Polypyrimidine tract-binding protein interacts with HnRNP L

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Abstract Polypyrimidine tract-binding protein (PTB) is involved in pre-mRNA splicing and internal ribosomal entry site (IRES)-dependent translation. In order to identify cellular protein(s) interacting with PTB, we performed a yeast two-hybrid screening. Heterogeneous nuclear ribonucleoprotein L (hnRNP L) was identified as a PTB-binding protein. The interaction between PTB and hnRNP L was confirmed in an in vitro binding assay. Both PTB and hnRNP L were found to localize in the nucleoplasm, excepting the nucleoli, in HeLa cells by the green fluorescent protein (GFP)-fused protein detection method. The N-terminal half of PTB (aa 1–329) and most of hnRNP L (aa 141–558) is required for the interaction between PTB and hnRNP L.

Key words: Polypyrimidine tract-binding protein; Heterogeneous nuclear ribonucleoprotein L; Protein-protein interaction

1. Introduction

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are abundant nucleoplasmic pre-mRNA-binding proteins which play important roles in the biogenesis of mRNA [1]. Polypyrimidine tract-binding protein (PTB), also known as hnRNP I, is a member of the hnRNP family [2–4]. PTB has been proposed as a splicing factor, since PTB was identified as a protein binding to the polypyrimidine tracts (Py-tracts) typically found near the 3′ end of introns [3,4]. Recently PTB has been implicated in the repression of the splicing of alternatively spliced introns through its binding to the Py-tracts [5–7].

Furthermore, when a 57 kDa protein was shown to bind specifically to the internal ribosomal entry sites (IRESs) of picornaviruses such as encephalomyocarditis virus (EMCV) [8], poliovirus [9], rhinovirus [10], and foot and mouth disease virus (FMDV) [11] and the IRES of hepatitis C virus [12], it was found to be identical to PTB [13]. The role played by PTB in IRES-dependent translation has been investigated. Mutational analyses have revealed a strong correlation between the binding affinity of PTB to the picornaviral IRESs and the translational efficiency of the corresponding mRNAs [8,11,14]. Depletion of endogenous PTB from HeLa cell lysates and rabbit reticulocyte lysates (RRL) selectively prevented the EMCV IRES-dependent translation [13]. Moreover, addition of recombinant PTB to PTB-depleted RRL restored the translation initiation directed by EMCV or FMDV IRES [15,16]. These results suggest that PTB plays an important role in the internal initiation of translation by binding to IRESs. Since PTB shuttles between the nucleus and the cytoplasm in a transcription-linked manner [17], it is possible that PTB may regulate alternative splicing in the nucleus and enhance translation via IRESs in the cytoplasm.

Three isoforms of PTB have been reported [2–4]. The prototype of PTB (PTB1) consists of 531 amino acids and has a molecular weight of 57 kDa. PTB2 and hnRNP I have insertions of 19 and 26 amino acids, respectively, after the 291st amino acid of PTB1. Four loosely conserved RNA recognition motifs (RRMs) are distributed throughout the PTB molecule (Fig. 1). Recently, the biochemical properties of PTB have been investigated [18–20]. The N-terminal region of PTB (aa 1–169), which contains RRM 1, was proposed to be a domain that responds to unknown cellular factor(s) in HeLa cells that enhance RNA binding of PTB [18]. A nuclear localization signal was found within 60 amino acids of the N-terminus of PTB [20]. RRM 2 plays a major role in the oligomerization of PTB. RRM 3 and RRM 4 play key roles in RNA-binding [18,19].

Here we report a cellular protein (hnRNP L) interacting with PTB. The PTB-hnRNP L interaction was first identified when using the yeast two-hybrid screening system [21] and then confirmed by biochemical methods. Moreover, both PTB and hnRNP L were shown to be localized in the nucleoplasm but excluded from the nucleoli in mammalian cells using the green fluorescent protein (GFP)-fused protein detection method. The domains responsible for the PTB-hnRNP L interaction were also determined.

