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Pyrimidine tract binding protein strongly stimulates in vitro encephalomyocarditis virus RNA translation at the level of preinitiation complex formation

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Abstract Cellular protein p57/58, now known to be identical to polypyrimidine tract binding protein (PTB), has earlier been shown to specifically bind to the internal ribosome entry sites (IRES) of encephalomyocarditis virus (EMCV) and some other picornaviral RNAs. To elucidate its relevance to the internal initiation, the effect of cloned purified PTB on EMCV IRES directed translation was studied in cytoplasmic extracts of Krebs-2 ascites carcinoma cells partially depleted of endogenous PTB. Addition of PTB to such extracts resulted in a strong stimulation of translation of a β-glucuronidase (GUS) reporter cistron fused to the EMCV IRES, but had no effect on translation of capped mRNAs, such as β-globin, and tobacco mosaic virus (TMV) RNAs. PTB was found to exert its effect at the level of 48S pre-initiation complex formation.

Key words: EMCV RNA translation; Pyrimidine tract binding protein

1. Introduction

Initiation of translation of picornaviral RNAs occurs by an unusual mechanism whereby ribosomes bind directly to an internal site (IRES) within the 5′-untranslated region (5′-UTR) of an mRNA instead of to its 5′-end [1]. Details of this mechanism are unknown but it is thought to involve specific proteins. One such factor is a 58 kDa cytoplasmic RNA-binding protein, recently found to be identical to the pyrimidine tract binding protein (PTB) [2,3], a predominantly nuclear polypeptide that binds single-stranded DNA and RNA containing pyrimidine-rich tracts [4–8]. Roles for PTB in transcription and/or splicing have been proposed [4,6,8].

PTB (p58) interacts specifically with the IRES elements of picornaviral RNAs [9–13] suggesting a role for this factor in the mechanism of internal initiation of translation. Some correlations were observed between the effects of IRES mutations on p58 (PTB) binding, and on the functional activities of the respective polynucleotides. However, the effects of these mutations on the IRES activity differed considerably in data of different research groups [10,11,14,15].

In this report, we have demonstrated that addition of purified recombinant PTB to the ascites cells extracts depleted of endogenous PTB exerts a strong enhancing effect on the activity of the EMCV IRES element in the internal initiation of translation.

2. Materials and methods

Plasmids of the pTE series, and preparation of RNA templates in T7 polymerase run-off transcription reactions are described elsewhere [10,16]. The isolation of PTB-1 was performed as described in [17]. In vitro translation experiments were performed either in the Krebs-2 cell-free translation system as described earlier [16] or in RRL as recommended by the manufacturer (Promega). β-Globin RNA was purchased from Sigma. Tobacco mosaic virus (TMV) RNA was a kind gift from Dr. N.P. Rodionova.

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The depletion experiments were conducted by incubating a competitor transcript with the appropriate cell-free translation mixture for 5 min at 30°C before addition of messenger RNA. All mRNAs were translated at sub-saturating concentrations determined by titration experiments. Quantitation of translation products resolved by SDS-PAGE was done using a CCD videocamera. Images in radioautographs were captured with CCD videocamera and the amounts of synthesized polypeptides were analyzed with software package for image analysis, itti, l.c., St. Petersburg, FL.

To prepare 48S pre-initiation complexes, extracts from ascites carcinoma cells Krebs-2 were supplemented with all necessary additions for in vitro translation, but GTP, which was replaced by 1 mM of its non-hydrolyzable analogue, guanosine 5′-[β,γ-imido]triphosphate (GMPPNP). The samples were then supplemented with ^{32}P-labelled mRNA to give a final volume of 25 μl and the 48S complex was formed for 7 min at 30°C. In competition assays, before adding the mRNA, the samples were first pre-incubated with a competitor transcript, taken in a 10–30-fold molar excess over the template. When analyzing the effect of PTB, this factor was added to the system before the template, but after the competitor RNA. The samples were run in a 10–30% sucrose gradient in an SW41 rotor (Beckman) at 24,000 rpm for 13 h at 4°C. The position of the 48S complex in the sedimentation profile was determined by parallel centrifugation in a separate tube of 50S ribosomal subunits from *E. coli*.

