–741 of the 5′ UTR RNA, as indicated. The interacting proteins were analysed by SDS-PAGE (10% gel) followed by phosphorimaging. Numbers at the bottom of the panel indicate the relative signal intensity. (c) 32P-labelled full-length CVB3 5′ UTR RNA was UV cross-linked to recombinant PTB in the presence of a 250- or 500-fold molar excess of unlabelled RNA corresponding to nt 1–250, 250–533 or 533–741 of the 5′ UTR (lanes 2–7, as indicated). Lane 1 shows cross-linking without any competitor RNA.

Fig. 2. Interaction of recombinant PTB with full-length and mutant 5′ UTR RNA. (a) Schematic diagram showing full-length 5′ UTR and deletion fragments representing nt 1–250, 250–533 and 533–741. (b) An increasing concentration of recombinant PTB was UV cross-linked to 32P-labelled probes corresponding to CVB3 full-length, nt 1–250, 250–533 or 533–741 of the 5′ UTR RNA, as indicated. The interacting proteins were analysed by SDS-PAGE (10% gel) followed by phosphorimaging. Numbers at the bottom of the panel indicate the relative signal intensity. (c) 32P-labelled full-length CVB3 5′ UTR RNA was UV cross-linked to recombinant PTB in the presence of a 250- or 500-fold molar excess of unlabelled RNA corresponding to nt 1–250, 250–533 or 533–741 of the 5′ UTR (lanes 2–7, as indicated). Lane 1 shows cross-linking without any competitor RNA.

In order to confirm the difference in relative affinity of interaction, 100- and 250-fold molar excesses of unlabelled RNA corresponding to the 5′ UTR fragments were allowed to compete with the full-length 5′ UTR riboprobe for binding to recombinant PTB protein. The result showed that all three deletions compete for binding to PTB, although with different affinities (Fig. 2c, compare lane 1 with lanes 2–3, 4–5 and 6–7), with fragment 533–741 of the 5′ UTR showing highest affinity (Fig. 2c, compare lane 6 with lanes 3 and 5).

In order to map the contact points of PTB interaction on CVB3 5′ UTR RNA more closely, a toe-printing experiment was performed using in vitro-transcribed 5′ UTR RNA and purified PTB protein. The results showed that addition of PTB causes the appearance of a significant number of unique reverse transcriptase pause (RT-pause) sites on the 5′ UTR RNA, including G603, A599, G596, C581, U571, A567 and U618 (Fig. 3a, compare lanes 2–3 with lane 1 and the schematic diagram in Fig. 3b). However, addition of BSA, a protein that does not bind to the 5′ UTR RNA, did not show any unique RT-pause sites (Fig. 3a, compare lanes 4 and 1). As the 3′ UTR of the CVB3 genome was also observed to interact with PTB, a toe-printing reaction with recombinant PTB was also performed with in vitro-transcribed 3′ UTR RNA and an end-labelled reverse primer that anneals to the 3′ end. The results showed the appearance of unique RT-pause sites at multiple positions on the 3′ UTR, corresponding to nt A7290, A7311, A7322, U7345, A7350, G7365 and to multiple positions between nt U7378 and C7383 (Fig. 3c, compare lanes 2–3 with lane 1). As expected, the addition of BSA did not show the appearance of any unique RT-pause sites (Fig. 3c, compare lane 4 with lane 1).