2. Materials and methods

2.1. Construction of plasmids

Plasmid pHCL3, containing the cDNA encoding the full length of hnRNP L, was kindly supplied by Dr. Maurice Swanson (University of Florida, Gainesville, FL). pSK/hnRNP L was constructed by inserting a *Pvu*II-*Eco*RI DNA fragment of pHCL3 into a pSK(−) vector treated with *Sma*I and *Eco*RI. To construct pTM/hnRNP L, a polymerase chain reaction (PCR) was performed to amplify a cDNA fragment (nucleotides 1–97) of hnRNP L using two primers: ACTGAATTCACATGGTGAAGATGGCGGCG and GTTGTCAGTCTTGAG. *Eco*RI- and *Pst*I-digested pTM1, *Eco*RI- and *Eag*I-digested PCR product, and *Eag*I- and *Pst*I-digested pSK/hnRNP L were ligated to generate plasmid pTM/hnRNP L. For the yeast two-hybrid system, plasmids pGBT9 and pGAD424 were used as sources of DNA-binding (DB) and transcription activation (Ac) domains, respectively. To prepare the insert encoding the full-length hnRNP L, plasmid pTM/hnRNP L was digested with *Eco*RI, filled in with Klenow fragment, and then digested with *Pst*I. Vectors pGBT9 and pGAD424 were digested with *Sma*I and *Pst*I. The vector fragments and the hnRNP L insert were ligated to form pGBT9/hnRNP L(1–558) and pGAD424/hnRNP L(1–558). Deletion mutants of hnRNP L were made from pGAD424, pSK/hnRNP L, and pTM/hnRNP L in order to determine the domain responsible for protein-protein interaction. pGAD424/hnRNP L(24–558), pGAD424/hnRNP L(141–558), and pGAD424/hnRNP L(294–558) were constructed by inserting DNA fragments of pSK/hnRNP L treated with *Eag*I-Klenow-*Pst*I, *Hinc*II-*Pst*I, and *Kpn*I-T4 polymerase-*Pst*I, respectively, into the pGAD424 vector treated with *Bam*HI-Klenow-*Pst*I. pGAD424/

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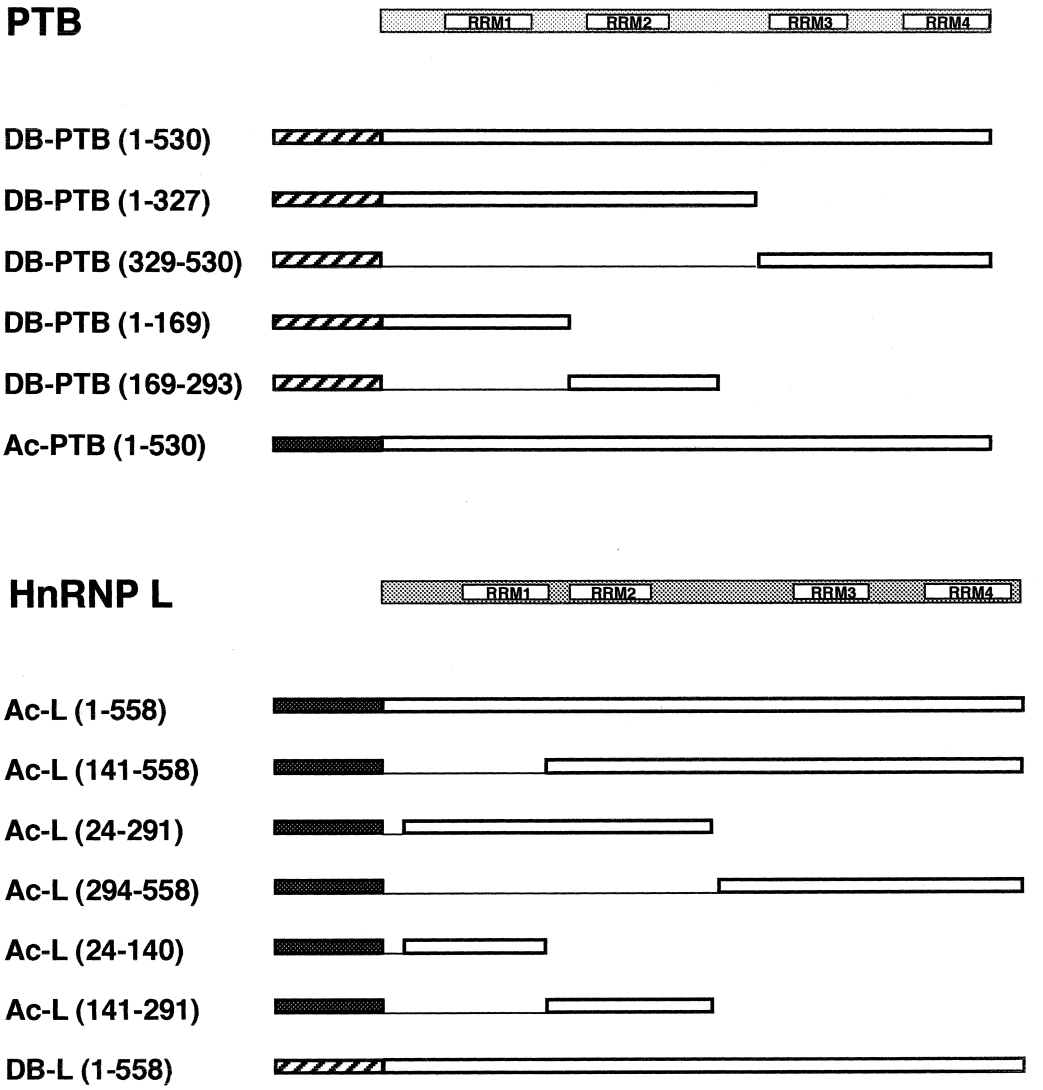