3. Results

3.1. Effect of PTB addition on EMCV IRES-dependent translation

To investigate the role of PTB in the initiation of translation by internal ribosome entry, the reporter coding sequence of β-glucuronidase was fused downstream of nt. 315–833 of the EMCV 5′-NTR, generating the EMCV IRES-dependent mRNA (E-GUS). Addition of PTB to cell-free extracts of Krebs-2 ascites carcinoma cells programmed with this message had a weak stimulatory effect on translation (Fig. 1A, lanes 1 and 2). Since a stronger effect was observed for RRL (data not shown), known to contain much lower PTB concentrations [17], it was suggested that PTB is not a limiting factor in the initiation of translation in Krebs-2 cells. Therefore, its effect may be best manifested in cell-free systems depleted of endogenous PTB.

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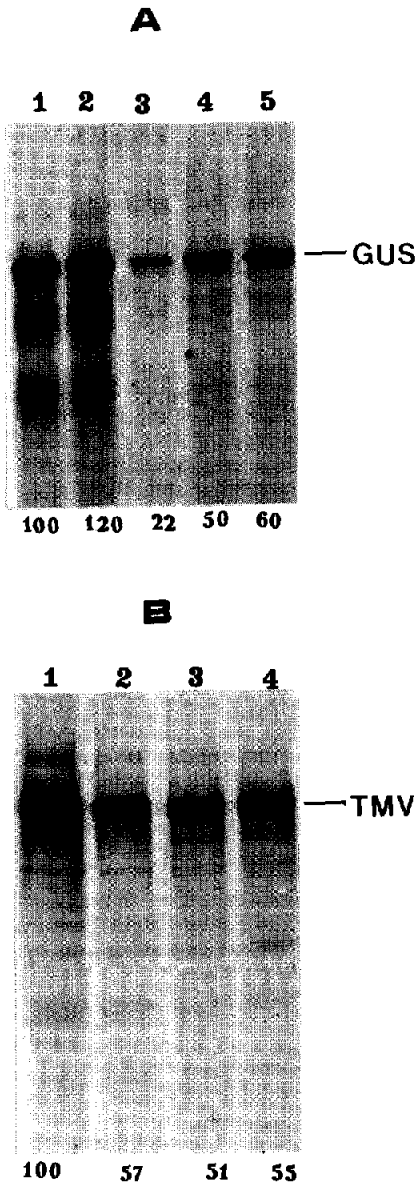

Fig. 1. Influence of PTB addition to the competitor-repressed Krebs-2 cell-free translation system on the translation of (A) E-GUS and (B) TMV RNAs. Translation was performed with 0.5 μg of E-GUS, and 1 μg of TMV RNA per assay (25 μl). (A) Lane 1, translation in the absence of either competitor or exogenous PTB; lane 2, 1.0 μg of exogenous PTB, no competitor; 3–5, translation in the presence of specific competitor; 3 – no PTB added; 4,5 – 0.5 μg and 1.0 μg of PTB, respectively; (B) lane 1, no competitor, no PTB added; lanes 2–4, translation in the presence of competitor RNA: 2 – no PTB; 3,4 – 0.5 μg and 1.0 μg of PTB, respectively. The numbers below the lanes indicate the yield of the synthesized polypeptide with respect to the control (no added PTB) taken for 100%. As a competitor RNA, the RNA 315–848 (Δ701–763) was used throughout these experiments.

3.2. *Inhibition of translation by EMCV IRES specific RNA transcripts*

RNA sequences within picornavirus IRES elements act as specific binding sites for *trans*-acting factors implicated in IRES function and can therefore act as specific competitors of IRES-dependent translation [13,15,16]. This observation suggested that sequestration of endogenous p58/PTB by competition with EMCV IRES-specific RNA transcripts should facilitate analysis of the role of PTB in internal initiation and circumvent problems associated with immunodepletion of p58/PTB from translation extracts [2,3].

Three RNA transcripts were used in competition experiments. The first transcript (tr. 315–484) contains just two p58/PTB binding sites [10,11], and the second transcript (tr. 315–848, 701–763) contains both these binding sites and an additional discontinuous downstream sequence. The third transcript (tr. 378–484, 414–417) does not bind p58/PTB. These three RNA transcripts are all inactive in translation [16] and do not form complexes with ribosomal 40S subunits (see below).

The competition properties of these 3 transcripts were assayed in ascites carcinoma cell-free extracts programmed with either E-GUS or TMV RNA (Table 1). Translation of TMV RNA results from the conventional cap-dependent initiation mechanism, and is thus an appropriate control template.

Competition with transcript 3 progressively impaired translation of both E-GUS and TMV RNA in a concentration-dependent manner. The inhibitory effect of this transcript is identical to that of other non-specific single-stranded polynucleotide competitors, and probably reflects sequestration of canonical initiation factors. The two RNA transcripts that contained p58/PTB binding sites inhibited E-GUS translation more strongly than translation of TMV RNA. These results support a role for p58/PTB in IRES-dependent initiation, and are consistent with reports that the 5′ half of the EMCV IRES contains high-affinity binding sites for this factor [10,11].