Toe-printing of the 3′ UTR RNA–PTB complex showed that the majority of the PTB-interaction sites lie at the 5′ and 3′ ends of the 3′ UTR RNA (Fig. 3d). To confirm this, deletion mutants of the 3′ UTR were generated by deleting either 15 nt from the 5′ end (nt 7301–7315; Δ5′) or 14 nt from the 3′ end (nt 7386–7399; Δ3′) (Fig. 4a). A UV cross-linking experiment with HeLaS10 extract using riboprobes corresponding to either the full-length or mutant 3′ UTRs showed a significant reduction in p58 binding in both the Δ3′ (Fig. 4a, compare lane 3 with lane 2) and Δ5′ (Fig. 4a, compare lane 4 with lane 2) riboprobes. Interestingly, in addition to the effect on p58 binding, interaction with a few other proteins was also observed to be affected in the deletion mutants. Both Δ5′ and Δ3′ showed reduced interaction with p95, p80, p45 and p39, whereas interaction with the other proteins remained unaltered. As the CVB3 genomic RNA is polyadenylated in vivo, we investigated the effect of a 30 nt poly(A) tail at the 3′ end of the Δ5′ or Δ3′ riboprobe on interaction with HeLaS10 proteins. The results suggest that addition of the poly(A) tail did not rescue the reduced p58–PTB binding in the mutants (compare Fig. 4b, lanes 2–4 with Fig. 4a, lanes 2–4).

Partial silencing of endogenous PTB RNA affects CVB IRES-mediated translation in HeLa cells

PTB has been shown to enhance translation via interaction with the IRES in picornaviruses. To investigate the possible role of PTB in CVB3 IRES-mediated translation, we transiently transfected plasmid DNA encoding a bicistronic mRNA containing the CVB3 5′ UTR into HeLa cells, with or without partial silencing of PTB using small interfering RNAs against the endogenous mRNA (Wagner & Garcia-Blanco, 2002). The bicistronic mRNA encodes Renilla luciferase (Rluc), translated by a cap-dependent mechanism, and firefly luciferase (Fluc), translated by the IRES element of the CVB3 5′ UTR, which is cloned between the two cistrons. The ratio of Fluc : Rluc was taken as the index of IRES activity. The result showed a significant reduction...
Reduced IRES activity in the BSC-1 cell line compared with HeLa cells

The BSC-1 cell line, derived from African green monkey kidney, is reported to have a lower level of endogenous PTB (Gosert et al., 2000). In order to compare the level of endogenous PTB in HeLa and BSC-1 cells, total cell lysate was immunoblotted using an anti-PTB monoclonal antibody; actin was immunoblotted as loading control. The result showed the level of endogenous PTB protein in BSC-1 cells to be remarkably lower than that in HeLa cells (Fig. 6a). In order to investigate whether this lesser amount of PTB would lead to a reduced efficiency of the CVB3 IRES activity, CVB3 bicistronic plasmid DNA was transiently transfected into the BSC-1 and HeLa cell lines. The ratio of Fluc (translated by CVB3 IRES) to that of Rluc (translated in a cap-dependent manner) was taken as the index of IRES activity. Comparison of activity in the two cell lines showed the CVB3 IRES to function in BSC-1 cell lines at only 40 % of the efficiency observed in HeLa cells (Fig. 6b, c). This supported the conclusion that PTB is an important ITAF for CVB3 IRES function.

Interestingly, transient co-transfection of the CVB3 bicistronic DNA construct along with the PTB protein expression plasmid (pCDHisC-PTB; a generous gift from Merkle et al., 2002).
Dr Garcia-Blanco, Duke University Medical Center, Durham, NC, USA) showed partial rescue of CVB3 IRES function (Fig. 6d).

**Mutation of PTB-interaction points in the CVB3 3′ UTR reduces translation enhancement in cis**

The 3′ UTR of picornaviruses has been shown to have a critical role in virus pathogenesis. In order to investigate a possible cis-acting role for the 3′ UTR in IRES-mediated translation, *in vitro*-transcribed uncapped monocistronic RNA, with or without the CVB3 3′ UTR downstream of the Fluc ORF (Fig. 7a, constructs 1 and 2), was translated *in vitro* in rabbit reticulocyte lysate. Capped Rluc RNA transcribed *in vitro* was translated simultaneously as a control. Fluc and Rluc activities were measured and plotted separately. Luciferase activity in the translation reaction containing 5′ UTR–Fluc RNA was taken as 100 % and those of others were plotted as fold excess of that. The result showed an almost 5-fold increase in the efficiency of CVB3 IRES translation with the addition of the 3′ UTR in cis (Fig. 7b, compare columns 2 and 1).

