FEBS 17120 FEBS Letters 388 (1996) 39–42

# Porcine polypyrimidine tract-binding protein stimulates translation initiation at the internal ribosome entry site of foot-and-mouth-disease virus

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Received 19 February 1996; revised version received 29 April 1996

**Abstract The cDNA for porcine polypyrimidine tract-binding protein (sPTB) was cloned. The sPTB amino acid sequence is highly homologous to the human PTB sequence (97% identity), and the sPTB sequence corresponds to that of the longest human PTB, PTB4. The specificity of binding in the UV-crosslink of sPTB to the internal ribosome entry site (IRES) of foot-and-mouth-disease virus (FMDV) is similar to that of human PTB. Purified recombinant sPTB efficiently stimulates internal translation initiation directed by the FMDV IRES in a rabbit reticulocyte lysate translation system from which the internal PTB had been depleted.**

*Key words:* Translation; Picornavirus; Foot-and-mouth-disease virus; Polypyrimidine tract-binding protein

## 1. Introduction

The positive-strand RNA of foot-and-mouth-disease virus (FMDV), a picornavirus, encodes a single large polyprotein, which is differentially processed into the final gene products. In contrast to the situation with normal cellular eukaryotic mRNAs, the initiation of translation from picornaviral RNAs is directed cap-independently from an internal section of the 5′-nontranslated region termed the internal ribosome entry site (IRES) [1–3]. Picornaviral IRES elements exhibit highly conserved secondary structures and contain two conserved *cis*-elements at their 3′ borders, an oligopyrimidine tract followed by an AUG triplet (for a review, see [4]).

Two cellular RNA-binding proteins not involved in cellular translation appear to support picornaviral IRES directed translation initiation. The 52 kDa La protein corrects translation initiation directed by the poliovirus IRES [5]. A 57 kDa protein binds to the IRES elements of FMDV [6,7], encephalomyocarditis virus (EMCV) [8], poliovirus [9] and rhinovirus [10]. This p57 is identical to polypyrimidine tract-binding protein (PTB) [9,10], which was initially characterized as a component of splicing complexes [11]. The FMDV IRES contains two separate binding regions for PTB, stem-loop 2 in the 5′ part, and a second PTB-binding site in the 3′ part including the oligopyrimidine tract [6,7]. Mutational analyses have revealed correlations between binding of PTB to the IRES and translational efficiency [7,8,12], and inhibition of translation initiation from the EMCV IRES after adding competitor RNAs to the translation extract could be compensated by PTB [13].

I report in this paper that the PTB protein cloned from swine (sPTB) effectively stimulates internal translation initiation directed by the IRES of FMDV in a translation system from which the internal PTB had been depleted, and thereby directly demonstrate that sPTB is an important component for translation from the FMDV IRES. sPTB is closely related to other PTBs, and the specificity of sPTB binding to the IRES of FMDV is similar to that of the human protein.

## 2. Materials and methods

Plasmid pSP449 [6] contains downstream of an SP6 promoter the complete FMDV $O_1K$ IRES sequence (pos. 363–831). pSP449Δ2 has the predicted IRES stem-loop 2 deleted (pos. 372–425), pSP449Δ3 stem-loop 3 (pos. 429–640), pSP449Δ4 stem-loop 4 (pos. 649–755), and pSP449Δ2,3 and -Δ3,4 contain double deletions [14,15]. pQE-9 was purchased from Qiagen. pD128 contains, in 5′ to 3′ direction, the SP6 promoter, the chloramphenicol-acetyltransferase (CAT) gene, the FMDV IRES (pos. 363–804) with the 11th ATG of FMDV fused to the luciferase gene, and SV40 splice and polyadenylation signals. pD4 was obtained during construction of pD128. It contains a linker flanked by SP6 and T7 promoters. Dicistronic mRNA for in vitro translation was transcribed from pD128 linearized downstream of the polyadenylation signal. Labelled IRES and mutant RNAs were transcribed using 2.5 μM [$\alpha$-$^{32}$P]UTP (400 Ci/mmol) from pSP449 and mutant plasmids linearized downstream of the IRES [14]. RNA Δpy was obtained from pSP449, RNA Δ2,py from pSP449Δ2 and RNA Δ3,4,py from pSP449Δ3,4, each linearized with *Ngo*MI upstream of the oligopyrimidine tract.