Fig. 1. Schematic diagram of PTB and hnRNP L and their derivatives used in the yeast two-hybrid system. Plasmids pGBT9 and pGAD424 were used as sources of DNA-binding (DB: hatched bars) domains and transcription activation (Ac: solid bars) domains, respectively. Deletion mutants of PTB were fused to the DNA-binding domain, and deletion mutants of hnRNP L were fused to the transcription activation domain. The RNA recognition motifs in PTB and hnRNP L are marked as RRM 1–4.

hnRNP L(24–291) and pGAD424/hnRNP L(24–140) were constructed by inserting DNA fragments of pSK/hnRNP L treated with *Kpn*I-T4 polymerase-*Eag*I and *Eag*I-*Hin*cII, respectively, into the pGAD424/hnRNP L(24–558) vector treated with *Pst*I-T4 polymerase-*Eag*I. To construct pGAD424/hnRNP L(141–291), pGAD424 vector treated with *Bam*HI-Klenow was ligated with pSK/hnRNP L treated with *Kpn*I-T4 polymerase-*Hin*cII. The construction procedures for plasmids pT7-7/PTB(1–530), pGAD424/PTB(1–530), pGBT9/PTB(1–530), pGBT9/PTB(1–327), pGBT9/PTB(1–169), pGBT9/PTB(169–293), and pGBT9/PTB(329–530) have been described elsewhere [17]. To construct pGEX/PTB, a ligation was performed using pGEX-KG vector and pT7-7/PTB(1–530) treated with *Sal*I-*Eco*RI. For the construction of GFP-fused proteins, pEGFP-C1 (Clontech) was used as vector. In order to construct pEGFP/PTB, pEGFP-C1 treated with *Bgl*II-Klenow-*Sal*I was ligated with pT7-7/PTB(1–530) treated with *Nde*I-Klenow-*Sal*I. pEGFP/hnRNP L was constructed by ligation of pEGFP-C1 treated with *Bgl*II-Klenow-*Pst*I and pTM/hnRNP L treated with *Eco*RI-Klenow-*Pst*I.

2.2. Yeast two-hybrid system

A yeast genetic system called 'two-hybrid screening' to identify protein(s) that can interact with PTB was used according to the manufacturer's protocol. Briefly, the yeast strain HF7c was first transformed with pGBT9/PTB and then with a rat brain cDNA library (Clontech). Transformants which grew on SD synthetic medium lacking leucine, tryptophan, and histidine were transferred to filter paper on which they were tested for expression of β-galactosidase. Liquid nitrogen was applied to the filter to break open the yeast cells. Then the filter was laid on top of a second filter paper presoaked with Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$) containing 0.82 mM 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The filters were kept at 30°C while they were monitored for color change indicating β-galactosidase activity. This yeast two-hybrid system was also used to confirm the PTB-hnRNP L interaction and to determine the domains responsible for PTB-hnRNP L interaction.