In order to investigate a possible role for PTB and 3′ UTR RNA interaction in 3′ UTR-mediated enhancement of IRES translation, the Δ5′ and Δ3′ mutants of the 3′ UTR were cloned downstream of CVB 5′ UTR–Fluc (Fig. 7a, constructs 3 and 4). *In vitro*-transcribed RNAs corresponding to the Fluc constructs (constructs 1–4) and capped Rluc were transiently transfected into HeLa cells. Fluc and Rluc activities were plotted separately. Luciferase activity from cells transfected with construct 1 (Fig. 7a) was taken as 100 % and those from the others plotted as fold excess of that. The results showed a significant reduction (almost 3-fold) of the 3′ UTR-mediated translational enhancement in the Δ5′ and Δ3′ 3′ UTRs (Fig. 7c).

Finally, as UV cross-linking of full-length and mutant 3′ UTRs with HeLaS10 proteins showed reduced interaction between p58 and the mutants, we carried out a similar study using recombinant PTB protein. As expected, the results showed a considerable decrease in PTB binding for...
both mutants compared with wild-type RNA (Fig. 7d, compare lanes 1–2, 3–4 and 5–6).

**DISCUSSION**

The IRES element in the 5' UTR of CVB3 RNA interacts with multiple host factors for translation initiation. The 3' UTR of the genomic RNA, containing extensive stretches of conserved residues, also contributes to IRES-mediated translation. In this report, we show the importance of a versatile cellular protein, PTB, in CVB3 IRES-mediated translation. The protein interacts with conserved residues on both the 5' and 3' UTRs of the genomic RNA, possibly leading to circularization of the genomic RNA, and thereby influences the efficiency of IRES-mediated translation.

As observed with PV, PTB showed multiple points of interaction on the 5' UTR, as well as the 3' UTR, of CVB3. A few interaction points on the 5' UTR were observed in the pyrimidine-rich region, whilst others were observed in AU-rich regions. Interestingly, a significant number of interaction points were found around the cryptic AUG (AUG<sub>591</sub>) on stem–loop H (SL-H) of the CVB3 IRES. Similar PTB contact points, rich in AU nucleotides, are also
observed on the 3' UTR at regions that are highly conserved among different strains. This suggests that the interaction between the 3' UTR and PTB might be a conserved feature among different coxsackievirus strains (Merkle et al., 2002).

Although PTB was observed to interact with both the 5' and 3' UTRs, the affinity of interaction with the former was observed to be higher than that with the latter. This could be because of the higher number of interaction points in the 5' UTR, although the implications of such a different affinity are not yet clear. It would be interesting to investigate which domains of the PTB protein are responsible for binding to the different UTRs of CVB3 RNA, and whether PTB is able to bind both of them simultaneously. Within the 5' UTR, the nucleotide fragment 533–741 showed highest affinity for binding to PTB. This was expected, as a significant number of PTB toe-prints were observed in this region. Interestingly, this region of the 5' UTR (containing the Shine–Dalgarno-like sequence, cryptic AUG and the spacer region of the 5' UTR) has also been observed to interact with almost all proteins in HeLa cytoplasmic extract as part of the full-length 5' UTR (data not shown). As observed with PTB, La-autoantigen...
has also been observed to interact specifically with the 5’ and 3’ UTRs of CVB3. It would be interesting to study whether PTB and La binding actually influence ribosome loading on the CVB3 IRES RNA.

Earlier studies have shown that the 3’ UTRs of HCV (Song et al., 2006) and FMoV (Serrano et al., 2006) enhance the IRES-mediated translation of respective IRES elements present in the 5’ UTR. The possible mechanism by which the 3’ UTR enhances IRES activity may be by bridging the 5’ to the 3’ end, through either RNA–RNA contacts or RNA–protein interaction. It has been shown for CVB3 that distal sequences, including 3’ UTR and poly(A) tail sequences, stimulate IRES function (Dobrikova et al., 2006). We have shown here that PTB binds specifically to the CVB3 5’ and 3’ UTRs. PTB interaction with the 3’ UTR may have a role in stimulation of IRES-mediated translation by the CVB3 3’ UTR. It is tempting to speculate that one or more of these host proteins may be involved in interacting simultaneously with the 5’ and 3’ UTRs, thus bringing about circularization of the mRNA. It is possible that some of this ITAF interaction, together with long-range RNA–RNA interaction, might contribute to the efficient IRES activity of CVB3 RNA.

