doi:10.1006/jmbi.2000.4179 available online at http://www.idealibrary.com on IDEAL® J. Mol. Biol. (2000) 304, 119–133

JMB

Polypyrimidine Tract-binding Protein Inhibits Translation of Bip mRNA

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Translation initiation of human Bip mRNA is directed by an internal ribosomal entry site (IRES) located in the 5′ non-translated region. No *trans*-acting factor possibly involved in this process has as of yet been identified. For the encephalomyocarditis virus and other picornaviruses, polypyrimidine tract-binding protein (PTB) has been found to enhance the translation through IRES elements, probably by interaction with the IRES structure. Here, we report that PTB specifically binds to the central region (nt 50-117) of the Bip 5′ non-translated region. Addition of purified PTB to rabbit reticulocyte lysate and overexpression of PTB in Cos-7 cells selectively inhibited Bip IRES-dependent translation. On the other hand, depletion of endogenous PTB or addition of an RNA interacting with PTB enhanced the translational initiation directed by Bip IRES. These suggest that PTB can either enhance or inhibit IRES-dependent translation depending on mRNAs.

Keywords: translation; Bip; IRES; PTB

Introduction

Translation of the majority of eukaryotic messenger RNAs is initiated through a cap and 5′ end-dependent mechanism termed ''ribosome scanning'' (Kozak, 1989). Binding of the small ribosomal subunit is mediated by interaction of a cap-binding protein complex to the methylated cap-structure at the 5′ end of an mRNA. Translation of some mRNAs, on the other hand, occurs in a cap and 5′ end-independent manner through an internal segment in the 5′ non-translated region (5′NTR). This RNA element has been termed ''internal ribosomal entry site'' (IRES) (Jang *et al.*, 1988, 1989; Jang & Wimmer, 1990). IRES elements were for the first time discovered on the mRNAs of the encephalomyocarditis virus (EMCV) and the poliovirus, both of which belong to the family Picornaviridae, which have long, highly structured 5′NTRs but lack a methylated cap structure at the 5′ end of their mRNAs (Jang *et al.*, 1988; Pelletier *et al.*, 1988). Other picornaviral mRNAs and hepatitis C mRNA were also found to contain IRES elements (Jang *et al.*, 1990; Jackson *et al.*, 1994, 1995).

Several cellular mRNAs also contain IRES elements. Human immunoglobulin heavy chain binding protein (Bip/GRP78) was the first cellular mRNA reported to harbor an IRES (Macejak & Sarnow, 1991). The latest list of IRES-containing cellular mRNAs includes human fibroblast growth factor 2 (FGF2/b-FGF; Vagner *et al.*, 1995), proto-oncogene c-myc (Nanbru *et al.*, 1997; Stoneley *et al.*, 1998), insulin-like growth factor II (IGF-II; Teerink *et al.*, 1995), platelet-derived growth factor 2 (PDGF2/*c-sis*; Jeanne *et al.*, 1997), *Drosophila antennapedia* and *ultrabithorax* (Oh *et al.*, 1992), the yeast transcription activators TFIID and HAP4 (Iizuka *et al.*, 1994), and the eukaryotic translation initiation factor eIF4G (Gan & Rhoads, 1996; Gan *et al.*, 1998).

Bip protein has been identified as an immunoglobulin heavy chain binding protein (Haas & Wabl, 1983; Bole *et al.*, 1986) that also binds transiently to a variety of nascent, wild-type secretory and transmembrane proteins and permanently to misfolded proteins that accumulate within the endoplasmic reticulum (ER). Suggested functions

Abbreviations used: EMCV, encephalomyocarditis virus; PTB, polypyrimidine tract-binding protein; Bip, immunoglobulin heavy chain binding protein; IRES, internal ribosomal entry site; RRL, rabbit reticulocyte lysate; hnRNP, heterogeneous nuclear ribonucleoprotein; 5′NTR, 5′ non-translated region; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; HSP70, heat-shock protein 70; ICS, intercistronic site; CAT, chloramphenicol acetyltransferase.

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0022-2836/00/020119–15 $35.00/0

for the Bip protein include the mediation of proper folding, the assembly of nascent proteins in the ER, and the scavenging of misfolded proteins in the ER (Bole *et al*., 1986; Pelham, 1986). Bip protein is also known as glucose-regulated protein 78 (GRP78). It is homologous to heat-shock protein 70 (HSP70; Munro & Pelham, 1986). The expression of Bip is regulated at the transcriptional level. Its synthesis can be induced by a variety of stress conditions such as glucose starvation (Lee *et al*., 1984), treatment with calcium ionophores, calcium-chelating agents such as EGTA (Wu *et al*., 1981; Welch *et al*., 1983; Lee *et al*., 1984), and tunicamycin or glucosamine, compounds that block cellular glycosylation (Olden *et al*., 1979). Bip mRNA is also induced after infection with paramyxovirus (Peluso *et al*., 1978; Stoeckle *et al*., 1988). Furthermore, several reports have suggested that the expression of Bip protein is further regulated at the level of translation (Sarnow,1989; Prostko *et al*., 1991; Ulatowski *et al*., 1993). For example, translation of Bip mRNA becomes enhanced when translation of most host cellular mRNAs is inhibited upon poliovirus infection (Sarnow, 1989).

Even though a number of cellular and viral IRES elements have been identified, little is known about the details of the mechanism of IRES-dependent translation except that some primary sequences and secondary structures of certain IRES elements are deemed to be crucial for efficient translation. It is generally believed that IRES-dependent translation requires most of the same initiation factors that are required for cap-dependent translation (Scheper *et al*., 1992). However, there are some RNA-binding proteins that interact specifically with IRES elements and that are not required for cap-dependent but are for IRES-dependent translation. For example, a 52 kDa protein known as the human La autoantigen has been shown to bind to and enhance translation of poliovirus mRNA in a rabbit reticulocyte lysate (RRL) system (Meerovitch *et al*., 1989, 1993; Svitkin *et al*., 1994). Another cellular protein with an apparent molecular mass of 57 kDa (p57) was shown to specifically interact with IRESs of the encephalomyocarditis virus (EMCV; Jang & Wimmer, 1990; Borovjagin *et al*., 1990, 1991), poliovirus (Hellen *et al*., 1994), and foot-and-mouth-disease virus (FMDV; Luz & Beck, 1991). It has been proposed that the role of p57 is to mediate IRES-dependent translation (Jang & Wimmer, 1990; Hunt & Jackson, 1999; Hunt *et al*., 1999). Depletion of endogenous p57 in HeLa cell lysate and RRL selectively hampered IRES-dependent translation, and addition of purified p57 to a p57-depleted RRL restored translation *via* EMCV and FMDV IRESs (Hellen *et al*., 1993; Kaminski *et al*., 1995; Niepmann, 1996). p57 was shown to be the same molecule as polypyrimidine tract binding protein/heterogeneous nuclear ribonucleoprotein (PTB/hnRNP I), which was assumed to be a splicing factor (Garcia-Blanco *et al*., 1989; Gil *et al*., 1991). Binding of PTB to the pyrimidine tract has recently been shown to control alternative splicing of introns (Garcia-Blanco *et al*., 1989; Gooding *et al*., 1998; Grossman *et al*., 1998; Lou *et al*., 1999; Singh *et al*., 1995; Southby *et al*., 1999). Therefore, it is possible that PTB may regulate alternative splicing in the nucleus and at the same time enhance translation *via* IRES in the cytoplasm. Recently, a protein of 97 kDa was found to specifically interact with the human rhinovirus 5′NTR and to stimulate synergistically HRV IRES-dependent translation together with PTB (Borman *et al*., 1993). On the other hand, no *trans*-acting factor has been reported in connection with cellular IRES functions so far.

