

Pyrimidine tract binding protein and La autoantigen interact differently with the 5' untranslated regions of lentiviruses and oncoretrovirus mRNAs

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Abstract Retrovirus genomic mRNA exhibits a several hundred nucleotides-long untranslated region (5' UTR) which encloses many control elements required for retrovirus replication. In addition, this 5' UTR contains translation regulatory elements, such as internal ribosome entry sites (IRESes) that have been described in oncoretroviruses, as well as in lentiviruses. UV cross-linking experiments suggested that the pyrimidine tract binding protein (PTB), a cellular protein known to regulate the activity of several picornaviral IRESes, binds to human T-cell leukemia virus (HTLV)-I RNA but not to lentiviral human immunodeficiency virus (HIV)-1, HIV-2 or simian immunodeficiency virus RNAs. To calculate the affinity of such RNA–protein interactions, we developed a new method based on the BIAcore technology. The absence of affinity of PTB for lentiviral RNAs was confirmed, whereas its affinity for HTLV-I RNAs was 1000-fold lower than for picornaviral RNAs. The BIAcore technology also revealed a significant affinity of the La autoantigen, previously described for its involvement in translational control of viral mRNAs, for HIV-1 and HTLV-I RNAs. Addition of recombinant PTB to in vitro translation experiments weakly enhanced translation initiation in the presence of HTLV-I IRES, suggesting that such an IRES requires additional *trans*-acting factors. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Retrovirus; Human immunodeficiency virus-1; Translation initiation; Internal ribosome entry site; Pyrimidine tract binding protein; BIAcore; Surface plasmon resonance; RNA–protein interaction

1. Introduction

Translation initiation of retroviral genomic mRNA has been shown to occur by an internal ribosome entry mechanism, however very little is known about the proteins involved

in the function of retroviral internal ribosome entry sites (IRESes).

In contrast, picornaviral IRESes have been shown to specifically bind cellular proteins (rather than viral factors) able to enhance their activity. The first proteins to be identified were the pyrimidine tract binding protein (PTB) and the La autoantigen [1–5]. More recently, the protein *unr* was shown to specifically activate the IRES of the human rhinovirus [6].

We report here the interactions of the picornavirus (PV) and retrovirus 5' untranslated region (5' UTR) (all containing IRESes) with cellular proteins studied by UV cross-linking experiments and BIAcore analysis, showing that only human T-cell leukemia virus (HTLV)-I UTR bound the PTB whereas HTLV-I and human immunodeficiency virus (HIV)-1 UTRs bound La protein. Addition of PTB had only a weak effect on the translation of a chimeric luciferase mRNA fused to the HTLV-I 5' UTR.

2. Materials and methods

2.1. Plasmid construction

Plasmid construction strategies are available upon request.

2.2. UV cross-linking experiments

S10 cytoplasmic extracts from different cell types were prepared as already described [7]. 1×10^5 cpm of 32 P-labelled RNA was incubated with 10 µg of S10 extract and UV-irradiated with an energy of 400 000 µJ/cm² at 254 nm. The samples were then treated with 2.5 U of RNase A (Sigma) and 10 U of RNase T1 (Calbiochem) at 37°C for 30 min, before 12.5% polyacrylamide gel electrophoresis (PAGE) analysis and autoradiography.

2.3. Recombinant protein purification

Human PTB and La were overexpressed in *Escherichia coli* (BL21 strain) as fusion proteins with GST and purified by glutathione-Sepharose chromatography (Pharmacia), as recommended by the manufacturer. Purification was monitored by SDS-PAGE followed by Coomassie brilliant blue R-250 staining of the gels. GST-PTB and GST-La preparations were estimated >90% pure and their concentration was 1 mg/ml.

2.4. BIAcore analysis of RNA–protein interactions

A BIAcore 3000 was used to perform binding studies. Research grade SA Chips coated with streptavidin were obtained from BIAcore (Uppsala, Sweden).

A 5' biotinylated single strand DNA probe (hybridizing with the first 36 firefly luciferase (LucF) nucleotides (nt)) was immobilized on each flow cell as capture molecule. The sequence was 3'-TACCTTCTGCGGTTTTTGTATTTCTTCCGGGCCGCG-biotinylated 5'.