CVB3 IRES-mediated translation was stimulated significantly by the 3’ UTR in cis, both in vitro in rabbit reticulocyte lysate and ex vivo in HeLa cells. Although the exact mechanism of this enhancement is not clear, it is possible that the rate of translation initiation mediated by the IRES element is increased by the 3’ UTR. The interaction points on the 3’ UTR were observed to be predominantly in the Z and X domains. Interestingly, partial deletion of either domain led to a significant drop in translation enhancement by the 3’ UTR. It may be possible that the interaction of PTB protein with the 3’ UTR RNA is responsible for the effect of the 3’ UTR on translation. Further, a simultaneous interaction of PTB protein with the 5’ UTR and the 3’ UTR might lead to circularization of the template RNA, leading to a higher rate of initiation. However, as deletion of the 3’ UTR domains leads to reduced interaction with additional proteins such as p80, the role of these proteins in 3’ UTR-mediated translation enhancement cannot be ruled out.

**METHODS**

**Cell lines and transfection.** HeLaS3 and BSC-1 cells (National Center for Cell Sciences, Pune, India) were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (both from Invitrogen). Transfections of CVB3 bicistronic plasmid cloned in the eukaryotic vector pcDNA3.1 and pSV40-Gal were carried out as described previously (Dhar et al., 2007). Essentially, 10⁶ cells were transfected with 1 μg bicistronic plasmid DNA using Tfx-20 reagent (Promega). Twenty-four hours post-transfection, cells were lysed as described previously (Bhattacharya et al., 2008) and the activity of luciferase enzymes was assayed by using the Dual Luciferase reporter assay system (Promega) in a TD-20/20 luminometer (Turner Biosystems). For overexpression of PTB in the BSC-1 cell line, 500 ng and 1 μg pCDHisC-PTB were transfected along with CVB3 bicistronic plasmid. Twenty-four hours post-transfection, cells were lysed and luciferase activity was assayed by using a TD-20/20 luminometer.

**Plasmid constructs and in vitro transcription.** CVB3 cDNA was obtained from Dr Nora Chapman, University of Nebraska, Omaha, NE, USA. The 5’ UTR (nt 1–741) was cloned between an upstream *Renilla* luciferase ORF and a downstream firefly luciferase ORF between *HindIII* and *EcoRI* sites of the pCDNA3.1 vector, to obtain the CVB3 bicistronic plasmid. A plasmid encoding β-galactosidase, pSV40-beta-Gal (Promega), was co-transfected to normalize transfection efficiency.

CVB3 nt 1–250, 250–533 and 533–741 were amplified by PCR using gene-specific primers. The 3’ UTR deletion mutants Δ5 (15 nt deleted from the 5’ end) and Δ3 (14 nt deleted from the 3’ end) were also amplified by PCR using specific primers. The CVB3 5’ UTR, 3’ UTR and the deletion mutants were cloned in the *HindIII* and *EcoRI* sites of plasmid pCDNA3, linearized with *EcoRI* and used for *in vitro* transcription using T7 RNA polymerase (Promega).

For monocistronic constructs, the CVB3 5’ UTR was cloned upstream of the firefly luciferase ORF in pCDNA3 to obtain CVB3 5’UTR–Firefly. The CVB3 3’ UTR and deletion mutants were subcloned downstream of firefly luciferase in the *Xhol* and *XbaI* sites of the monocistronic plasmid. All monocistronic plasmids were linearized with *Xbal* and used for *in vitro* transcription using T7 RNA polymerase.