2.3. In vitro binding studies

From the plasmids pGEX-KG and pGEX/PTB, GST protein and GST-PTB fusion protein were expressed in *Escherichia coli*. ^{35}S-Labeled proteins were synthesized in a rabbit reticulocyte lysate in vitro translation system as described by the manufacturer. ^{35}S-Labeled proteins were incubated with GST or GST-fusion proteins bound to glutathione Sepharose 4B resin (Pharmacia) in 1 ml of binding buffer (20 mM Tris (pH 8.0), 300 mM NaCl, 1 mM EDTA, and 0.25% NP-40) containing 25 μg of RNase A for 2 h. Samples were washed four

times with 1 ml of the binding buffer, and the resin-bound proteins were resolved on a 12% SDS-polyacrylamide gel.

2.4. Fluorescence microscope analysis

HeLa cells were maintained in DMEM supplemented with 10% bovine calf serum. For fluorescence analysis, transfection was performed at 80% confluence on autoclaved coverslips in 35-mm culture dishes employing the electroporation method as described previously [22]. 20 μg of plasmid DNA was used for each transfection. Two days post-transfection at 32°C, the cells were washed three times with phosphate buffered saline (PBS) and fixed with 3.7% (w/v) paraformaldehyde (Sigma) on the coverslips at room temperature for 20 min. To stain the DNA in the nucleus, the cells were treated with 0.1 mg/ml of Hoechst 33258 solution (Sigma) for 2 min. The cells on coverslips were placed on a glass slide and sealed with transparent nail polish. They were then examined with a fluorescence microscope, equipped with an FITC filter set for fluorescence detection. For detection of the nuclear staining by Hoechst 33258, a DAPI filter set was used. Photographs were taken with Kodak 400 color film.

3. Results

3.1. Identification of cellular proteins interacting with PTB

In order to identify PTB-binding proteins, yeast two-hybrid screening was performed using PTB as the 'bait' and a rat brain cDNA library as the 'prey'. Two positive clones were obtained and sequenced. One of them was rat PTB. This supports the conclusion that PTB exists in oligomeric form as well as monomeric form [18]. The second clone turned out to encode rat hnRNP L protein which is homologous to PTB. The interaction between PTB and hnRNP L was then further confirmed using the full-length hnRNP L clone and its derivatives. As shown in Fig. 1, full-length and truncated PTB cDNAs were fused to the GAL4 DNA-binding domain, and full-length and truncated hnRNP L cDNAs were fused to the GAL4 transcription activation domain. Full-length PTB and hnRNP L cDNAs fused separately to the GAL4 transcription activation domain and the GAL4 DNA-binding domain, respectively, were also constructed.

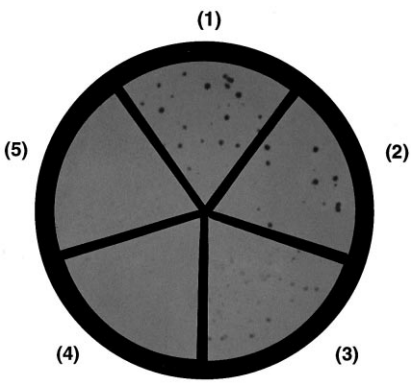

Fig. 2. In vivo analysis of PTB-hnRNP L interaction using the yeast two-hybrid system. The upper panel shows β-galactosidase activities in the yeast cells containing the plasmids shown in the lower panel. The β-galactosidase activity was tested by filter paper assay. Dots indicate yeast colonies with β-galactosidase activity.

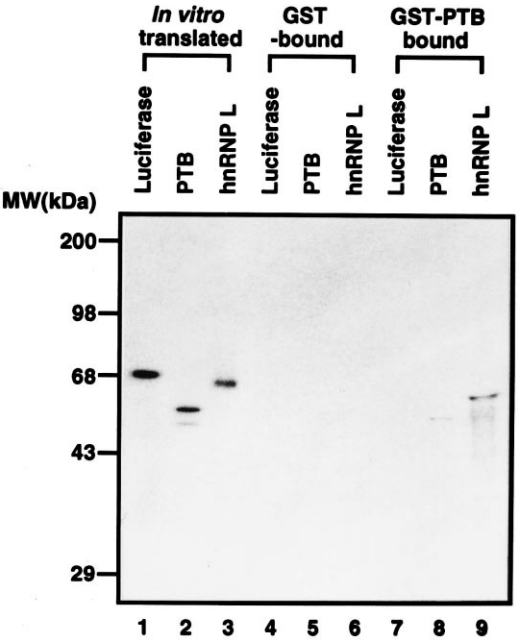

Fig. 3. In vitro analysis of PTB-hnRNP L interaction. The in vitro translated proteins luciferase, PTB, and hnRNP L are shown in lanes 1, 2, and 3, respectively. These ^{35}S-labeled proteins were incubated with resin-bound GST (lanes 4, 5, and 6) or GST-PTB (lanes 7, 8, and 9). After washing the samples with the binding buffer, the resin-bound proteins were resolved on a 12% SDS-polyacrylamide gel.