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Abbreviations: EMCV, encephalomyocarditis virus; HIV, human immunodeficiency virus; HTLV, human T-cell leukemia virus; IRES, internal ribosome entry site; MuLV, murine leukemia virus; nt, nucleotide; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PTB, pyrimidine tract binding protein; RRL, rabbit reticulocyte lysate; SIV, simian immunodeficiency virus; UTR, untranslated region

The immobilization steps were carried out at a flow rate of 20 $\mu\text{l}/\text{min}$ in HEPES buffer from BIAcore (10 mM HEPES, 150 mM NaCl, 3 mM EDTA and 0.005% P20 surfactant). An average of 1000 relative units (RU) of DNA was immobilized on each flow cell.

The mRNA tested was produced from plasmids pKS-JPE (see above). The mRNAs were then filtered on RNeasy Mini kit from Qiagen, according to the manufacturer's instructions. RNA transcripts were then quantitated by absorbance at 260 nm, and their integrity verified by ethidium bromide staining on agarose gel. They were then hybridized over biotinylated DNA at a flow rate of 5 $\mu\text{l}/\text{min}$ in modified HEPES buffer (10 mM HEPES, 400 mM NaCl, 3 mM EDTA and 0.005% P20 surfactant), and the quantities of bound RNA (i.e. the RU) were monitored. Binding experiments were performed at a flow rate of 5 $\mu\text{l}/\text{min}$. The instrument was equilibrated at 25°C and the running buffer used was the one obtained from BIAcore. For each experiment, a flow cell was coated with an mRNA used as a negative control of the interaction, pJPE T(–). The response from the control surface flow cell was subtracted from the other three flow cells to correct the refractive index changes and non-specific binding.

3. Results

3.1. Cell proteins cross-linked to the retroviral RNA leader sequences

A previous report had shown that PTB binding was correlated to the activity of the Mo-murine leukemia virus (MuLV) IRES [7]. This prompted us to analyze the cellular proteins able to bind to the retrovirus leader sequences by UV cross-linking. ^{32}P -labelled RNA probes corresponding to PV (ence-

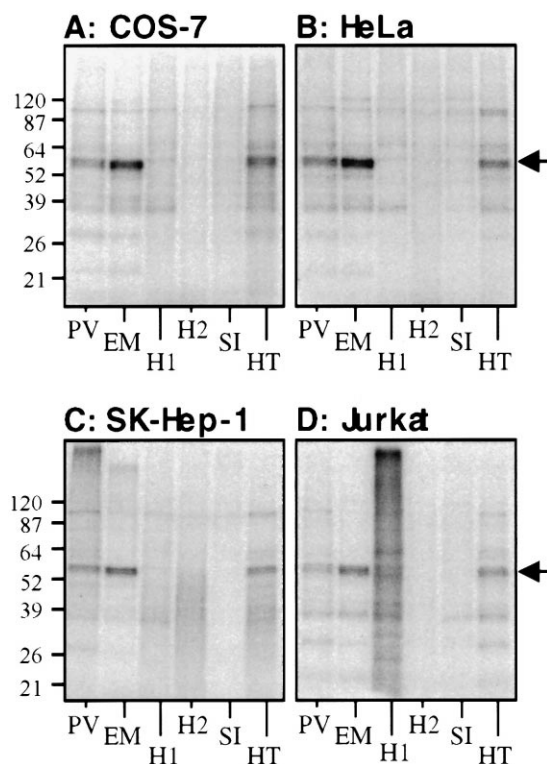


Fig. 1. Analysis of RNA–protein interactions by UV cross-linking. (A) COS-7, (B) HeLa, (C) SK-Hep-1 and (D) Jurkat cells S10 extract were incubated with RNA probes (10^5 cpm) corresponding to the different PV (EMCV nt 261–837, poliovirus) or retrovirus leaders (HIV-1, HIV-2, SIV and HTLV-I). UV irradiation was performed as described in Section 2. Samples were treated with RNases A and T1 before analysis by SDS–PAGE. The name of the probe is indicated under each lane: PV (poliovirus), EM (EMCV), H1 (HIV-1), H2 (HIV-2), SI (SIV), HT (HTLV-I). Migration of the size standards (kDa) is shown. The arrow indicates PTB migration.