For cDNA preparation, porcine liver was crushed in liquid nitrogen, dissolved in GTC buffer (5 M guanidine isothiocyanate, 50 mM Tris-Cl pH 7.5, 10 mM EDTA, 5% 2-mercaptoethanol) at room temperature, homogenized and passed through a 20 gauge needle. Nucleic acids were precipitated with isopropanol and redissolved in GTC buffer. RNA was purified by centrifugation through 5.7 M CsCl, 10 mM EDTA in a Beckman SW40 rotor at 150 000 × *g* at 20°C for 20 h. Poly(A)$^+$ RNA was selected by two passages over oligo(dT)-cellulose columns. An oligo(dT) primer was used for cDNA first strand synthesis, and the RNase H method [16] for the second strand. cDNAs were size-selected on a Sephacryl S-500 column (Pharmacia) and an agarose gel.

Polymerase chain reactions (PCR) were performed with the following primers. PTB-E includes the first 25 nucleotides of the human PTB (hPTB) coding sequence (GCGCGGATCCGCCATGGACG-CATTGTCCCAGATATAGC; PTB sequences are underlined), PTB-D 18 nucleotides of the hPTB C-terminus (GGGGGATC-CTAGATGGTGGACTTGGA(G/A)AA) in antisense orientation. PTB-N includes 3′-nontranslated PTB sequences in antisense orientation (TCTGATTCGTCGACTAAGGTCACTTCAGCTG). PTB-K includes internal sPTB sequences in sense orientation (CGTATGCAGGAGCTGGTTTC, sPTB pos. 920–939), PTB-F in antisense orientation (GTACTTGATGTTGAGGTTGGTGAGC-TTGGA, sPTB pos. 801–772). PTB-M includes PTB sequences immediately preceding the start codon (GAATCAGGAAGCTT-CCCGCGGTCTGCTCTGTGTGCC), PTB-B internal sPTB antisense sequences (AACTGGTTGTTCTTGGT(A/G)AA, sPTB pos. 665–646). Sequencing was performed with these and additional primers.

sPTB was expressed from pQE-9-sPTB as N-terminal $(His)_6$ fusion protein, purified [17] and dialyzed against storage buffer (50 mM Tris-Cl pH 7.5, 5% glycerol, 1 mM DTT). hPTB was expressed as glu-

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*PII* S0014-5793(96)00509-1

tathione S-transferase fusion protein [18] from pGEX-2TKhuPTB (kindly provided by M. Garçia-Blanco, Durham, NC). The glutathione S-transferase moiety was cleaved off with thrombin, removed on a second glutathione sepharose column, and hPTB (plus 13 additional N-terminal amino acids) was collected and dialyzed against storage buffer.

UV crosslinks were performed as described [7]. For depletion of internal PTB from the rabbit reticulocyte lysate (RRL, Promega), 1 ml RRL was adjusted to 250 mM K-acetate and incubated twice with 75 μl poly(U)-sepharose (Pharmacia) for 30 min at 4°C with gentle agitation. In vitro translations included 50% complete or depleted RRL, 135 mM K-acetate and 30 μM amino acids in 10 μl and were supplemented with purified sPTB as indicated. Reactions were incubated with dicistronic mRNA for 60 min at 30°C. Luciferase activity was measured at room temperature in a Berthold Lumat 9501 luminometer (Bad Wildbad, Germany) by injecting 100 μl substrate solution (25 mM glycylglycine pH 7.8, 15 mM $MgSO_4$, 0.1 mM EDTA, 33 mM DTT, 1 mM ATP, 85 mM coenzyme A, 120 mM beetle luciferin) and measuring light intensity for 20 s. CAT activity was measured using [$^{14}$C]acetyl-coenzyme A [19].