Since PTB can form multimers [17], yeast cells expressing two fusion proteins of full-length PTB showed β-galactosidase activity (Fig. 2, filter 1). When fusion proteins of full-length hnRNP L and PTB were expressed in yeast cells, β-galactosidase activity was detected regardless of the fused domains (Fig. 2, filters 2 and 3). On the other hand, hnRNP L did not induce β-galactosidase activity in cells that contained only either the GAL4 DNA-binding domain (Fig. 2, filter 4) or the GAL4 transcription activation domain (Fig. 2, filter 5). The data therefore suggest that PTB specifically interacted with the human hnRNP L in the yeast cells.

To confirm the hnRNP L-PTB interaction, a direct in vitro binding assay was carried out using the GST-PTB fusion protein and in vitro translated hnRNP L as shown in Fig. 3. Both PTB and hnRNP L proteins coprecipitated with GST-PTB (Fig. 3, lanes 8 and 9). On the other hand, these proteins could not be precipitated by GST alone (Fig. 3, lanes 5 and 6). Luciferase, used as a negative control, did not bind to either GST-PTB or GST by itself (Fig. 3, lanes 4 and 7). To exclude the possibility that binding of PTB to hnRNP L is mediated by RNA-protein interaction, which could have been possible if the protein source was contaminated with RNA, RNase A was included in all reaction mixtures. The in vitro results, therefore, indicate that the binding of PTB to hnRNP L is by direct protein-protein interaction.

3.2. Both PTB and hnRNP L are localized in the nucleus but excluded from the nucleolus

The GFP-fusion technique was used to study the subcellular localization of PTB and hnRNP L in mammalian cells. Since