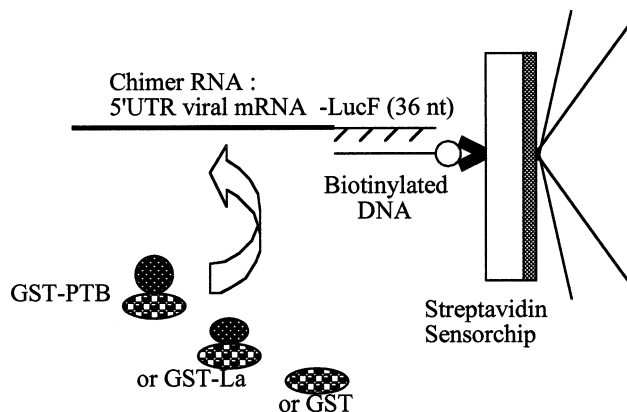


Fig. 2. Scheme of RNA–protein interaction on BIAcore 3000. The different viral RNA 5' UTRs (transcribed using the MEGAscript[®] T7 kit from Ambion) were fused to the first 36 nt of LucF ORF. These RNAs were bound to a streptavidin sensorchip by the intermediary of a biotinylated DNA oligonucleotide complementary to the 36 nt of LucF ORF. Binding experiments were performed on BIAcore 3000 using purified GST-PTB, GST-La or GST alone as a control.

phalomyocarditis virus (EMCV)) or retrovirus 5' UTRs (HIV-1, HIV-2, simian immunodeficiency virus (SIV), HTLV-I) were used in UV cross-linking experiments using acellular extracts from COS-7, HeLa, SK-Hep-1 and Jurkat cells (Fig. 1A–D). The four kinds of cell extracts gave similar cross-linking profiles for each 5' UTR. A major 60 kDa migrating band was visible using poliovirus and EMCV IRESes (Fig. 1, PV, EM), probably corresponding to the PTB. This was confirmed by immunoprecipitation using anti-PTB antibodies (data not shown). This 60 kDa protein was clearly bound to HTLV-I 5' UTR (Fig. 1, HT), but not to HIV-1, HIV-2 or SIV 5' UTR (Fig. 1, H1, H2, SI). Bands were detectable, although less clearly, between 40 and 52 kDa using EMCV, poliovirus, HIV-1 and HTLV-I probes (Fig. 1, PV, EM, H1, HT), but not with the HIV-2 and SIV leaders (Fig. 1, H2, SI). As expected from the literature, poliovirus, EMCV, HIV-1 leaders should bind the La protein (47–52 kDa) [3,8,9]: this suggested that one of the cross-linked proteins between 40 and 52 kDa could correspond to La.

3.2. BIAcore analysis of retrovirus RNA leader interactions with PTB and La

We developed a new approach to characterize and quantify RNA–protein interactions using the BIAcore technology, mainly proposed for the study of protein–protein interactions [10]. Bacterial fusion proteins GST-La and GST-PTB were purified (as described in Section 2) to study their interactions with picornaviral and retroviral RNAs immobilized on streptavidin chips bearing a biotinylated DNA with a 3' end complementary to the different RNAs (Fig. 2).

The results clearly showed that GST-PTB interacted with EMCV, poliovirus and HTLV-I RNAs, but not with HIV-1, HIV-2 and SIV RNAs (Fig. 3). GST-La showed affinity with EMCV, poliovirus, HIV-1 and HTLV-I, but not with HIV-2 and SIV RNAs. These data not only confirmed the UV cross-linking experiments, but enabled us to calculate the apparent affinity constants (K_D) for each interaction. We measured a K_D of 4 and 5 pM for GST-PTB interactions with EMCV and PV RNAs, respectively, and a K_D of 5.3 nM for its interaction with HTLV-I RNA. GST-La bound to HIV-1 RNA with a