Here, we present our investigation of the effect of PTB protein on translation *via* Bip IRES. We found that PTB binds to the central region (nt 50-117) of the Bip 5′NTR. To our surprise, PTB inhibited Bip IRES-dependent translation rather than enhance it, as has been observed for the viral IRESs. This dual role of PTB on IRES-dependent translation is discussed.

Results

Several cellular factors are known to bind to viral IRESs. Among them, PTB bound to all picornavirus IRESs tested (Borman *et al*., 1993; Hellen *et al*., 1994). It has been suggested that PTB enhances cap-independent translation of picornavirus mRNA (Jang & Wimmer, 1990; Hellen *et al*., 1993; Kaminski *et al*., 1995). On the other hand, a role for PTB in the translation of cellular mRNAs containing IRESs has not yet been demonstrated. We investigated the effect of PTB on the translation of Bip mRNA, which contains a cellular IRES element.

The Bip 5′NTR can direct translation *via* internal ribosome binding in a rabbit reticulocyte lysate system

We tested whether the Bip 5′NTR can function as an IRES in an *in vitro* RRL translation system. A capped dicistronic mRNA construct [CATΔ-Bip IRES-CAT] was used as the reporter mRNA. In this construct CAT genes of different lengths were used as reporters (Figure 1(a), CATΔ-BCAT). Since the mRNAs used in this experiment contain a cap structure at their 5′ end, the first cistron (CATΔ) is most likely translated by ribosome scanning. The second cistron (CAT), on the other hand, is likely to be translated by internal initiation directed by the Bip IRES. The translational efficiency of the second cistron (directed by the Bip IRES) was below that of the first cistron directed by ribosome scanning) by a factor of 2.3 (Figure 1(b), lane 2). The effect of a hairpin structure on the translation of the first and the second gene was investigated to test for the possibility that expression of the second cistron (the full-length CAT gene) was

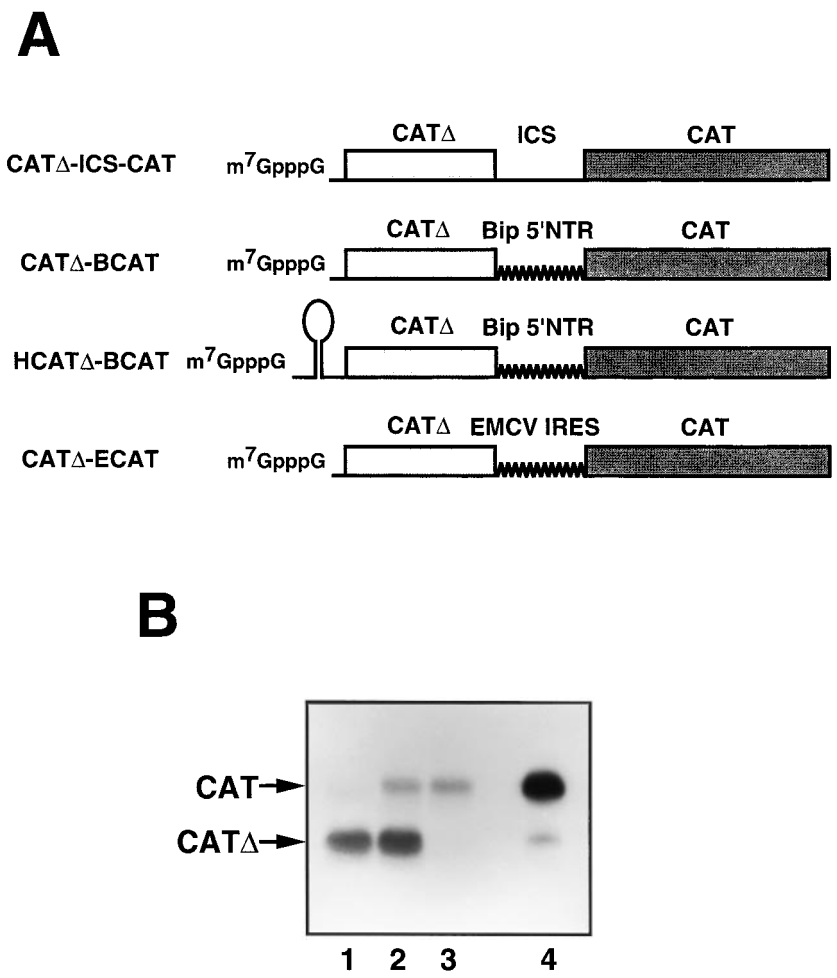
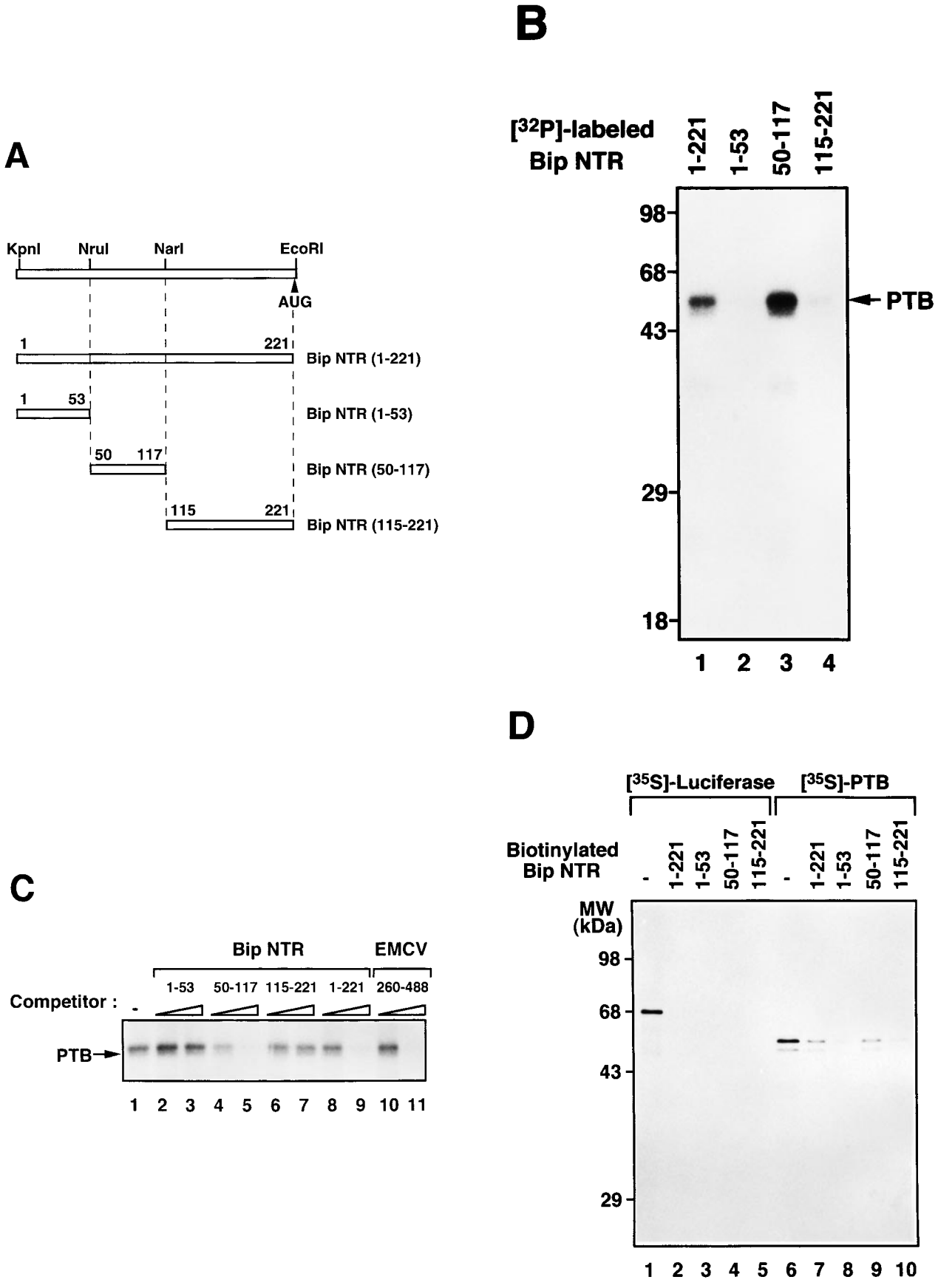


