2.6. In vitro transcription and translation

Plasmids were linearized by digestion with XmnI (pNII5′), HpaI (pBSECAT) and NheI (p(M1)T7) and transcribed into RNA by Megascript^{TM} T7 RNA polymerase kit (Ambion). RNAs were treated with DNase I, precipitated with LiCl, and quantitated by the Spectrophotometer DU64 (Beckman).

Synthetic RNAs (pNII5′ RNA; 1.0 pmol, pBSECAT; 1.8 pmol, p(M1)T7; 0.36 pmol and they were optimized for the linear phase in translation activity) were translated in HeLa S10 lysates at 37 °C for 30 min with [^{35}S]-Methionine (ICN), as described previously[5] . Translation products were analyzed using 7.5–15% gradient SDS-PAGE.

2.7. Restoration assay

Purified recombinant PTB, bovine serum albumin and ribosome salt wash (RSW) were dialyzed to S10 dialysis buffer, and added to PTB depleted or non-treated HeLa S10. RSW (total 6.7 ml) was prepared from 6.1 l of HeLa S10, as described previously [5] (kindly supplied by Dr. H. Toyoda).

3. Results

3.1. Fifty-seven and 60 kDa doublet protein bound HCV, EMCV and PV RNA

HeLa cytoplasmic proteins that were detected by UV cross-linking to ^{32}P-UTP labeled RNA derived from the HCV, EMCV, and PV 5′UTR were compared (Fig. 1). Total counts of binding proteins in HCV RNA was five times lower than those of EMCV RNA, and three times lower than those of PV-RNA (PSL; HCV 21536.9, EMCV 105622.8, PV 59307.9). Among these cytoplasmic proteins, 57 and 60 kDa doublet bands on HCV RNA,EMCV RNA and PV RNA have been identified to be PTB (Fig. 1, indicated by asterisk). According to band intensities of the 57 and 60 kDa proteins, PTB bound to EMCV IRES most abundantly, and more diminished amount of PTB bound to PV and HCV IRESs (Fig. 1).

3.2. Identification of P57/60 kDa doublet protein on HCV-RNA as PTB

HCV-IRES-binding proteins with molecular weight of 57/60 kDa were further characterized. The recombinant PTB protein was expressed in *E. coli.* in the presence of IPTG, purified by glutathione sepharose and polyU sepharose column (Fig. 2A), and reacted with affinity purified anti-PTB IgG (Fig. 2B), as described in Section 2. Labeled HCV RNA 5′UTR was cross-linked to HeLa S10 lysate, and immunoprecipitated by affinity purified anti-PTB IgG (Fig. 3). The 57 and 60 kDa doublet bands were specifically reacted to the anti-PTB IgG (Fig. 3, lane HeLa). The recombinant PTB protein was cross-linked with HCV RNA 5′UTR and precipitated with anti-PTB IgG (Fig. 3, lane PTB). These results strongly indicate that PTB

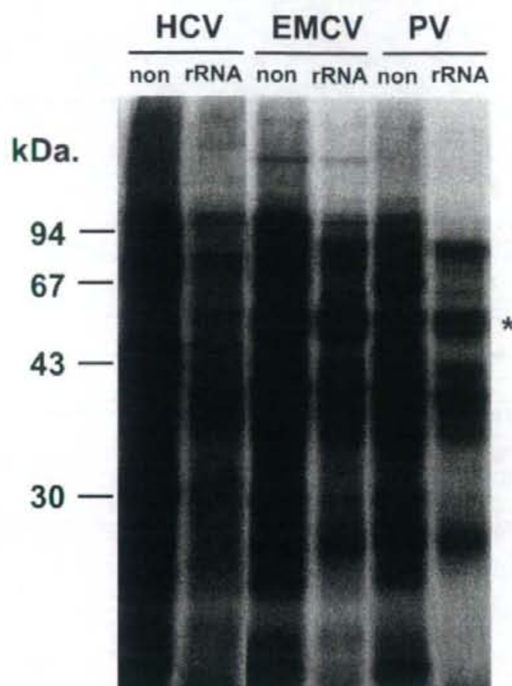

Fig. 1. UV-cross-linking analysis of binding factors to HCV, EMCV and PV-IRES RNAs. Each reaction without competitor indicates "non", and with competitor rRNA indicates rRNA on the top of the lanes. Asterisk indicates the position of PTB proteins. An asterisk indicates PTB binding.

specifically bound to HCV RNA 5′UTR, and observed as doublet protein with molecular weight of 57 and 60 kDa.