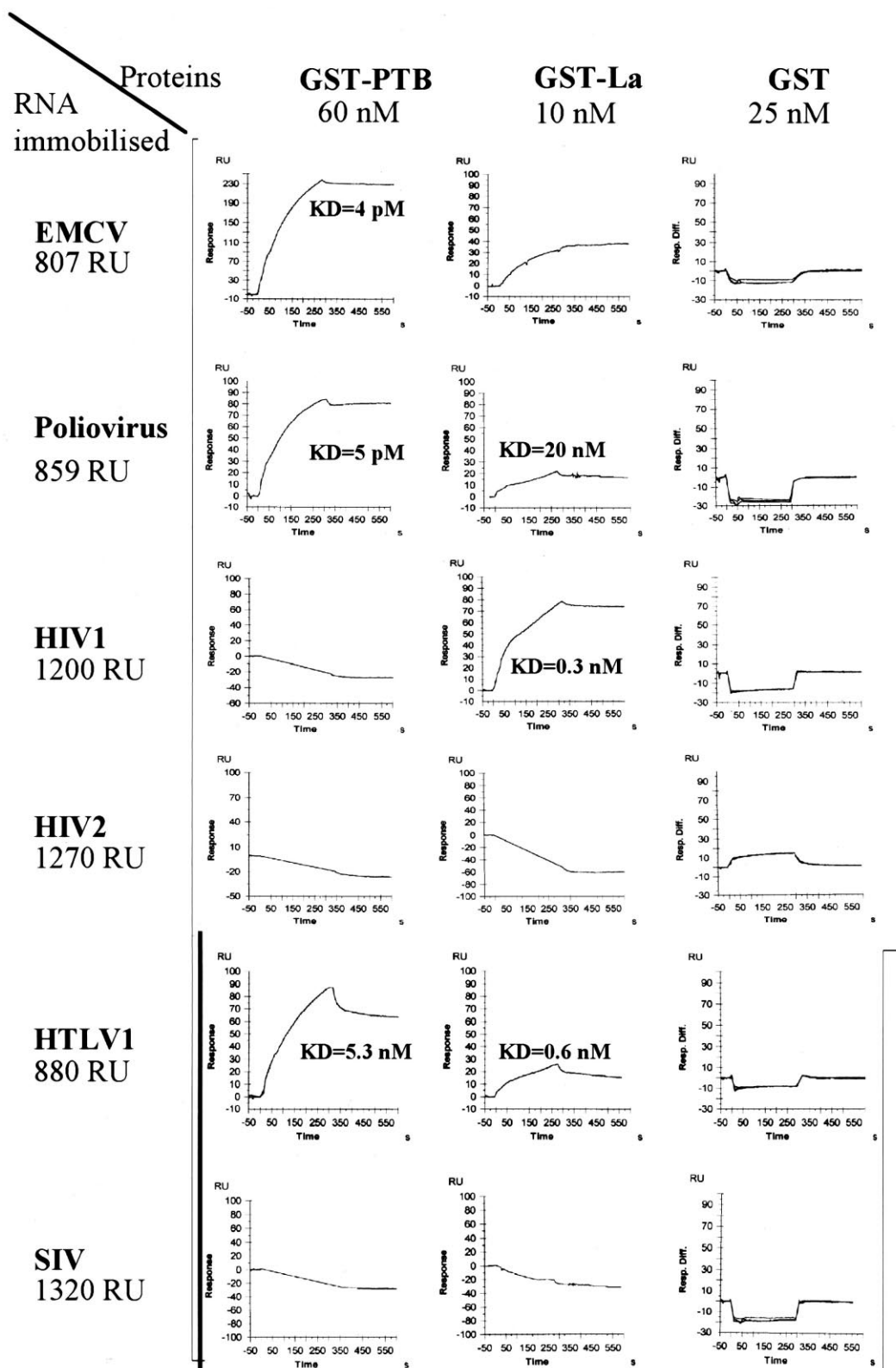


Fig. 3. BIAcore analysis of viral RNA leader interactions with GST-PTB and GST-La. Analysis of mRNA interactions with proteins by surface plasmon resonance. The sensograms show the relative responses for the interaction tested. Amounts of mRNA captured onto biotinylated single strand DNA probe are indicated (as described in Section 2). GST proteins were injected over the flow cell at the indicated concentrations. Apparent values for K_D were calculated using Biaevaluation 3.1 from BIAcore.

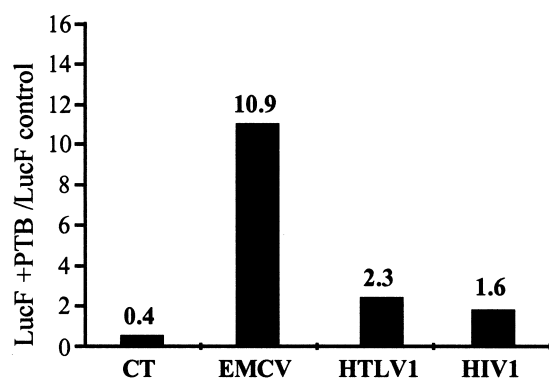


Fig. 4. Effect of GST-PTB addition on translation efficiency of EMCV-Luc, HTLV-I-Luc or HIV-1-Luc mRNAs in vitro. Uncapped monocistronic mRNAs corresponding to either LucF mRNA (CT), or mRNAs bearing fusions of EMCV, HTLV-I or HIV-1 leaders with LucF ORF were translated in RRL supplemented or not with GST-PTB at a concentration of 50 μ g/ml [15]. Translation efficiency was analyzed by measuring luciferase activity. The histogram represents, for each tested RNA, the ratio of luciferase activity obtained in the presence of PTB (black boxes) divided by the luciferase activity in the non-supplemented RRL. The origin of the leader fused to LucF is indicated under the histograms (EMCV, HTLV-I, HIV-1).