Figure 1. Internal initiation *via* the Bip 5′NTR in the *in vitro* RRL translation system. (a) Schematic diagrams of the dicistronic RNA transcripts used. CAT and CATΔ represent a full-length CAT gene and a CAT gene with a deletion at the C terminus, respectively. The positions of the 7-methyl guanosine cap structure (m^7GpppG), the Bip IRES, and the EMCV IRES are indicated. (b) Translations of the capped dicistronic mRNAs shown in (a) were performed in RRL: CATΔ-ICS-CAT (lane 1), CATΔ-BCAT (lane 2), HCATΔ-BCAT (lane 3), and CATΔ-ECAT (lane 4). The CAT and CATΔ products are indicated by arrows.

directed by leaky scanning or termination-reinitiation. For this purpose, a 78 bp inverted tandem repeat sequence was placed upstream of the first cistron (Figure 1(a), HCATΔ-BCAT). The free energy of the hairpin structure was calculated to be −77.1 kcal/mol by the Mfold program (version 3.0) developed by Zuker & Turner (1999). This bonding is considerably more stable than the −50 kcal/mol that has been shown to be sufficient to stop scanning by the small ribosomal subunit (Kozak, 1986). If the expression of the CAT gene had been due to leaky scanning, suppression of CATΔ translation by the hairpin structure should also inhibit the translation of CAT. On the other hand, if expression of the CAT gene was directed by internal ribosome binding, suppression of CATΔ translation should not affect CAT translation. The hairpin structure upstream of the first cistron (CATΔ) did suppress CATΔ translation but not CAT translation (Figure 1(b), lane 3). Moreover, capped CATΔ-ICS-CAT mRNA, which had no IRES element at the intercistronic site (ICS), showed only very little expression of the second cistron (CAT) (Figure 1(b), lane 1). As an IRES-dependent mRNA control we used the CATΔ-ECAT mRNA (Figure 1(b), lane 4). The results thus indicate that the Bip 5′NTR can indeed support internal ribosome binding and direct *in vitro* translation in the RRL system. Compared with the EMCV IRES, the translational efficiency of the Bip IRES is very poor. This might be due to the limitation of cellular factor(s) in RRL required for Bip IRES-dependent translation.

PTB binds to the central region of the Bip 5′NTR

In order to investigate whether PTB interacts with the Bip 5′NTR, we performed UV cross-linking analyses using purified PTB and ^{32}P-labeled RNA corresponding to Bip NTR sequences (1-221), (1-53), (50-117), and (115-221), which are depicted in Figure 2(a). Whole PTB protein bound strongly to the full-length Bip NTR and to Bip NTR (50-117) (Figure 2(b), lanes 1 and 3). On the other hand, PTB bound only poorly to Bip NTR (1-53) and Bip NTR (115-221) (Figure 2(b), lanes 2 and 4). The smaller bands of about 40 kDa and 25 kDa visible in Figure 2(b) are most likely degradation products of PTB generated during the UV cross-linking process, since polypeptides of these sizes were not



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detected in a Coomassie-stained gel containing purified PTB (data not shown).

The PTB-binding activity of the Bip IRES was also analyzed by competition assay with cold competitor RNAs corresponding to different portions of the Bip 5′NTR (Figure 2(c)). ^{32}P-labeled Bip 5′NTR (1-221) was UV cross-linked to purified PTB in the presence of cold competitor RNA. Addition of a 50-fold molar excess of Bip NTR (50-117), Bip NTR (1-221), and EMCV NTR (260-488) strongly inhibited binding of PTB to the probe (Figure 2(c), lanes 5, 9 and 11). On the other hand, Bip NTR (1-53) and Bip NTR (115-221) showed little competition, if any, for the PTB-RNA interaction at the same competitor RNA concentrations (Figure 2(c), lanes 3 and 7). These observations, therefore, match the UV cross-linking results shown in Figure 2(b).

The PTB/Bip RNA interaction was also confirmed by an RNA affinity resin-binding assay (Figure 2(d)). RNAs of full length or truncated Bip IRES were synthesized in the presence of biotinylated UTP. ^{35}S-labeled luciferase and PTB, synthesized in the *in vitro* RRL translation system, were the target proteins (lanes 1 and 6 in Figure 2(d), respectively). After incubating the *in vitro* translation products with a mixture of the biotinylated RNAs, RNA-binding proteins were precipitated with streptavidin/acrylamide beads and analyzed by SDS-PAGE. The ^{35}S-labeled PTB preferentially precipitated together with the full-length Bip NTR (1-221) and Bip NTR (50-117) as seen in lanes 7 and 9 in Figure 2(d). On the other hand, PTB bound only weakly to Bip NTR (1-53) and (115-221) (Figure 2(d), lanes 8 and 10). The ^{35}S-labeled luciferase did not bind to any of the RNA molecules (Figure 2(d), lanes 2 to 5). These binding affinities of PTB to the Bip 5′NTR correlate well with the UV cross-linking results described above. All the data, therefore, strongly suggest that nucleotides between positions 50 and 117 of the Bip 5′NTR specifically interact with PTB.

PTB selectively inhibits Bip IRES-dependent translation

The role of PTB in Bip IRES-dependent translation was examined in the RRL translation system (Figure 3). A capped dicistronic mRNA (CATΔ/Bip IRES/CAT) was used as the reporter mRNA (Figure 1(a), CATΔ-BCAT). Translation reactions were performed in the presence of various amounts of purified PTB. It is surprising that the translational efficiency of the second cistron, the CAT gene, was drastically inhibited by PTB in a dose-dependent manner (Figure 3(a), lanes 2 to 6). Addition of 1 μg of PTB completely inhibited translation directed by the Bip IRES (Figure 3(a), lane 6), while the translation of the first cistron (CAT) was not affected (Figure 3(a), lane 6). In order to investigate whether the inhibitory effect of PTB on the internal initiation is specific for the Bip IRES, we compared this inhibitory effect of PTB to its effect on the translational efficiency directed by the EMCV IRES, which is known to require PTB for efficient internal initiation. A dicistronic mRNA (CATΔ/EMCV IRES/CAT) composed of a truncated CAT gene in the first cistron, which is translated by ribosome scanning, and a CAT gene in the second cistron, which is translated through EMCV IRES (see CATΔ-ECAT in Figure 1(a)), was translated in an RRL in the presence of various amounts of purified PTB (Figure 3(a), lanes 7 to 11). The data show that the translation directed by the EMCV IRES remained unaffected by the addition of PTB (Figure 3(a), lanes 7 to 11). This result together with previous reports indicates that the endogenous PTB in the RRL is sufficient for the internal initiation at the EMCV IRES because of the high affinity of PTB to the EMCV IRES and that extra PTB does not inhibit EMCV IRES-dependent translation (Jackson, 1986; Jang & Wimmer, 1990). The result, therefore, suggests that the inhibitory effect of PTB is specific for the Bip IRES. When we investigated the effect of truncated PTBs on the Bip IRES-dependent translation, we saw no change in the translation of Bip mRNA (data not shown) after the addition of the PTBs, which were truncated (N-terminal, central, or C-terminal) but still retained the RNA-binding domains (Oh *et al*., 1998). This suggests that almost the entire PTB is needed for the inhibitory effect.