3.3. Depletion of PTB in HeLa S10 lysate

Previous results indicated the possibility that other factors than canonical eukaryotic translation initiation factors (eIFs) are working in cap independent translation. PTB is one of the candidates and when the PTB might be commonly used in several kinds of IRESs, it might play the central role in internal initiation. To compare the significance of PTB in translation initiation in HCV and other Picorna virus IRESs, PTB in HeLa S10 lysate was depleted by affinity purified anti-PTB IgG. For the depletion of PTB, pre-coating of Affi-gel protein A beads was necessary to block the non-specific adsorption, as described in Section 2. Pre-coated beads were reacted with anti-PTB IgG. From the preliminary experiments, more than 100 times higher molar ratio of anti-PTB IgG to PTB in S10 lysates was required for the over 90% depletion, as described in Materials and methods. We performed the PTB depletion, and 94.5% of PTB was depleted by anti-PTB IgG and 26.3% of PTB was depleted by pre-immune IgG (Fig. 4). We further examined the effect of PTB

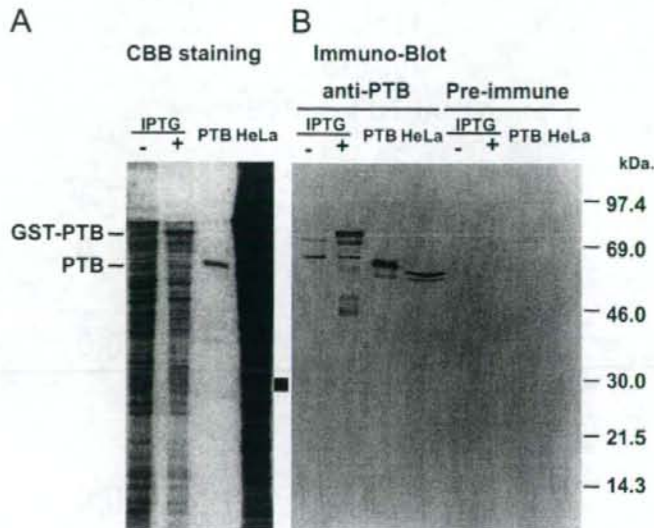

Fig. 2. Expression of recombinant PTB protein fused with GST in *E. coli*: (A) Expression of PTB protein was induced by IPTG, purified by glutathione sepharose column and stained with CBB. (B) Expressed recombinant PTB was transferred to membrane and reacted with specific antibody by WB.

depletion to the binding of cellular factors to three IRESs (Fig. 5). In PTB depleted lysates, binding of 57 and 60 kDa doublet protein was decreased, especially in PV-RNA. However, binding of other factors was not influenced significantly, other than 28 kDa protein (Fig. 5, indicated by an arrow).

3.4. Effect of PTB depletion in translation

Influence of PTB depletion was examined in HCV, EMCV and PV-RNA (Fig. 6A, Table 1). The reaction curves of each RNA were different from each other (data not shown), and the optimum quantity of each RNA used in this study was different from each other (Table 1). From the comparison of translation activity in PTB depleted S10 lysates, translation of PV-RNA was significantly decreased in 4 and 8 µl lysates (22–4.5%, 15–0.9%, Table 1, Fig. 6A). Translation of EMCV-IRES was significantly decreased after PTB depletion (53–44% (4 µl), 28–11% (8 µl)), but this suppression was not as much as PV-IRES. Activity of HCV-IRES was almost similar between pre-immune IgG-treated and anti-PTB IgG-treated S10 lysates. Because the optimal RNA quantities for translation are different in each IRESs, therefore, we calculated the ratio of PTB quantity per template RNA molecules (PTB/RNA) (ng/pmol; Table 1). In PV-IRES, translation activity was significantly reduced after depletion (4.5%, 0.9%) and the PTB/RNA ratio was 1.4 and 0.56. EMCV-IRES and HCV-IRES activity. Influence of PTB depletion to HCV-IRES activity was much lower than those of PV and EMCV.