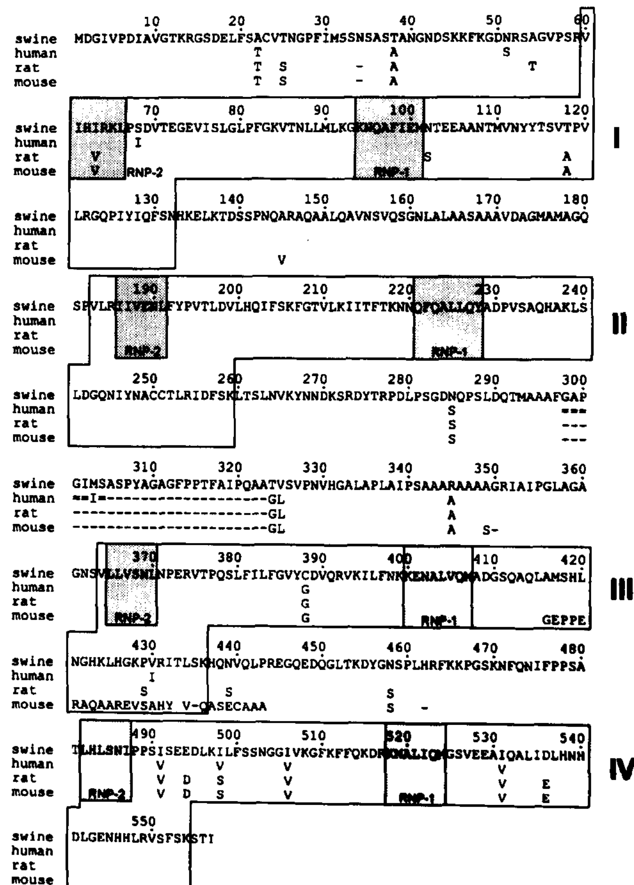

Fig. 1. Amino acid sequence of porcine PTB. The PTB sequences from swine (this study), human [20], rat [21] and mouse [22] were compiled using the HUSAR 'multalign' program (Deutsches Krebsforschungszentrum, Heidelberg). The sPTB sequence is shown completely, changes compared to sPTB for the other PTBs. Gaps are indicated by dashes. For human PTB, three forms are known, the long PTB4 without a gap between pos. 298–323, PTB2 with a gap between pos. 298–304 (double dashes), and the short PTB with a gap between pos. 298–323 (single plus double dashes). In PTB4, pos. 303 is changed to isoleucine compared to sPTB. Large open boxes represent putative RNA recognition motif domains [23] indicated by Roman numerals, and small gray boxes RNP-1 and RNP-2 motifs [24].

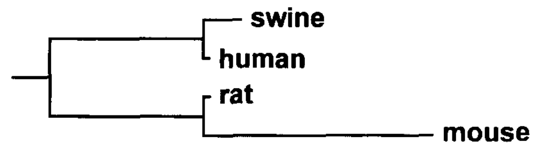


Fig. 2. Evolutionary distances between known PTB amino acid sequences calculated using the HUSAR program 'tree' [27].