Recently, Svitkin *et al*. (1996) demonstrated that general RNA-binding proteins can inhibit translation of uncapped mRNA, prevent spurious initiation at aberrant translation start sites, and

Figure 2. Determination of the PTB-binding site on the Bip 5′NTR. (a) Diagram of the entire Bip 5′NTR and of the parts used in UV cross-linking assays. The positions of the nucleotides in the 5′NTR are indicated by numbers above the boxes representing each RNA transcript. AUG denotes the initiator AUG codon. (b) UV cross-linking of Bip 5′NTR RNAs with purified PTB. The RNA probes used in the UV cross-linking experiments are indicated on top. The position of PTB is marked by an arrow. (c) Competition for PTB binding by Bip 5′NTR. Competition experiments were carried out by adding cold competitor RNAs in fivefold (lanes 2, 4, 6, 8, and 10) and 50-fold (lanes 3, 5, 7, 9, and 11) molar excess. The competitor RNAs used were Bip NTR (1-53), (50-117), (115-221), (1-221), and EMCV IRES (260-488) as indicated on the top of the panel. ^{32}P-labeled full-length Bip 5′NTR and 1 μg of purified PTB were used as probe and protein source, respectively. Lane 1 shows a control reaction with no competitor. (d) Co-precipitation patterns of PTB and RNA transcripts corresponding to different parts of the Bip 5′NTR. The biotinylated RNAs Bip NTR (1-221), (1-53), (50-117), and (115-221) were used in lanes 2 and 7, 3 and 8, 4 and 9, and 5 and 10, respectively. *In vitro* translation products of luciferase and PTB were loaded as controls in lanes 1 and 6, respectively.

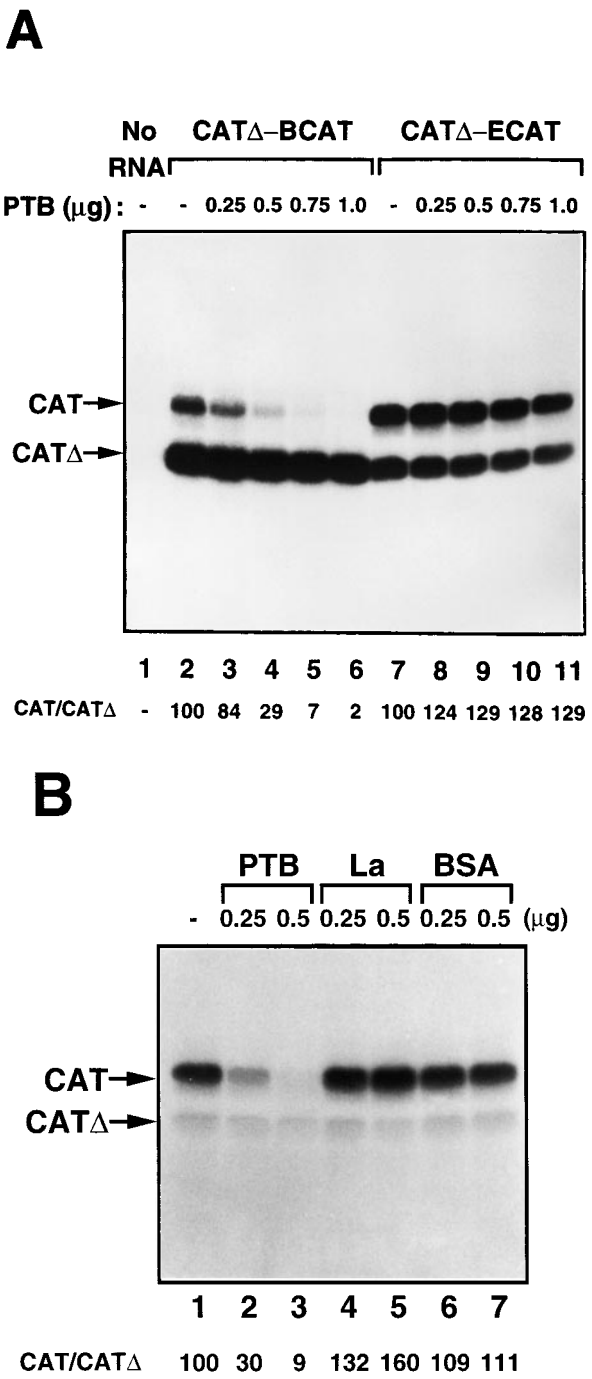

Figure 3. Effect of PTB on the internal initiation by Bip IRES. (a) Effect of PTB on the translation of cap, Bip IRES, and EMCV IRES-dependent mRNA. The RNAs used in the translation reactions were CATΔ-BCAT (lanes 2 to 6) and CATΔ-ECAT (lanes 7 to 11). The amounts of PTB added to the translation reaction mixtures are indicated on the top of the panel. No RNA was added to the reaction mixture in lane 1. (b) Effects of PTB, La, and BSA on Bip IRES-dependent translation. *In vitro* translations were performed using the capped mRNA HCATΔ-BCAT shown in Figure 1(a). The proteins added to the respective reactions were purified PTB (lanes 2 and 3), La (lanes 4 and 5), and BSA (lanes 6 and 7). The amounts of protein added to the reactions are indicated on the top of the panel. No protein was added to the sample loaded in lane 1. The film had to be overexposed in order to detect the CATΔ product. The intensities of the bands were measured using a densitometer. The relative translational efficiencies were normalized setting the relative ratio of the CAT/CATΔ product of the translation reaction containing the dicistronic mRNA (HCATΔ/Bip IRES/CAT) with a stable hairpin structure upstream of the first ORF was used as the reporter mRNA (see HCATΔ-BCAT in Figures 1(a)). Recombinant PTB specifically inhibited the Bip IRES-dependent translation, whereas BSA and La did not affect or even slightly enhance the internal initiation at the Bip IRES, respectively (Figure 3(b)). In case of La, its binding to Bip IRES was confirmed by UV cross-linking assays and co-precipitation with a biotinylated Bip IRES (data not shown). This indicates that the inhibition of the Bip IRES-dependent translation was not a non-specific inhibitory effect of Bip IRES-binding proteins but a specific effect of PTB on the Bip IRES.

Depletion/sequestration of endogenous PTB enhances translation directed by Bip IRES

PTB is not a limiting factor in the RRL (Jackson, 1986; Jang & Wimmer, 1990). About 5 μg of PTB was shown to be present in 1 ml of RRL (Kaminski *et al*., 1995). We investigated, therefore, the effect of endogenous PTB on the Bip IRES-dependent translation by depleting or sequestering the PTB in the RRL.

An RNA segment corresponding to the EMCV IRES (nt 393-488), which contained a strong PTB-binding site (Jang & Wimmer, 1990), was used as a competitor molecule to sequester the endogenous PTB in the RRL (Figure 4(a)). It has been well documented that this EMCV 5′NTR RNA fragment (nt 393-488) can inhibit translation directed by EMCV IRES, and that the inhibition can be reversed by addition of purified PTB (Borovjagin *et al*., 1994). A capped dicistronic mRNA (HCATΔ-BCAT) was used as reporter. The Bip IRES-dependent translation was enhanced upon addition of a 100-fold molar excess of competitor RNA corresponding to the EMCV 5′NTR (393-488) (compare lane 1 with lane 4 in Figure 4(a)). This indicates that the endogenous PTB partially inhibited the Bip IRES-dependent translation. On the other hand, addition of an RNA segment corresponding to the EMCV IRES (nt 422-488), which does not

contain a PTB-binding site (Jang & Wimmer, 1990), at a 100-fold molar excess showed little or no effect on the translation directed by Bip IRES (compare lane 1 with lane 3 in Figure 4(a)). Moreover, addition of RNA transcript from a pSK(−) vector linearized with *Not*I, a non-specific control RNA of equal length, also had no effect on Bip IRES-dependent translation (data not shown). Addition of PTB inhibited the translation directed by Bip IRES (Figure 4(a), lane 2).