Polyadenylated RNAs were synthesized *in vitro* by using T7 RNA polymerase. For this purpose, the template DNA was generated from the respective DNA constructs by PCR using the T7 forward primer and a reverse primer containing the sequences corresponding to 20 nt of the 3’ end of the gene, followed by 30 nt of poly(A) sequences. The PCR-amplified products were gel-eluted and used as template in the *in vitro* transcription reaction as described above.

For synthesis of riboprobes, [α-32P]UTP was used in *in vitro* transcription by T7 RNA polymerase (Promega).

**HeLaS10 preparation and UV cross-linking.** HeLaS10 extract preparation and RNA–protein interaction studies were performed as described previously (Bhattacharya et al., 2008). Briefly, 32P-labelled riboprobes were incubated with 20.0 μg HeLaS10 extract in binding buffer (5 mM HEPES (pH 7.6), 25 mM KCl, 2 mM MgCl₂, 3.8% glycerol, 2 mM dithiothreitol and 0.1 mM EDTA) and incubated at 30 °C for 10 min. The RNA–protein complexes were UV cross-linked and analysed by SDS-PAGE (10% gel) followed by phosphorimaging.

**Purification of recombinant PTB.** The expression of recombinant PTB from pET28a-PTB, transformed in *Escherichia coli* (BL21-DE3), was induced by 0.6 mM IPTG. His-tagged protein was purified by using Ni-NTA–agarose (Qiagen) as described previously (Grover et al., 2008).

**Immunoprecipitation of ribonucleoprotein complexes from HeLa cells.** Ribonucleoprotein complexes were immunoprecipitated from HeLa cells as described previously (Grover et al., 2008). Briefly, cells from 35 mm dishes transfected with CVB3 bicistronic plasmid were resuspended in 1 × RIPA buffer. To cell extracts, 8 μl anti-PTB (a gift from Dr Garcia-Blanco) or pre-immune serum was added and incubated for 4 h at 4 °C. The immunocomplexes were precipitated by Protein A–CL Sepharose beads (Sigma) for 4 h at 4 °C. The beads were washed extensively with 1 × RIPA buffer. RNAs associated with antigen–antibody complexes were isolated by proteinase K digestion. DNase treatment was performed to avoid any DNA contamination. Phenol/chloroform extraction was performed, followed by alcohol precipitation. The extracted RNA was analysed by RT-PCR using primers specific for the CVB3 5’ UTR.
Toe-printing assay. The RNA toe-printing assay was performed as described previously (Bhattacharyya & Das, 2006). Essentially, unlabelled CVB3 5’ UTR RNA (10 pmol) was incubated in the absence or presence of an increasing concentration of recombinant PTB protein and then annealed to an end-labelled primer (100 fmol) with sequence complementary to nt 624–640 of the RNA. The RNA–protein complex was reverse-transcribed with 3 U avian myeloblastosis virus reverse transcriptase (AMV-RT; Promega). Similarly, toe-printing with PTB on the CVB3 3’ UTR was performed by using a labelled primer complementary to the 3’ end of the RNA.

PTB silencing and Western blotting. Co-transfection of siPTB (pre-characterized and reported by Wagner & Garcia-Blanco, 2002) with bicistronic plasmid was performed as described previously (Bhattacharyya & Das, 2006). For transfection, 100 and 150 nm siPTB (Dharmacon) and 1 μg bicistronic DNA were co-transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Thirty-six hours post-transfection, the cells were lysed and luciferase activity was assayed by using the DLR assay system (Promega). PTB expression was analysed by using a monoclonal anti-PTB antibody (Calibiochem) and tubulin was detected by using a monoclonal anti-tubulin antibody (Sigma). The signal was detected by using an ECL detection kit (Amersham Pharmacia).

In vitro translation. Rabbit reticulocyte lysate was used for in vitro translation of monocistronic RNAs. One microgram of CVB3 5’ UTR-containing monocistronic firefly luciferase RNA and 0.5 μg m7-G-capped Renilla luciferase RNA were translated in a rabbit reticulocyte lysate system (Promega) supplemented with all amino acids, according to the manufacturer’s protocol. Luciferase activity was assayed by using the DLR assay system (Promega).

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