## 3. Results

For cloning of the porcine PTB, cDNA was prepared from porcine liver and size-selected for fragments ≥700 bp. The sPTB coding sequence was amplified by PCR using primers PTB-E and PTB-D, which contained the nucleotide sequences corresponding to the N- and C-termini of human PTB, and the resulting sPTB open reading frame with the modified ends was cloned into the *Bam*HI site of pD4. Three independent clones were sequenced. Since the oligonucleotides used for the initial PCR contained human PTB sequences, the authentic termini of the sPTB open reading frame had to be identified. For obtaining the authentic C-terminal sequence, primer PTB-N, which matches to a conserved sequence in the 3′-nontranslated regions of the human, rat and mouse PTB mRNAs, and primer PTB-K were used. For cloning the authentic N-terminal sequence of the sPTB open reading frame, a specific cDNA was prepared from porcine liver poly(A)$^+$ RNA using primer PTB-F for first strand synthesis. The second strand was synthesized using the RNase H method [16], and the cDNA was size-selected for fragments of 500–1200 bp. From this specific cDNA, the authentic N-terminal sequence of the sPTB open reading frame was amplified using primer PTB-M, which corresponds to a conserved sequence directly upstream of the AUGs of the known PTB genes, and PTB-B matching to an internal sPTB sequence. No nucleotide exchanges compared to hPTB were found in the N-terminal sPTB coding sequence, and only two silent nucleotide exchanges in the C-terminal coding sequence covered by primer PTB-E in the initial cloning of sPTB. Thus, these sequences were not replaced prior to expression of sPTB protein.

The derived sPTB amino acid sequence is shown in Fig. 1 compared to the human [20], rat [21] and mouse [22] PTB sequences. The PTB sequences are highly conserved, with a minimum of 91% similarity. sPTB is most closely related to the human PTB4 isoform (97% identity). In several amino acid positions different in any of the genes, the sPTB sequence is identical to that of human PTB but different from both rat and mouse. In other positions, sPTB differs from the human, rat and mouse PTB. There are some exchanges in the RNA recognition motif (RRM) domain I, but no exchanges at all in RRM domain II. In domain III, the mouse PTB sequence is extensively changed in a limited area. In domain IV, there are some conservative exchanges. In a genetic distance tree (Fig. 2), swine and human PTB are in a group separate from rat and mouse PTB, with a certain distance between rat and mouse PTB, mainly due to the extensive changes in mouse PTB domain III.

In order to analyze sPTB binding to the FMDV IRES and the effect of sPTB on translation initiation, the sPTB sequence was cloned into pQE-9, and recombinant sPTB protein was expressed (Fig. 3B, lane 1). The binding of sPTB to the FMDV IRES and different deletion mutant RNAs in the

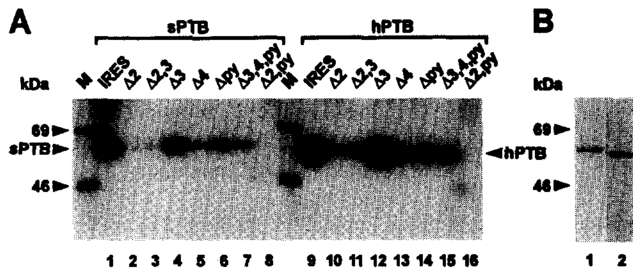


Fig. 3. (A) UV crosslinking experiment with porcine and human PTB. 0.1 μg of sPTB (lanes 1–8) or hPTB (lanes 9–16) were UV-crosslinked to complete or mutant IRES RNA as indicated at the top. Excess RNA was digested with RNase A, the samples boiled in sample buffer and loaded onto a 12.5% SDS gel. M, marker proteins. (B) Coomassie-stained gel with 0.1 μg of purified sPTB (lane 1) and PTB (lane 2).

UV crosslink assay (Fig. 3A, lanes 1–8) is similar to that of human PTB (lanes 9–16). Both proteins are strongly labelled by complete IRES or Δ3 RNA. Deletion of stem-loop 2, the PTB 5′ binding site in the IRES, nearly abolishes sPTB and hPTB binding (Δ2 and Δ2,3). Mutations affecting the 3′ binding site (Δ4, Δpy, Δ3,4,py and Δ2,py) also reduce sPTB and hPTB binding to the IRES.