K_D of 20 nM, whereas it was only 0.3 and 0.6 nM with HIV-1 and HTLV-I RNAs.

These data indicated that GST-PTB binds to HTLV-I RNA and GST-La to HIV-1 and HTLV-I RNAs, but with much lower affinity than to the picornaviral RNAs, which was not visible on the cross-linking autoradiogram. Furthermore they provided evidence that PTB does not bind to HIV-1, HIV-2 and SIV RNAs and that La does not bind to HIV-2 and SIV RNAs.

3.3. The effect of PTB addition on translation efficiency in vitro is correlated to its affinity for RNA

The results obtained from the cross-linking and BIAcore experiments suggested that PTB could be a *trans*-acting factor able to promote or enhance the retrovirus IRES activities depending on their binding to these IRESes. To test this hypothesis, in vitro translation experiments were performed with monocistronic mRNAs bearing EMCV, HTLV-I or HIV-1 5' UTR fused to the LucF open reading frame (ORF). GST-PTB protein was added to the translation reactions and luciferase synthesis compared in the absence or presence of the protein. The results are reported in Fig. 4: for each mRNA, the effect of the protein is expressed by an activation factor corresponding to the ratio of Luc activity in the presence versus absence of GST-PTB. A negative control without any IRES was also used (Fig. 4, CT). PTB was able to activate EMCV IRES efficiency by an 11-fold factor (Fig. 4, EMCV). In contrast, PTB was only able to activate translation in the presence of the HTLV-I 5' UTR by a 2.3-fold factor in the same conditions (Fig. 4, HTLV-I). The effect of PTB addition was still lower in the presence of HIV-1 5' UTR ($\times 1.6$, Fig. 4, HIV-1).

These experiments showed that PTB had different effects on translation of the LucF mRNA, depending on the presence of EMCV, HTLV-I or HIV-1 5' UTRs. Such differences reflected the different affinities of PTB for the three RNAs, shown in Fig. 3.

4. Discussion

We show here, by the BIAcore technology, that the 5' UTRs of picornaviral and retroviral mRNAs have different affinities for PTB and La proteins. The most striking differences are observed with PTB, and are correlated with the ability of the protein to enhance translation in the presence of these different 5' UTRs in vitro.

On a technological point of view, these results show that surface plasmon resonance (BIAcore) provides a powerful approach for measuring RNA–protein affinity. Whereas the UV cross-linking experiments provided a qualitative information as regards the ability of PTB to bind to the different RNAs, the BIAcore analysis revealed a 1000-fold difference between the affinity of PTB for PV and HTLV-I RNAs, whereas it confirmed the absence of binding of PTB to the HIV-1 5' UTR RNA.

The better affinity of PTB for a PV 5' UTR than for a retrovirus 5' UTR RNA has already been observed for MoMuLV, in competition experiments involving UV cross-linking assays [7]. The affinity of the La protein for HIV-1 UTR is also lower than its affinity for poliovirus (K_D of 0.3 and 20 nM, respectively). This is consistent with a previous report describing a 6-fold higher affinity of poliovirus RNA for La than of HIV-1 TAR RNA [8]. The bigger K_D difference observed in our experiments can be explained by the fact that we used the complete HIV-1 5' UTR.

IRESes have been identified in the genomic mRNA 5' UTR of both lenti- and oncoretroviruses [7,11–13] whereas PTB seems to bind to the mRNA 5' UTR of oncoretroviruses only [7] (and this report). This suggests that PTB might be involved in the function of oncoretrovirus, but not lentivirus IRESes. However the supplementation of rabbit reticulocyte lysate (RRL) with purified GST-PTB (Fig. 3), fully active on EMCV IRES activation (11-fold), results in a weak enhancement of HTLV-I IRES (2.3-fold), that is barely superior to the enhancement observed for HIV-1 IRES (1.6) which has no affinity for the protein (see above). We may hypothesize that PTB is not a determinant *trans*-acting factor for the oncoretrovirus IRES function, or that it is not sufficient alone to enhance IRES activity, another protein being required. Supporting the second hypothesis, we observed in co-transfection experiments that PTB activated the expression of a monocistronic HTLV-I-CAT (chloramphenicol acetyltransferase) mRNA in COS-7 cells (data not shown). Furthermore a recent report shows that foot and mouth disease virus IRES requires both PTB and another factor, ITAF(45), acting as RNA chaperones [14]. Thus, the weak enhancing effect of PTB on translation efficiency in the presence of the oncoretrovirus IRES suggests the requirement of a co-factor for optimal IRES activity.

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