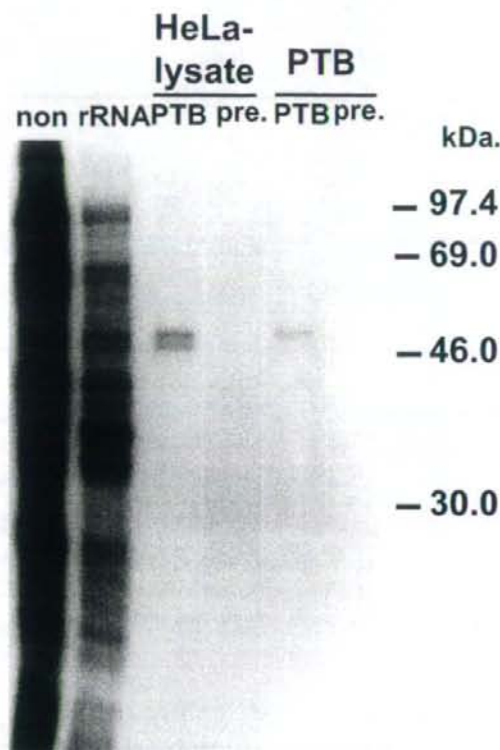

Fig. 3. HCV-IRES cross-linked S10 and PTB was immuno-precipitated by purified anti PTB antibody and pre-immune antibody. The 57 and 60 kDa doublet bands were specifically reacted to the anti-PTB IgG (lane HeLa). The recombinant PTB protein was cross-linked with HCV RNA 5′UTR and precipitated with anti-PTB IgG (lane PTB). Pre-immune antibody did not reacted to both Hela S10 and PTB.

The IRES activity of EMCV and PV-RNA was decreased by treatment of pre-immune IgG, however, treatment of pre-immune IgG did not influence significantly to the IRES activity of HCV-RNA.

3.5. Restoration of PTB to depleted S10

To clarify the effect of immuno-depletion was mainly caused by the decreased quantity of PTB, the purified recombinant PTB or RSW was added to depleted S10 (Fig. 6B). The IRES activity of PV-RNA in depleted S10 lysate (6 μl) was increased by the addition of PTB in dose-dependent manner. The EMCV-IRES activity was recovered even in the presence of 1 μg of PTB in depleted S10 lysate (4.0 μl). When too much quantity of PTB was added to the S10, translation activity of PV, EMCV and HCV decreased (over 10 times of PTB in PV, over 300 times in EMCV and over 500 times in HCV RNA, data not shown).

Translation activity of PV and EMCV-RNA became higher after the addition of RSW to anti-PTB IgG depleted S10 (150% and117%, respectively) (date not

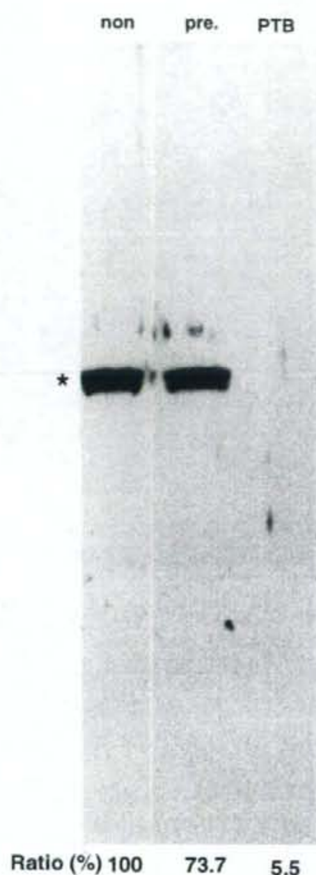

Fig. 4. Depletion of HeLa S10 by pre-immune IgG and affinity purified anti-PTB IgG. They were reacted with anti-PTB antibody by WB. Asterisk indicates the position of PTB proteins. Anti-PTB IgG deplete 94.5% of PTB (lane PTB) and pre-immune IgG deplete 26.3% of PTB (lane pre).

shown). This might indicate the existence of several translation factors other than PTB, which were lost during the treatment of IgG.

Taken together, results of this study strongly indicate that significance of PTB was highest in PV-IRES and was lowest implication in HCV-IRES.