The inhibitory activity of endogenous PTB proteins on Bip IRES-dependent translation in an RRL was also examined by PTB depletion from the RRL. Endogenous PTB was separated out using chromatography columns containing biotinylated EMCV (nt 260-488) RNA. A UV cross-linking assay with ^{32}P-labeled full-length EMCV IRES as the RNA probe indicated that the PTB-depleted RRL retained an amount of PTB protein that was less than 1% of that in the original RRL (Figure 4(b)). In the PTB-depleted RRL, translation of EMCV mRNA, which requires PTB for translation, was curtailed by about 60% (compare lane 1 with lane 2 in Figure 4(c)). On the other hand, cap-dependent CATΔ protein translation remained unchanged (Figure 4(c)). Addition of PTB restored translation of the EMCV mRNA (Figure 4(c), lanes 3 and 4). These data together with previous reports indicate that PTB enhances the translation of EMCV mRNA under these conditions (Jang & Wimmer, 1990; Borovjagin *et al*., 1994; Kaminski *et al*., 1995). It is interesting that, and quite opposite, depletion of endogenous PTB enhanced the translation of Bip mRNA by twofold (Figure 4(d), lane 2), while addition of PTB inhibited the translation of Bip mRNA (Figure 4(d), lane 3). More PTB was required to achieve a similar extent of inhibition of Bip mRNA translation in comparison to undepleted RRL (compare lanes 1 and 2 in Figure 3(b) with lanes 1 and 3 in Figure 4(d)). These results strongly suggest that PTB modulates Bip IRES-dependent translation.

Overexpression of PTB inhibits Bip IRES-dependent translation *in vivo*

Finally, the inhibitory effect of PTB on Bip IRES-dependent translation was examined *in vivo* by using a transient expression system. For reporters, three dicistronic vectors were constructed to contain different ICS between the CAT and LUC genes. pECL contains only an ICS as a negative control. pECBL and pECEL contain the Bip IRES or the EMCV IRES, respectively (Figure 5(a)). The reporter plasmids were co-introduced into Cos-7 cells with or without effector vector pEGFP-PTB. CAT and luciferase activities were measured to monitor translation of the reporter genes (see Materials and Methods). Expression of the downstream cistron in the pECBL was stimulated 2.6-fold as compared to pECL containing the negative control ICS only (Figure 5(b), compare lanes 1 and 2). Co-expression of PTB reduced the relative ratio of LUC to CAT 1.6-fold (Figure 5(b), lane 3). On the other hand, co-expression of PTB did not affect the EMCV IRES-dependent translation under the same conditions (Figure 5(b), compare lanes 4 and 5). To monitor the expression levels of PTB and GFP-fused PTB in the transfected cells, Western blot analyses were performed with anti-PTB monoclonal antibody (Figure 5(c)). Densitometric measurement of PTB-related bands showed that the level of GFP-PTB production is lower than that of endogenous PTB by seven- to tenfold. Considering transfection efficiency (10 ∼ 15%), the level of GFP-PTB is similar to that of endogenous PTB. This indicates that the inhibitory effect of PTB *in vivo* is close to that of PTB *in vitro*.

Discussion

The mechanism underlying translation *via* IRES elements is still poorly understood. However, there are several lines of evidence suggesting that RNA-binding proteins facilitate translation initiated through viral IRESs (Meerovitch *et al*., 1989, 1993; Borman *et al*., 1993; Hellen *et al*., 1994; Svitkin *et al*., 1994; Ali & Siddiqui, 1995). Some cellular factors are known to bind to viral IRES elements. La (a human autoantigen; Meerovitch *et al*., 1989, 1993; Svitkin *et al*., 1994) and PTB (Jang & Wimmer, 1990; Borman *et al*., 1993; Hellen *et al*., 1994; Ali & Siddiqui, 1995) are well characterized proteins that enhance IRES-dependent translation of viral mRNAs. However, the effects of La and PTB on different types of IRESs differ (Jang *et al*., 1990; Meerovitch *et al*., 1993). PTB is a member of the heterogeneous nuclear ribonucleoprotein (hnRNP) family shuttling between nucleus and cytoplasm in a transcription-sensitive manner (Michael *et al*., 1995). It has been suggested that PTB has at least two unrelated functions: pre-mRNA maturation and cap-independent translation. Therefore, it is possible that PTB may regulate alternative splicing in the nucleus, while it modulates translation *via* IRES in the cytoplasm. Recently, translational modulatory functions have been reported for other hnRNPs. For example, hnRNP E2 enhances translation directed by the poliovirus IRES (Blyn *et al*., 1997). It has also been reported that translational inhibition of human papillomavirus type 16 L2 mRNA was mediated *in vitro* through interactions with hnRNP K, hnRNP E1, and hnRNP E2 (Collier *et al*., 1998).

Here, we describe a novel function of PTB as a negative regulator of the cap-independent translation of Bip *via* its IRES. PTB binds to the central region (nt 50-117) of the Bip 5′NTR (Figure 2). Addition of purified PTB inhibits translation directed by the Bip IRES (Figure 3) in the RRL system, and the depletion or sequestration of the endogenous PTB in the RRL enhances the internal