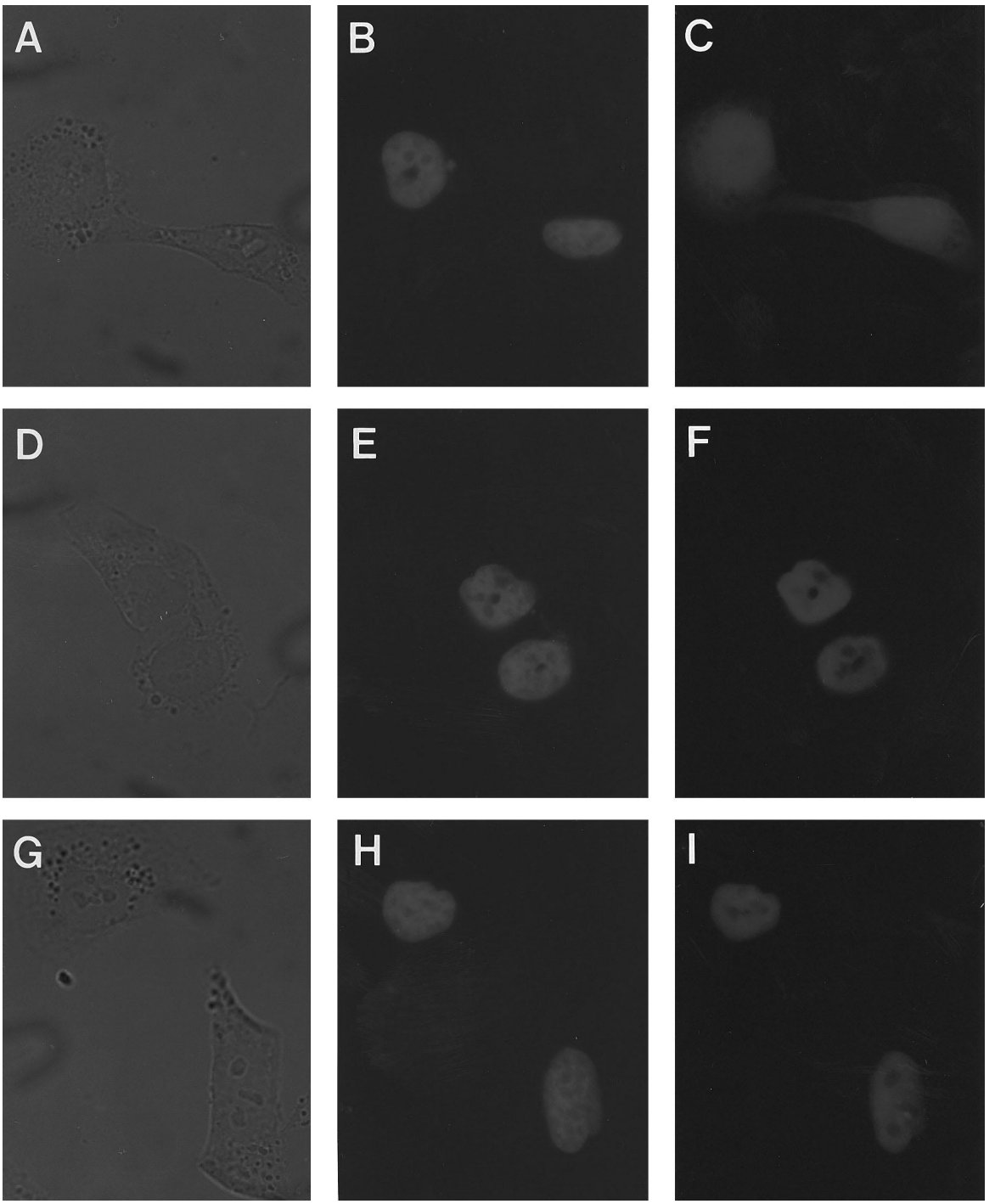

Fig. 4. Subcellular localization of PTB and hnRNP L. HeLa cells transfected with plasmids were observed with a light (A, D, and G) or a fluorescence (B, C, E, F, H, and I) microscope. The Hoechst 33258-stained images are also shown in panels B, E, and H. Panels C, F, and I show the distribution patterns of GFP by itself, GFP-PTB, and GFP-hnRNP L, respectively.

GFP is stable and less photobleaching and since it follows the localization signal on the protein it is fused to, it has been used frequently as a tag in localizing proteins in living cells [23,24]. HeLa cells were transfected with the appropriate plasmid DNAs and examined through a fluorescence microscope as described in Section 2. As seen in Fig. 4, the GFP protein by itself is distributed throughout the cell rather evenly in the cytoplasm as well as the nucleus (Fig. 4C). On the other hand, both GFP-fused PTB and GFP-fused hnRNP L are localized distinctly in the nucleoplasm with the exclusion of the nucleoli (Fig. 4F and 4I, respectively). The similar distribution pattern of PTB and hnRNP L which we observed differs from previous reports which observed a punctate pattern in the nucleus when using indirect immunohistochemical methods [2,25]. The discrepancy may be due to the difference in methods used in determining subcellular localization of proteins. The colocal-