In order to demonstrate directly that sPTB itself exerts a stimulatory function in FMDV IRES-dependent translation, a system was developed that made it possible to measure the effect of sPTB on translation efficiency. Rabbit reticulocyte lysate is competent for FMDV IRES-driven translation, but it contains considerable amounts of internal PTB [6]. From this RRL, the internal 57 kDa protein corresponding to PTB [6,9] was depleted using poly(U)-sepharose in two cycles to levels below the detection limit of the UV crosslink assay (Fig. 4B, lane 3), while another protein of 80 kDa (corresponding to initiation factor eIF-4B [14]) was less affected. This RRL deficient in internal PTB was used to analyze the effect of added purified recombinant sPTB. The dicistronic reporter mRNA (Fig. 4A) contains the CAT gene monitoring 5′-dependent initiation and the luciferase gene monitoring internal initiation driven by the FMDV IRES placed between the two genes. In complete RRL, reasonable levels of CAT and luciferase were expressed (Fig. 4C, upper panel, lane 1). With incomplete RRL, luciferase expression was considerably reduced, whereas CAT expression remained unchanged (lane 2). 3 ng sPTB caused a stimulation of luciferase expression (lane 3), and increasing amounts of added sPTB further increased the efficiency of internal initiation (lanes 4–6). The lower panel of Fig. 4C shows the ratios of the standardized luciferase expression levels divided by the CAT expression levels. In the depleted RRL, the level of internal initiation is reduced to 27% of the original level (lane 2). With 3 ng sPTB in 10 μl reaction (5 nM, lane 3), the luciferase expression is restored to 54%, with 10 ng sPTB to 88% (17 nM, lane 4), and with 30 ng to 93% (53 nM, lane 5). Although only 84% of the original translation efficiency was obtained in the reaction with 100 ng sPTB, obviously the original level of translation initiation was essentially completely recovered.

In conclusion, the porcine PTB binds to the FMDV IRES like the human PTB, and sPTB stimulates the translation initiation directed by this picornaviral IRES element without the addition of other factors to the translation system from which internal PTB had been depleted.

The nucleotide sequence of sPTB is available in the EMBL, GenBank and DDBJ nucleotide sequence databases under accession number X93009.

## 4. Discussion

In order to investigate the interaction of the FMDV IRES with a PTB protein from a natural host of the virus and its role in the initiation of translation, I analyzed the function of a PTB cloned from swine. The sPTB coding sequence is very similar to the PTB sequences already known. The sPTB protein binds to the FMDV IRES like the human PTB, and, more important, sPTB effectively stimulates translation initiation from the FMDV IRES.