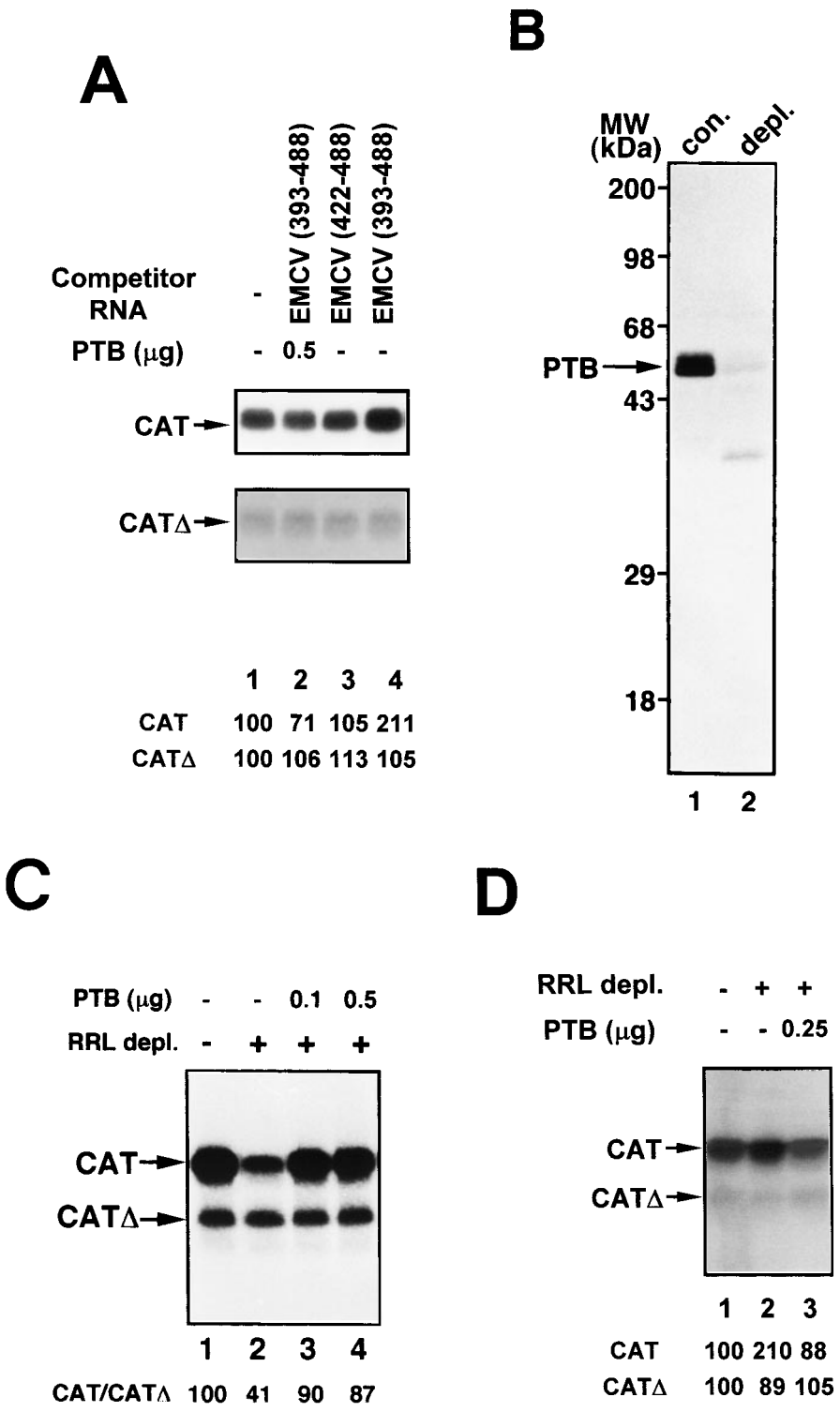

Figure 4 (*legend opposite*)

initiation directed by Bip IRES (Figure 4). It is worth noting that the Bip mRNA was not efficiently translated in HeLa cell extracts, where the concentration of PTB is significantly higher than in reticulocyte lysates (data not shown; Borovjagin *et al*., 1990; Luz & Beck, 1991; Borman *et al*., 1993). Furthermore, overexpression of PTB in mammalian cells reduced internal initiation *via* Bip IRES (Figure 5).

Svitkin *et al*. (1996) demonstrated that general RNA-binding proteins inhibit translation of uncapped mRNA and render translation cap-

dependent in the *in vitro* RRL translation system. Several lines of evidence suggest that the inhibition of Bip mRNA translation by PTB is not the result of a non-specific inhibitory effect comparable to the effects of general RNA-binding proteins. First, Bip mRNA translation occurs in the RRL in an IRES-dependent manner as demonstrated in Figure 1(b). Second, La protein and truncated PTB (329-530), which both bind to RNA (Perez *et al*., 1997; Oh *et al*., 1998), had nevertheless no inhibitory effect on the Bip IRES-dependent translation (data not shown and Figure 3(b)). Also, PTB did not inhibit translation of EMCV mRNA, which contains a different IRES element. These phenomena led us to conclude that PTB specifically inhibits the translation of Bip mRNA.

It is not clear how PTB inhibits the translation of Bip mRNA. It is very likely that the inhibition is mediated by protein-RNA interaction between PTB and the central region (nt 50-117) of the Bip 5′NTR. According to a report by Yang & Sarnow (1997), this region is excluded from the minimal IRES element of Bip mRNA, which is located between nucleotides 129 and 220 of the Bip 5′NTR. Based on these facts, we can come up with some speculations about the inhibitory mechanism of PTB on Bip mRNA translation. The bound PTB might block the binding of a factor essential for Bip IRES function. However, addition of PTB to the RRL did not affect the interaction of the two major Bip 5′NTR-binding proteins with the apparent molecular mass of 46 kDa and 67 kDa in UV cross-linking experiments (data not shown). Still, we cannot rule out the possibility that PTB might block the binding of another essential translational factor which was not detected by the UV cross-linking technique employed. Moreover, the inhibitory effect of PTB is most likely not due to a non-specific prevention of binding of a translation factor to the Bip IRES or due to a contaminant which may have become included during the PTB purification, since the truncated PTBs, which were expressed and purified in a similar way as the full-length PTB, did not retain the inhibitory effect (data not shown). Alternatively, PTB-binding might induce a conformational change in the Bip IRES resulting in an impaired IRES which cannot be recognized by the translational machinery. Finally, PTB by itself or in concert with other protein(s) might actively block the binding of the translational machinery to the Bip IRES. Investigations into the details of the mechanism of the inhibition of Bip mRNA translation by PTB are underway.

We can also speculate on the physiological role of the inhibition of Bip mRNA translation by PTB based on data in previous reports about Bip and PTB. Bip protein is regarded as a key regulator of homeostasis of newly synthesized proteins. Bip expression is induced by a variety of chemicals and conditions that damage proteins, promote protein misfolding, or inhibit protein processing along with other stress responses (Welch, 1987; Lee, 1992; Price *et al*., 1992; Wong *et al*., 1993; Morris *et al*., 1997). The translational expression of PTB, on the other hand, is higher in actively growing cells than in resting cells, like 5′-terminal oligopyrimidine (5′TOP) containing mRNAs (Camacho-Vanegas *et al*., 1997). One possible regulatory mechanism of the reciprocal expression of Bip and PTB may be as follows. Under normal growth conditions, high levels of PTB may down-regulate the expression of Bip protein by blocking the translation of Bip mRNA (Welch, 1987; Lee, 1992; Price *et al*., 1992; Wong *et al*., 1993; Morris *et al*., 1997). Under stressed conditions, on the other hand, a lower level of PTB may increase expression of the Bip protein by relieving Bip mRNA from translational inhibition, thus mediating the proper folding and assembly of misfolded or nascent proteins accumulated in the ER during stress conditions.

It is important to note that PTB can either enhance translation of some mRNAs such as EMCV and rhinovirus or inhibit translations of others such as the Bip mRNA. This inverse effect may provide a specific regulatory mechanism for gene expression under varying conditions. One of the interesting facts about PTB is that it shuttles between the nucleus and the cytoplasm in a cell cycle-dependent manner. The majority of PTB is

Figure 4. Effect of sequestration and depletion of endogenous PTB on Bip-IRES function. (a) Effect of a competitor RNA corresponding to nt 393-488 of EMCV on the translation of Bip mRNA. *In vitro* translations were performed using the capped HCATΔ-BCAT mRNA. Sequestration experiments were carried out by adding RNA corresponding to EMCV IRES (nt 393-488) (lanes 2 and 4) or EMCV IRES (nt 422-488) (lane 3) in a 100-fold molar excess. The amounts of PTB added to the translation reactions are indicated at the top of the panel. Lane 1 shows a control translation reaction with no competitor RNA added. (b) The amount of PTB in the control RRL and the PTB-depleted RRL was checked with the UV cross-linking method: 75 μg of control RRL (lane 1) or of PTB-depleted RRL (lane 2) was UV cross-linked to a ^{32}P-labeled full-length EMCV IRES probe. The position of PTB is marked by an arrow. (c) Effect of PTB depletion on EMCV IRES-dependent translation. The capped CATΔ-ECAT mRNAs were translated in control RRL (lane 1) and PTB-depleted RRL (lanes 2 to 4). The amounts of PTB added to the translation reaction mixtures are indicated on the top of the panel. (d) Effect of PTB depletion on Bip IRES-dependent translation. The capped HCATΔ-BCAT mRNA was translated in control RRL (lane 1) and PTB-depleted RRL (lanes 2 and 3). The intensities of the bands were determined with a densitometer. The relative translational efficiencies were normalized taking the intensity of CAT or CATΔ from the translation mixture containing the dicistronic mRNA without addition or depletion of PTB as 100 %.