4. Discussion

In present study, the significance of PTB in HCV, EMCV and PV IRESs has been compared. From the immuno-depletion experiment (Table 1), PTB-Ig depleted S10 (4.0 μl) contained 0.009 molecule of PTB per 1 molecule of RNA, in which PV IRES activity is 0.9%. This may indicate that almost one PTB molecule should be required

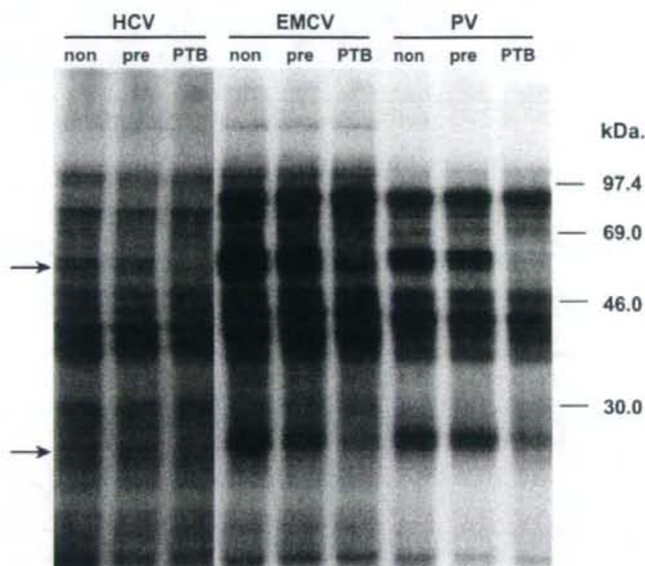

Fig. 5. UV-cross-linking analysis of HCV, EMCV and PV RNA with non-treated, pre-immune IgG-treated, and anti-PTB IgG-treated HeLa S10 lysate (lane non, pre, PTB). Upper arrow indicates the binding of PTB. Doublet protein (57 and 60 kDa) was decreased, especially in PV-RNA. Lower arrow indicates 28 kDa protein.

for 100% activity of PV IRES-RNA. In the case of EMCV IRES-RNA, 0.002 molecule of PTB per RNA gave 11% of EMCV IRES activity, and that of HCV IRES, 0.0025 molecule of PTB gave 31% of HCV IRES activity. Therefore, requirement of PTB for IRES activity was highest in PV, and less in EMCV and HCV IRES-RNA.

From the results in this study, we can compare the requirement amount of PTB in IRES activity with those of canonical eIFs. The most limiting initiation factor in cells is eIF4E, with estimates in rabbit reticulocyte lysates ranging from 0.02 copies [23] to 1 copy [24] per ribosome. The concentration of ribosomes has been estimated to be approximately 2 μM [25]. From the results of *in vitro* translation experiment, PTB should work at 0.1–0.15 M in each IRESs at maximum activity (Table 1). Therefore, working concentration of PTB for IRES activity should show almost similar to those of eIFs.

During the immuno-depletion experiment, treatment of normal IgG conjugated beads decreased the IRESs activity; 89 (6.5 μl) or 33 (3.5 μl)% in HCV IRES, 53 or 28% in EMCV IRES, and 22% or 15% in PV-IRES (Table 1). This may suggest the existence of unknown factors, which could be inactivated during the process of immuno-depletion experiment, and these effects in PV-IRES were highest among the IRESs. PV-IRES is classified into the type I [26], and the canonical eIFs with the exception of cap-binding protein eIF4E [27] and PTB [26], La [26] and 39 kDa poly(rC)-binding protein [26] are working. In EMCV IRES (type II), eIF4G was