In its overall organization, the sPTB amino acid sequence is

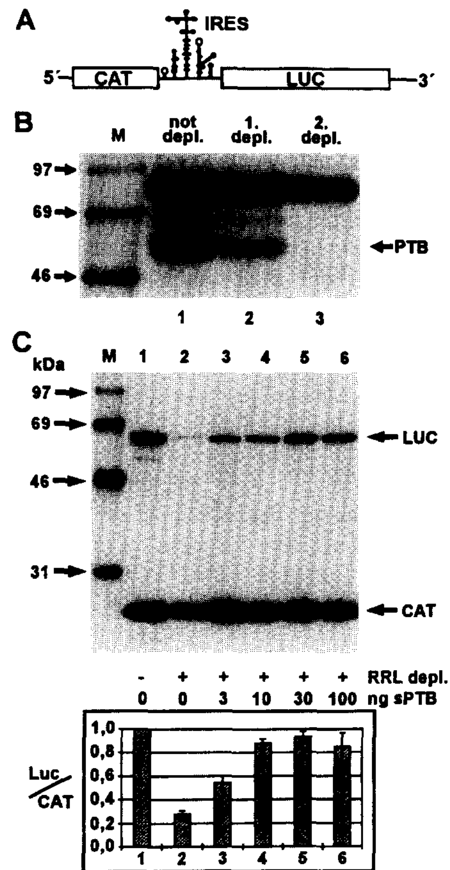


Fig. 4. sPTB stimulates FMDV IRES-dependent translation. (A) Schematic representation of dicistronic mRNA. (B) Depletion of internal PTB from rabbit reticulocyte lysate, checked by UV crosslinks using IRES RNA with each 2 μl of normal RRL (lane 1) and RRL after one (lane 2) and two (lane 3) cycles of poly(U)-sepharose treatment. (C) Upper panel: Stimulation of translation by sPTB. Dicistronic mRNA was translated in normal RRL (lane 1) and in RRL from which internal PTB was depleted (lanes 2–6). Purified recombinant sPTB was added before translation as indicated. The $^{35}$S-labelled translation products were separated on an SDS gel. Lower panel: Summary of results from three experiments. The use of RRL and sPTB in lanes 1–6 is as indicated in the upper panel. Luciferase (LUC) and CAT activity were measured using the corresponding enzyme assays. Readings were standardized setting reaction 1 as 100%. In order to normalize luciferase expression with respect to the translational capacity of the extract, % luciferase expression was divided by % CAT expression. Standard deviations are indicated.

closely related to that of the other species. Particularly, no gross changes are present in the four putative RNA recognition motif domains, and there are no amino acid exchanges in the ribonucleoprotein (RNP) motifs, except for one conservative exchange in the RNP-2 motif of RRM domain I. The four putative RRM domains in PTB had been identified by sequence similarities between PTB and the splicing factor hnRNP L [20,23], and by alignment to an RNA binding domain of the snRNP protein U1A [24]. In the C-terminal part of the mouse PTB RRM domain III, a stretch of 27 amino acids differs widely from the other PTB sequences. This may reflect an individual mutational event that occurred in the mouse PTB gene rather than a large genetic distance between rat and mouse PTB genes, since in many cases of amino acid exchanges elsewhere in the protein the amino acid in question is common to the rat and mouse PTB but different from swine and human PTB. The gap between RRM domains II and III in the rat, mouse and some human PTB sequences is most likely the result of differential splicing of the PTB pre-mRNA that leads to different hPTB isoforms [20,23]. The porcine PTB characterized here corresponds to the longest of these PTB isoforms, PTB4 (97% identity). No evidence was obtained whether sPTB also exists in different forms, but it cannot be excluded that shorter sPTB forms exist. The high level of overall conservation of the PTB sequences indicates that PTB may have some basic function in the cell.

When the function of sPTB was investigated using a dicistronic mRNA expression system, the cloned recombinant sPTB effectively stimulated the initiation of translation directed by the FMDV IRES. By adding increasing amounts of sPTB to a translation system from which the internal PTB had been deleted, the IRES-dependent translation was restored approximately to the original level. Thus, sPTB itself is a cellular factor important for efficient FMDV translation. The stimulation was not dependent on addition of other cofactors that could have been suspected to represent the actual stimulating factors, perhaps acting in a complex with PTB and other proteins. Also the human PTB protein efficiently stimulates FMDV IRES-dependent translation initiation, and the original translation efficiency is completely recovered after addition of PTB (unpublished results), supporting the idea that the PTB proteins are cellular factors important for efficient FMDV translation. These data are in accordance with earlier results that suggested a correlation between PTB binding and translation efficiency [7,8,12], and the finding that inhibition of translation initiation from the EMCV IRES after adding competitor RNAs to the translation extract could be compensated by addition of PTB [13].

PTB was characterized as a component of splicing complexes [11] and found to be involved in splice site selection with some mRNAs, e.g. β-tropomyosin mRNA [25]. Nonetheless, in the picornaviral replication cycle the PTB proteins appear to be used by viral IRES elements as factors stimulating translation initiation. The translational machinery may be essentially the same in cap-dependent and internal initiation, including standard initiation factors like eIF-4B which binds to the FMDV IRES 3′ region [14]. Internal entry of the ribosomal preinitiation complex on the viral RNA is supported by the IRES and additionally recruited host factors, in a way that the preinitiation complex is enabled to encounter the RNA in a 'starting window' at the 3′ border of the IRES [4,26]. The PTB proteins appear to be important components of this specially modified translational machinery.

*Acknowledgements:* I thank Ewald Beck for helpful discussions. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 272).

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