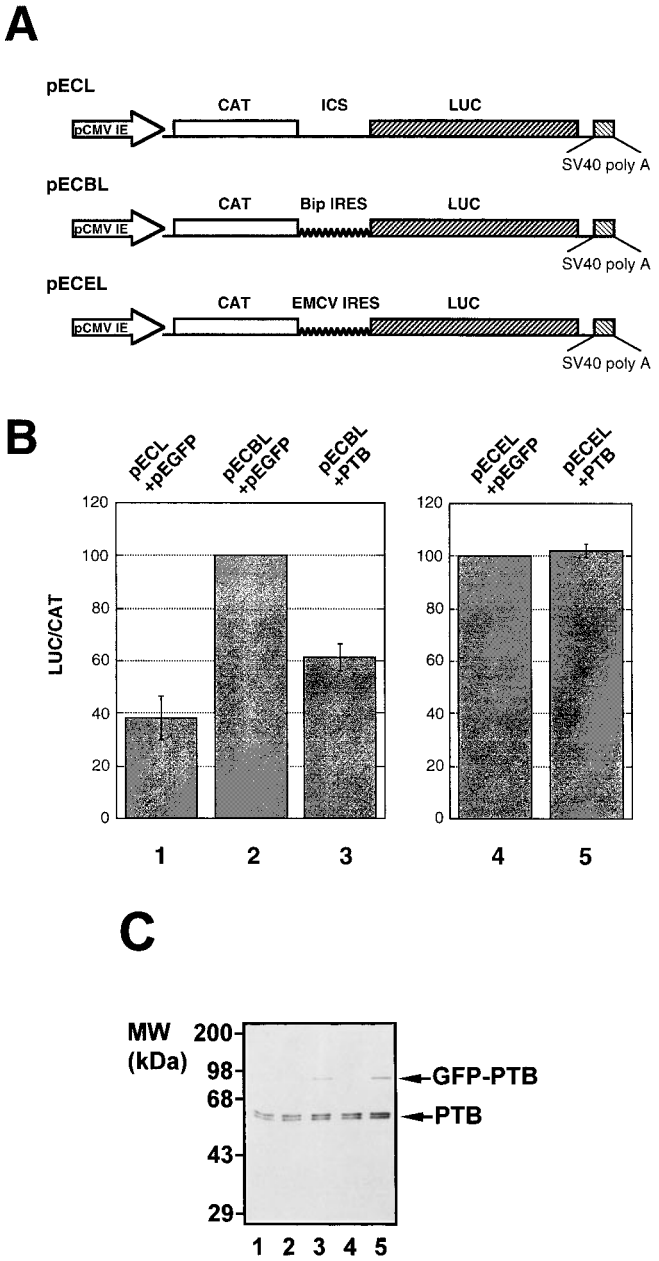

Figure 5. Effect of PTB overexpression on the internal initiation *via* the Bip 5′NTR in cultured cells. (a) Schematic diagram of the dicistronic vectors. Three dicistronic vectors containing CAT and luciferase reporter genes downstream of a CMV promoter were constructed. (b) Effect of exogenously expressed PTB on internal initiation *via* Bip 5′NTR: 0.3 μg of reporter plasmid pECBL (lanes 2 and 3), pECEL (lanes 4 and 5), or pECL (lane 1) and 5 μg of effector plasmid (pEGFP-PTB) expressing PTB (lanes 3 and 5) or control plasmid pEGFP-C1 (lanes 1, 2, and 4) were co-introduced into Cos-7 cells. Fourty-eight hours after transfection, CAT and luciferase expression was measured as described in Materials and Methods. The relative ratio of LUC to CAT in each cell extract was compared to that of cells transfected with pECBL + pEGFP (lanes 1 to 3) or with that of cells transfected with pECEL + pEGFP (lanes 4 and 5). The columns and bars represent the means and standard deviations of triplicate experiments. To monitor the expression levels of GFP-fused PTB in the transfected cells, Western blot analyses were performed with anti-PTB monoclonal antibody. The GFP-fused PTB and endogenous PTB protein are indicated by arrows.

confined to the nucleus at interphase, but it is dispersed in the cytoplasm at metaphase (Ghetti *et al.*, 1992). This change in the PTB level in the cytoplasm may regulate the translation of certain mRNAs in a cell cycle-dependent manner as a consequence. It is possible that PTB pro mRNAs are translated well in the metaphase, while PTB con mRNAs are not translated in the metaphase. More investigations into this aspect will be required.

Materials and Methods

Plasmid construction

Enzymes used for cloning and modifying DNA were purchased from New England Biolabs, Inc. and Boehringer Mannheim. Plasmid pSK(+)/Luc-BCAT (BCAT stands for Bip 5′NTR followed by the chloramphenicol acetyltransferase gene) was constructed by ligating a *Kpn*I/Klenow-filled-*Xho*I fragment of pSK(+)Bip-CAT (kindly provided by Dr P. Sarnow) to a *Kpn*I fragment of the PCR-amplified luciferase gene. The PCR reaction was carried out with the pGL2-control (Promega) and two oligonucleotides (5′-CCTGGTACCAAAAGCTTGG-CATTCCGG-3′ and 5′-GTTACATTTTACAATTTGG-3′) as template and primers, respectively. In order to extend the gap between the stop codon of the first cistron and the 5′ border of the Bip 5′NTR, a plasmid (pSK(+)/Luc-PBCAT) was constructed by inserting the *Xho*I fragment of pRSETb-PLD PCR1 into the *Xho*I-digested pSK(+)/Luc-BCAT. Plasmid pRSETb-PLD PCR1 had been constructed by ligation of *Xho*I-digested pRSETb and a Klenow-filled-in fragment of phospholipase D (PLD) amplified by PCR with oligonucleotides 5′-GCC CGGGTATGTATAGCAGGAACGA-3′ and 5′-GCCCG GGTTAGACATCCTTTGCCATTAA-3′. In the pSK(+)/Luc-PBCAT construct, an inversely oriented PLD sequence was inserted. Plasmid pSK(+)/CATΔ-BCAT containing a T7 promoter followed by a dicistronic mRNA coding sequence was generated by ligating an *Eco*RV/Klenow-filled-*Hin*dIII fragment of pSK(+)/Luc-PBCAT to a *Sac*I/*Ssp*I fragment of plasmid pCV (Tsukiyama-Kohara *et al*., 1992). Upon *in vitro* transcription by T7 RNA polymerase, plasmid pSK(+)/CATΔ-BCAT produces a dicistronic mRNA containing a CAT gene with a C-terminal truncation followed by the human Bip 5′NTR and a full-length CAT gene. To generate plasmid pSK(+)HCATΔ-BCAT (HCATΔ stands for a hairpin followed by CATΔ), a 40-bp *Kpn*I-*Hin*dIII fragment of pSK(−) was inserted in the form of an inverted tandem repeat into the unique *Kpn*I site in the 5′NTR of CATΔ in plasmid pSK(+)/CATΔ-BCAT. The mRNA transcribed from this construct contains a stable RNA hairpin structure (−77.1 kcal/mol). Plasmid pSK(+)/CATΔ-ECAT was constructed by replacing the Bip 5′NTR sequence of pSK(+)/CATΔ-BCAT with the EMCV 5′NTR sequence from pBS-ECAT (Jang *et al*., 1988). For a negative control, plasmid pSK(+)/CATΔ-ICS-CAT was constructed by replacing the Bip 5′NTR sequence of pSK(+)/CATΔ-BCAT with the *Hin*dIII-*Xho*I fragment of the pGL2-control.

Plasmids with serial deletions in the Bip 5′NTR were derived from pSK(+)Bip-CAT. Plasmid pSK(+)Bip-CAT(50-117) was constructed by self-ligation of the *Nru*I/Klenow-filled-*Acc*I fragment of pSK(+)Bip-CAT. Plasmid pSK(+)Bip-CAT(115-221) was generated by self-ligation of pSK(+)Bip-CAT after digestion with *Nar*I and *Acc*I.