3.3. *Restoration of EMCV IRES function in vitro by PTB following sequestration of endogenous p58 by competitor RNAs*

Addition of PTB to ascites cell-free extracts partially reversed the inhibition of EMCV IRES-dependent translation caused by prior addition of competitor RNAs containing p58 binding sites (Fig. 1A, lanes 3–5). For example, the presence of a 30-fold molar excess of competitor transcript 2 over E-GUS

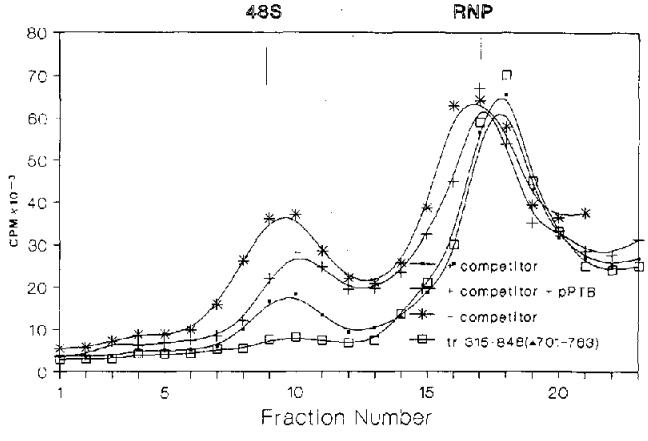

Fig. 2. Effect of PTB on the efficiency of the 48S pre-initiation complex formation in Krebs-2 cell-free translation system programmed by a fragment of EMCV RNA. The ^{32}P-labelled transcript (nts. 315–1,155), carrying the N-terminal coding sequence of the EMCV polyprotein up to nt. 1,155 was used. The complex was formed with 0.5 μg of the message in 25 μl for 7 min at 30°C. Before addition of PTB (0.5 μg/25 μl), the system was repressed by tr. 315–848 (Δ701–763) (2 μg/25 μl). For other conditions, see section 2.

Table 1
Competition effect of different derivatives of the EMCV IRES in Krebs-2 cell-free translation system programmed by the E-GUS and TMV RNAs

EMCV IRES derivatives used as competitors	[^{35}S]Met incorporation (%)					
	E-GUS RNA			TMV RNA		
	Competitor RNA (μg/25 μl)					
	1	2	4	1	2	4
tr. 1: 315–484	71	57	46	85	77	69
tr. 2: 315–848 (Δ701–763)	64	44	25	86	67	53
tr. 3: 378–484 (Δ414–427)	85	78	66	86	74	67

The translation efficiency is expressed as % of control translation with no competitor RNA added. Synthesized protein was quantitated by trichloroacetic acid-insoluble radioactivity after 60 min of incubation at 30°C. 0.5 μg of E-GUS RNA and 1 μg of TMV RNA per 25 μl of assay were used in each case. The values represent an average of four independent experiments using different preparations of Krebs-2 extracts. A standard deviation was about 10%.

mRNA reduced translation to 20%, but addition of saturating amounts of PTB-1 (40 μg/ml) restored GUS translation up to 60% of the control level. Inhibition of E-GUS translation under conditions of lower competitor excess was less severe, and PTB addition resulted in near-total restoration of IRES activity (data not shown). The effect of PTB addition is specific, because addition of PTB had no effect on TMV translation in the same cell-free extract in the presence of competitor RNA (Fig. 1B). In similar experiments, addition of PTB did not stimulate translation of β-globin mRNA (data not shown). The failure to fully restore EMCV IRES activity, particularly under conditions of large competitor excess, is consistent with earlier observations that a proportion of the inhibition caused by competitor transcript 2 is non-specific (see above). It may be due to sequestration of canonical initiation factors involved in both cap-dependent and IRES-dependent mechanisms of translation initiation.

3.4. Influence of PTB on 48S pre-initiation complex formation

To identify the step in the initiation process at which PTB functions, formation of 48S pre-initiation complex was assayed under conditions corresponding to those used in earlier in vitro translation assays. Formation of 48S pre-initiation complexes was monitored by incorporation of [^{32}P]UTP-labelled mRNA. 48S pre-initiation complexes were formed efficiently on RNA templates corresponding to EMCV nts. 315–1155, but did not form on the corresponding EMCV RNA template containing an internal deletion (nts. 701–763) (Fig. 1). This deletion is distant from both the 5′ terminus and the authentic initiation codon (AUG-11 at nt. 834), and by definition, cannot affect canonical 5′-end dependent initiation of translation [18]. These pre-initiation complexes therefore result from internal ribosome entry. Preincubation with excess competitor transcript 2 (which contains two p58 binding sites) resulted in a three-fold reduction in pre-initiation complex formation; this reduction was partially reversed by addition of PTB.