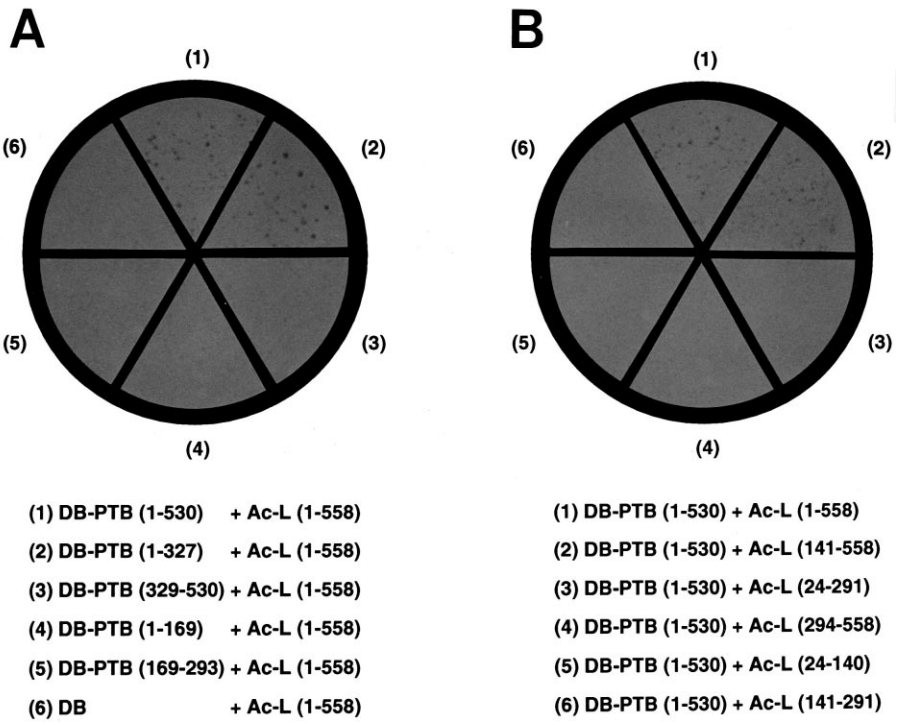

Fig. 5. Determination of the protein-protein interacting domains in PTB and hnRNP L. A series of PTB deletion mutants (A) and hnRNP L deletion mutants (B) were tested for their ability to interact with each other using the yeast two-hybrid system. The β-galactosidase activity was tested by filter paper assay. Dots indicate yeast colonies with β-galactosidase activity.

ization of PTB and hnRNP L would suggest possible complex formation between PTB and hnRNP L in the nucleus of mammalian cells.

3.3. *Determination of the protein-protein interaction domains in PTB and hnRNP L*

In order to determine the PTB domain required for interaction with hnRNP L, a series of PTB deletion mutants were tested for their ability to interact with hnRNP L using the two-hybrid system. As shown in Fig. 5A, DB-PTB(1–327) and bigger PTB constructs exhibited β-galactosidase activity. This indicates that the amino acid residues 1–329 of PTB are sufficient for the interaction with hnRNP L. Likewise a series of hnRNP L deletion mutants were constructed and tested for their binding ability to PTB. As shown in Fig. 5B, most of the hnRNP L protein (aa 141–558) was required for the interaction with PTB. When two polypeptides, DB-PTB(1–329) and Ac-hnRNP L(141–558) were coexpressed in the yeast cell, β-galactosidase activity was detected (data not shown). The results thus indicate that the N-terminal half of PTB (aa 1–329) and most of the hnRNP L (aa 141–558) are involved in the interaction between PTB and hnRNP L.

4. Discussion

Two independent functions have been suggested for PTB, one in the regulation of alternative splicing which occurs in the nucleus and another in the regulation of IRES-dependent translation which is carried out in the cytoplasm. These PTB actions are likely to be accomplished with the assistance of other cellular factors. In order to understand the molecular mechanisms of the PTB activities, we searched for cellular factors interacting with PTB using the yeast two-hybrid system. The genes for PTB and hnRNP L were isolated by screening about 3 million yeast colonies. Cloning of the PTB gene confirmed PTB-PTB interaction as shown by Oh et al. [18]. The PTB-hnRNP L interaction was newly discovered and confirmed by biochemical techniques in this study.

HnRNP L is a 68 kDa protein which belongs to the hnRNP family [25]. Sequence comparison revealed that human hnRNP L shares significant homology (55% similarity, 29% identity) with PTB. Moreover, both PTB and hnRNP L contain four loosely conserved RRMs in which sequence identity reaches 32%. However, the p*I* values of PTB and hnRNP L are quite different. PTB is a basic protein (p*I* 9.95), while hnRNP L is a nearly neutral protein (p*I* 6.71). HnRNP L was shown to bind to a *cis*-acting RNA sequence element that enhances intron-independent gene expression [26]. The binding of hnRNP L to the element resulted in accumulation of the RNA in the cytoplasm. Based on these data, the authors suggested that hnRNP L may facilitate mRNA transport to the cytoplasm.

Considering our results and reported functions of PTB and hnRNP L, it is possible to speculate about the role for the PTB-hnRNP L interaction. The PTB and hnRNP L may function concertedly in RNA-processing, mRNA transport from the nucleus to the cytoplasm, and/or translation of some mRNAs. Neither hnRNP L's involvement in pre-mRNA splicing and translation nor PTB's role in mRNA transport has been reported so far. The PTB-hnRNP L interaction may link the pre-mRNA maturation process with the translation of processed mRNA. Alternatively, a PTB-hnRNP L complex may have an RNA-binding specificity different from PTB and/or hnRNP L alone and thus perform a completely different function. Investigations into the roles of PTB, hnRNP L, and the PTB-hnRNP L complex should shed light

on the mechanisms of the consecutive processes of pre-mRNA splicing, mRNA transportation, and IRES-dependent translation.

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