Plasmid pECBL was constructed by placing the Bip 5′NTR between the chloramphenicol acetyltransferase (CAT) and luciferase reporter genes downstream of the CMV promoter of pEGFP-C1 (Clontech). Plasmid pECEL containing the EMCV IRES between the CAT and luciferase genes was constructed by replacing the Bip 5′NTR sequence in pECBL with the EMCV IRES sequence. To construct plasmid pECL, the Bip 5′NTR was eliminated from plasmid pECBL. Construction of the pEGFP-PTB has been described (Hahm *et al*., 1998).

In vitro transcription and translation

Plasmid DNAs were purified by the polyethylene glycol precipitation method (Sambrook *et al*., 1989) and linearized with appropriate restriction enzymes. The linearized DNAs were then treated with phenol/chloroform and ethanol-precipitated. Transcription reactions were performed with T7 RNA polymerase (Boehringer Mannheim) at 37 °C for 90 minutes as described by the manufacturer. To produce capped mRNAs, 1 mM m^7GpppG (Pharmacia Biotech Inc.) was included in the transcription reaction mixture. The concentration of the RNA transcripts was determined with a UV spectrophotometer. Plasmids pSK(+)/CATΔ-BCAT, pSK(+)/HCATΔ-BCAT and pSK(+)/CATΔ-ECAT digested with *Bam*HI and pSK(+)/CATΔ-ICS-CAT digested with *Xba*I were used to generate the dicistronic mRNAs CATΔ-BCAT, HCATΔ-BCAT, CATΔ-ECAT, and CATΔ-ICS-CAT, respectively. To produce mRNAs with serial deletions in the Bip 5′NTR (Bip NTR (1-221), -(1-53), -(50-117), and -(115-221)), we used *Eco*RI-digested pSK(+)Bip-CAT, *Nru*I-digested pSK(+)/Bip-CAT, *Nar*I-digested pSK(+)Bip-CAT(50-117), and *Eco*RI-digested pSK(+)Bip-CAT(115-221) in transcription reactions. The *Hin*dIII-digested pBS-ECAT, pBS-ECAT393, pBS-ECAT422 and *Bal*I-digested pBS-ECAT plasmids were used to generate EMCV(260-488), EMCV (393-488), EMCV (422-488) and full-length EMCV IRES, respectively (Jang *et al*., 1988). *Sal*I-digested pTM1-PTB1 was used as template for *in vitro* transcription of PTB1 mRNA (Oh *et al*., 1998). Luciferase mRNA was purchased from Promega. Radioactive RNA probes and biotinylated RNAs were synthesized by including [α-^{32}P]UTP (NEN) or biotin-labeled UTP in the transcription reaction (Clontech).

In vitro translations in micrococcal nuclease-treated RRL (Promega) were performed in 20 μl reaction mixtures containing 6 nM (for EMCV IRES) or 30 nM (for Bip IRES) mRNA with the addition of purified proteins dialyzed against HT buffer (16.2 mM Hepes-KOH (pH 7.5), 36 mM KCl, 160 mM KOAc, 1.24 mM MgOAc, 1.6 mM DTT, 2.8 mM β-mercaptoethanol) Translation reactions were carried out at 30 °C for one hour in the presence of [^{35}S]methionine (NEN). Competitor RNAs were additionally included in the translation-competition assays. Translation products were analyzed by SDS-15 % PAGE. The intensity of the autoradiographic images was enhanced by fluorography using salicylic acid. The gels were dried and exposed to Kodak XAR-5 or Agfa Curix RP1 for 12 to 18 hours. The efficiencies of the translations were quantified using a densitometer (B. I. System Corp., Bioimage 50S Series).

Purification of PTB and its derivatives

The purification steps taken in the purification of the recombinant PTB and its deletion mutants are described elsewhere (Oh *et al*., 1998). All purified proteins were dialyzed against HT buffer. The purification steps for the La protein will be described elsewhere (Kim & Jang, 1999).

UV cross-linking of labeled RNA and protein

UV cross-linking reactions were performed essentially as described by Meerovitch *et al*. (1989) with only slight modifications. RNAs labeled with [α-^{32}P]UTP (NEN) were used as probes in the UV cross-linking reactions.

RNA probes (2×10^5 to 4×10^5 cpm) purified using push columns (Stratagene) were incubated at 30 °C for 30 minutes along with 1 μg of purified protein or 75 μg of RRL (30 μl total volume of the cross-linking reaction). After RNA-binding, the reaction mixtures were irradiated with UV light on ice for 30 minutes using the UV-Stratalinker (Stratagene). Unbound RNAs were removed by digestion with 20 μg of RNase A, 200 units of RNase T_1, and one unit of RNase V_1 (cobra venom nuclease; Pharmacia Biotech, Inc.) at 37 °C for 20 minutes. The RNA-protein complexes were analyzed by SDS-12 % or 15 % PAGE followed by autoradiography. In the competition assays, unlabeled competitor RNAs were included in the RNA-protein binding reactions.

Streptavidin/acrylamide precipitation of PTB with biotinylated RNA

This experiment was performed as described by Boelens *et al*. (1993) with slight modification. [^{35}S]methionine-labeled PTB or luciferase (translated in RRL) was mixed with 1.25 μg of biotinylated RNA and 10 μg of yeast tRNA (Boehringer Mannheim) in 15 μl of KHN buffer (150 mM KCl, 20 mM Hepes (pH 7.9), 0.05 % (v/v) Nonidet P-40, 0.2 mM DTT). The binding reaction was carried out at 4 °C for one hour. The reaction mixture was further incubated at 4 °C for one hour after addition of 1 ml of KHN buffer and 70 μl of streptavidin/acrylamide beads (Pierce). The beads were collected by centrifugation, washed three times with 1 ml of KHN buffer, resuspended in 20 μl of sample buffer, and then boiled for four minutes. After centrifugation, 20 μl of the supernatant was analyzed by SDS-12 % PAGE followed by autoradiography.

Depletion of endogenous PTB

Endogenous PTB in the RRL was depleted by letting it react with biotinylated RNA corresponding to the EMCV IRES (nt 260-488), which strongly binds PTB. Briefly, 100 μl of streptavidin/acrylamide beads were incubated with 250 μg of biotinylated EMCV IRES (260-488) and then washed four times with KHN buffer: 500 μl of RRL was incubated with the RNA-bead complexes at 4 °C for one hour and then centrifuged at 7000 rpm. The supernatant was used in the translation reaction. Control RRL was incubated with streptavidin/acrylamide beads without biotinylated RNA.

Cell culture and transfection

Cos-7 cells were cultured and transfected using the electroporation procedure. Cells were cotransfected with 0.3 μg of dicistronic plasmid and 5 μg of PTB expression vector. About 48 hours after transfection, the cells were harvested and lysed by freeze-thawing. Cell extracts were analyzed for CAT activity according to Neumann *et al*. (1987) and for luciferase activity following the supplier's instructions (Promega).

To monitor the expression level of PTB, cell extracts were resolved by SDS-12 % PAGE and then transferred to a nitrocellulose paper (Amersham). The membrane was blocked overnight with 5 % (v/v) skim milk in TBS buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 % (v/v) Tween-20) and then incubated with anti-PTB monoclonal antibody (kindly provided by Dr E. Wimmer) for two hours. Horseradish peroxidase-conjugated anti-mouse IgG was used as secondary antibody. To visualize bands, the membrane was developed employing the ECL method following the supplier's instructions (Amersham).

Acknowledgments

We thank Dr Peter Sarnow at Stanford University for plasmid pSK(+)/Bip-CAT and Dr Eckard Wimmer for anti-PTB monoclonal antibody. The present study was supported in part by the G7, NRL, the Molecular Medicine Research Group Programs of MOST, and by HMP-98-B-3-0020.

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Edited by K. Nagai

(Received 18 July 2000; received in revised form 20 September 2000; accepted 27 September 2000)