4. Discussion

We have presented here direct evidence that (PTB) p58 plays an essential role in EMCV IRES function, and have found that it strongly stimulates EMCV translation and promotes 48S pre-initiation complex formation on EMCV templates. p58/PTB is not a limiting factor in some cell-free extracts, and the effects of exogenous PTB are therefore most clearly manifested if endogenous p58 is depleted, or, as in the experiments described here, sequestered by competition with translationally inactive RNA transcripts containing p58/PTB binding sites. The effects of PTB on EMCV translation are specific, and PTB had no effect on translation or 48S complex formation using TMV and β-globin mRNAs.

PTB is not itself sufficient to promote interaction of 40S ribosomal subunits with EMCV RNA, since the second specific RNA competitor used here (which lacks nt. 701–763 of the IRES) did not act as template for formation of 48S pre-initiation complex even (Fig. 2) though it binds p58/PTB and contains the authentic initiation codon. RNA transcripts containing this nts. 701–763 segment of the IRES exert a potent inhibitory effect on both IRES-dependent and cap-dependent modes of translation initiation [15,16], suggesting that they may sequester a trans-acting factor involved in both mechanisms. Experiments to analyze functional interactions between both canonical, and non-canonical initiation factors and PTB are in progress.

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References

[1] Jackson, R.J., Howell, M.T. and Kaminski, A. (1990) Trends Biochem. Sci. 5, 477–483.
[2] Hellen, C.U.T., Witherell, G.W., Schmid, M., Shin, S.H., Pestova, T.V., Gil, A. and Wimmer, E. (1993) Proc. Natl. Acad. Sci. USA 90, 7642–7646.
[3] Borman, A., Howell, M.T. and Jackson, R.L. (1993) J. Gen. Virol. 74, 1775–1788.
[4] Garcia-Blanco, M.A., Jamison, S.F. and Sharp, P.A. (1989) Genes Dev. 3, 1874–1886.
[5] Gil, A., Sharp, P.A., Jamison, S.F., Garcia-Blanco, M.A. (1991) Genes Dev. 5, 1224–1236.
[6] Patton, J.G., Mayer, S.A., Tempst, P. and Nadal-Ginard, B. (1993) Genes Dev. 5, 1237–1251.
[7] Bothwell, A.L.M., Ballard, D.W., Philbrick, W.M., Lindwall, G., Maher, S.E., Bridgett, M.M., Jamison, S.F. and Garcia-Blanco, M.A. (1991) J. Biol. Chem. 266, 24657–24663.
[8] Brunel, F., Alzari, P.M., Ferrara, P. and Zakin, M.M. (1991) Nucleic Acids Res. 19, 5237–5245.
[9] Borovjagin, A.V., Evstafieva, A.G., Ugarova, T.Yu. and Shatsky, I.N. (1990) FEBS Lett. 261, 237–240.
[10] Borovjagin, A.V., Ezrokhi, M.V., Rostapshov, V.M., Ugarova, T.Yu., Bystrova, T.F. and Shatsky, I.N. (1991) Nucleic Acids Res. 18, 4999–5005.

[11] Jang, S.-K. and Wimmer, E. (1990) Genes Dev. 4, 1560–1572.
[12] Luz, N. and Beck, E. (1991) J. Virol. 65, 6486–6494.
[13] Pestova, T.V., Hellen, C.U.T. and Wimmer, E. (1991) J. Virol. 65, 6194–6204.
[14] Kuhn, R., Luz, N. and Beck, E. (1990) J. Virol. 64, 4625–4631.
[15] Duke, G.M., Hoffman, M.A. and Palmenberg, A.C. (1992) J. Virol. 66, 1602–1609.
[16] Evstafieva, A.G., Ugarova, T.Yu., Chernov, B.K. and Shatsky, I.N. (1991) Nucleic Acids Res. 19, 665–671.
[17] Hellen, C.U.T., Pestova, T.V., Litterst, M. and Wimmer, E. (1994) J. Virol. 68, 941–950.
[18] Kozak, M. (1989) J. Cell Biol. 108, 229–237.