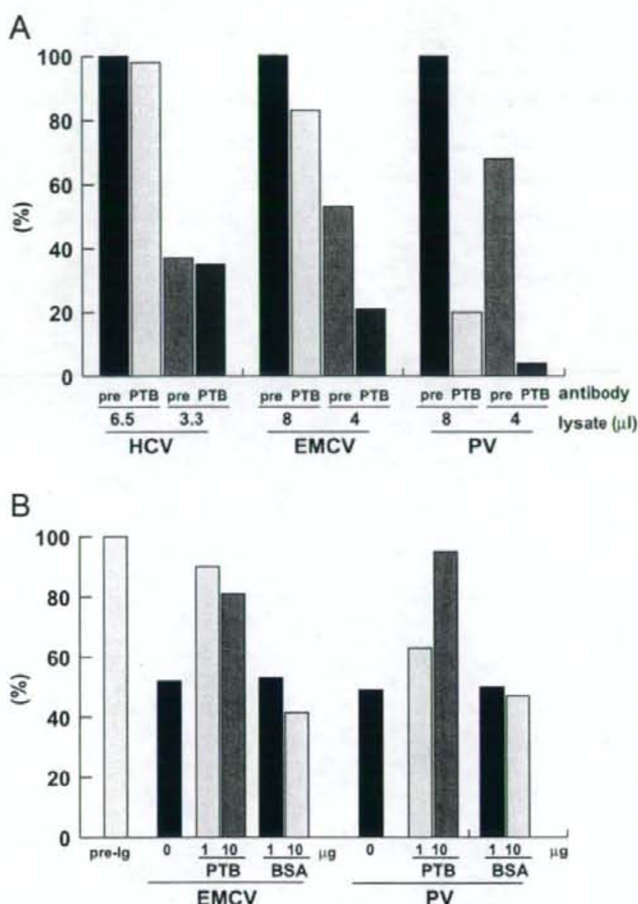

Fig. 6. (A) Effect of PTB depletion in HCV, EMCV and PV IRES. IRESs were translated in pre-immune IgG-depleted and anti-PTB IgG depleted S10 lysates (3.3, 6.5 μl in HCV-IRES, 4.0, 8.0 μl in EMCV- and PV-IRES. Translated products in SDS-PAGE were measured by image analyzer, and the quantity (PSL) of pre-immune IgG-treated S10 lysate was calculated as 100%. (B) Recovery of translation in PTB depleted S10 lysate by addition of recombinant PTB protein (1 and 10 μg. Translated products in SDS-PAGE were measured by image analyzer, and the quantity (PSL) of pre-immune IgG-treated S10 lysate was calculated as 100%.

directly bound and eIF4A and eIF4B can recruit 43S preinitiation complex which is composed of 40S ribosomal subunit and eIF3, eIF2, GTP and initiator tRNA[13]. Recent findings indicated the dependence of EMCV IRES on PTB for activity [28]. The HCV IRES possesses striking difference from type I and II IRESs, it recruits 43S preinitiation complex to initiation codon to form a 48S complex without involvement of eIF4A, 4B or 4F [29]. Thus, HCV IRES is simple and does not require most of eIFs, and might not be influenced by the depletion experiment using normal IgGs.

Table 1
Effect of PTB depletion in HCV, EMCV and PV IRES

RNA	RNA quantity (pmol)	S10 (μl)	PTB (ng)	Molar ratio of PTB to RNA	Ratio of translation (%)[a]
HCV	1.0	*Untreated*			
		6.5	7.2	0.12	100
		3.5	3.6	0.06	63
		Pre-im.-IgG			
		6.5	5.2	0.085	89
		3.5	2.6	0.045	33
		αPTB-IgG			
		6.5	0.4	0.005	87
		3.5	0.2	0.0025	31
EMCV	1.8	*Untreated*			
		8.0	8.8	0.08	100
		4.0	4.4	0.04	57
		Pre-im.-IgG			
		8.0	6.4	0.06	53
		4.0	3.2	0.03	28
		αPTB-IgG			
		8.0	0.5	0.005	44
		4.0	0.2	0.002	11
PV	0.36	*Untreated*			
		8.0	8.8	0.4	100
		4.0	4.4	0.2	65
		Pre-im.-IgG			
		8.0	6.4	0.3	22
		4.0	3.2	0.15	15
		αPTB-IgG			
		8.0	0.5	0.02	4.5
		4.0	0.2	0.009	0.9

[a]Ratio of translation products was quantitated by image analyzer.

Recent riboproteomic approach revealed the novel interacting proteins to IRESs [30], other than PTB, such as actin, forming homolog overexpressed in spleen, and microtuble interacting protein that associates with TRAF3. These factors should be characterized as novel ITAFs and comparative aspects in different IRESs should be addressed in the future work to clarify the character of each IRESs.

Acknowledgments

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